Pergamon

Tetrahedron Letters 42 (2001) 2625–2629

TETRAHEDRON LETTERS

Synthesis of peptide-diazeniumdiolate conjugates: towards enzyme activated antitumor agents

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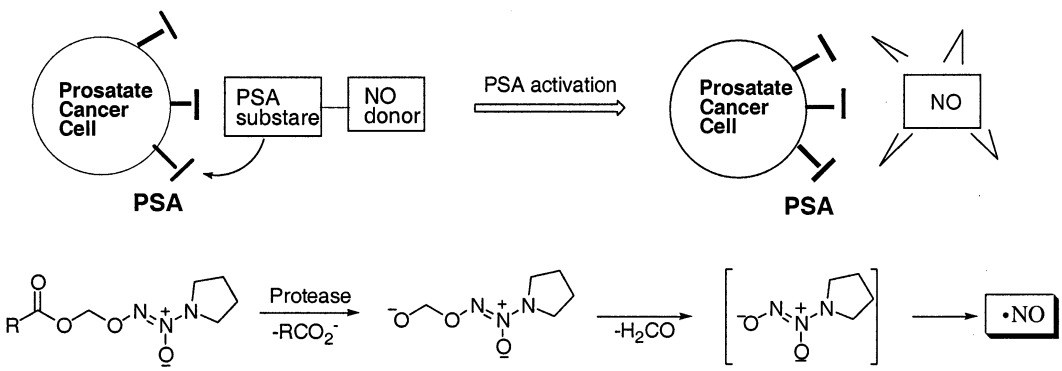
Received 22 January 2001; accepted 18 February 2001

Abstract—The development of NO donors with site-specific and time-controlled properties is of great interest. We have designed a novel prodrug class as possible agents against metastatic prostate cancer by coupling a diazeniumdiolate to the terminal carboxyl groups of amino acids or peptides, such as Ser-Ser-Tyr-Tyr, Ser-Ser-Phe-Tyr, and Gly-Ile-Ser-Ser-Phe-Tyr. These prodrugs can be activated by α-chymotrypsin or prostate specific antigen and are potentially potent compounds for prostatic cancer. © 2001 Published by Elsevier Science Ltd.

There is currently no effective therapy for men with metastatic prostate cancer with relapse after androgen ablation.[1] We recognized the need to develop new strategies for the delivery of cytotoxic agents specifically to sites of metastatic prostate cancer while avoiding systemic toxicity. One such approach would be to develop prodrugs that are inactive when given systemically but become activated when processed proteolytically within prostate cancer metastases by a prostate specific antigen (PSA).[2–4] Recently reported conjugates of peptide with doxorubicin[2,3] or mustard agents[4] were designed according to this strategy. PSA is a serine protease with chymotrypsin-like substrate specificity;[5] it is synthesized and secreted in large quantities by normal and malignant prostatic epithelial cells.[6] The elevated levels of PSA in serum have been correlated with the benign and metastatic disease, making PSA a useful marker for the growth of prostate cancer.[7] However, PSA in blood serum is inactive due to the presence of high concentrations of natural inhibitory proteins, such as α_1-antichymotrypsin and α_2-macroglobulin.[8]

Nitric oxide (NO) has been identified as a major mediator in many physiological processes.[9–11] In addition to its well-documented vasoactive,[12,13] and neurotransmitter properties,[14,15] NO produced by macrophages, Kupffer cells, natural killer T-cells, and endothelial cells participate in tumoricidal activity against many types of tumors.[16,17] It may interact with oxygen-derived radicals to generate molecules that can nitrosate proteins, modify their functions and mediate damage to DNA.[18] Although NO can be endogeneously produced from



Scheme 1. PSA activated peptide-diazeniumdiolate prodrug targeting prostate cancer.

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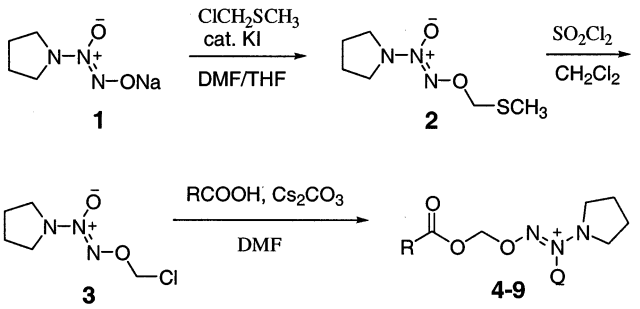
PII: S0040-4039(01)00263-5



Scheme 2. General synthetic scheme for PSA-activated prodrugs.

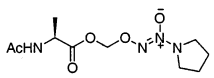
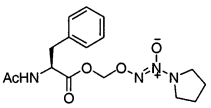
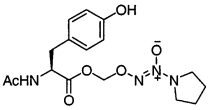
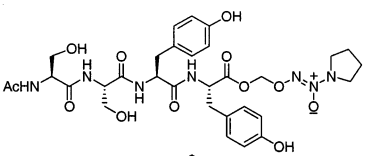
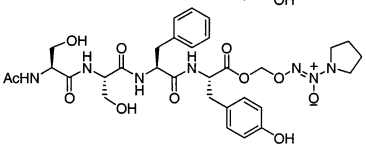
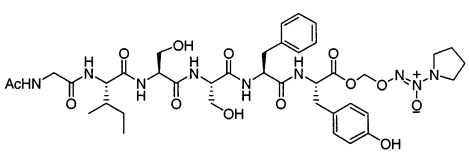
L-arginine, mediated by nitric oxide synthase (NOS),[19,20] exogenous sources of NO have been widely used as therapeutic agents.[21–25] Diazeniumdiolates (NONOates) are compounds containing the $[N(O)NO]^-$ structural unit, this class of compound is known as an excellent source for a controlled release of NO both in vitro and in vivo.[26,27] Keefer's group has recently introduced a new class of diazeniumdiolate prodrugs which are acetoxymethylated at the anionic oxygen of the parent NONOates.[28] These esterase-sensitive compounds reveal significant antileukemic activity in vitro.

We are interested in the design of PSA substrate-nitric oxide donor conjugates as potential enzyme activated antitumor agents. This class of conjugate prodrugs, if properly designed, will be inactive until they reach the prostate cancer cell, where it will be specifically hydrolysed by PSA and release toxic NO radicals to destroy the cancer cells (Scheme 1). The work described herein chooses a diazeniumdiolate, PYRRO/NO, as the specific NO donor. Although a number of high-affinity substrates for PSA have been identified, peptides containing amino acid sequence Ser-Ser-(Tyr/Phe)-Tyr were initially selected as the PSA substrate based on synthetic consideration.[29] These units will be linked together via an 'acetal' linkage, which was previously used in our study of cupferron prodrugs.[30]

As shown in Scheme 2, the sodium salt of PYRRO/NO (**1**) was first *O*-alkylated with chloromethyl methylsulfide, and the resulting compound (**2**) was subsequently treated with sulfuryl chloride. Such in situ generated chloride (**3**) can be coupled to the C-terminal carboxylic acids of the peptides in the presence of cesium carbonate to give the desired prodrugs. Three single amino acids (Ala, Phe, and Tyr) were chosen to test the feasibility of the coupling strategy. Then three peptides (Ser-Ser-Tyr-Tyr, Ser-Ser-Phe-Tyr, and Gly-Ile-Ser-Ser-Phe-Tyr) were successfully coupled to the diazeniumdiolate moiety. All of the peptide-diazonemdiolate conjugates (**4–9**) were obtained in very good yield (Table 1). The peptides were prepared by standard solid phase peptide synthesis techniques using Fmoc strategy.[31]

Enzymetic assays were performed to study the hydrolysis of prodrugs by the α-chymotrypsin (EC 3.4.21.1)

Table 1. The synthetic result of peptide-diazonemdiolate prodrugs (**4–9**)

Compounds	Structure	ROOH	yield %
4		Ac-A-OH	60%
5		Ac-F-OH	60%
6		Ac-Y-OH	54%
7		Ac-SSYY-OH	71%
8		Ac-SSFY-OH	41%
9		Ac-GISSFY-OH	45%

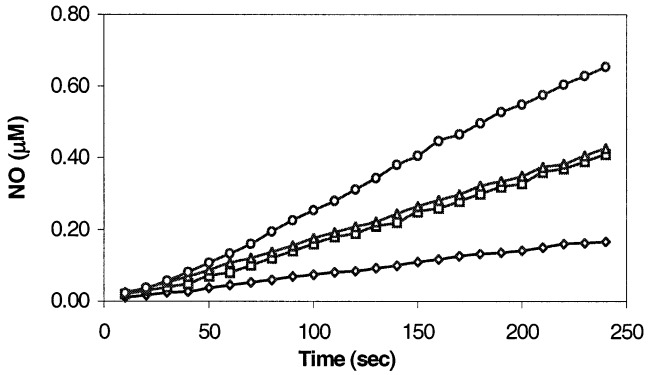


Figure 1. Enzymatic release of NO from prodrugs **5** (◇), **6** (□), **8** (△) and **9** (○) in the presence of PSA (substrate: 25 μM, PSA: 20 μg/mL).

and purified PSA (Cortex Biochem. Inc., San Leandro, CA) at room temperature. Each prodrug was first dissolved in 1.0 mL of anhydrous methanol, a portion of the solution was then diluted with 10 mL of 0.1 M Tris–Cl buffer (pH 7.0) followed by the addition of an aqueous solution of pure enzyme. The generation of NO from the hydrolysis could be quantitatively measured with an Electrochemical ISO-NO Mark II Isolated Nitric Oxide Meter manufactured by World Precision Instruments, Inc. (Saraota, Florida). All the prodrugs were essentially stable at neutral pH, and no detectable amount of NO was released even after several hours. When the prodrug was treated either with pure PSA (Fig. 1) or α-chymotrypsin (Fig. 2), the NO release was significantly accelerated and a substantial amount of NO was detected. The difference in the NO release rate between the prodrugs may reflect different binding affinity to the enzyme.

In summary, we have developed an efficient synthetic strategy to synthesize peptide-diazeniumdilote conjugates. Such prodrugs can be activated by cellular proteases, such as α-chymotrypsin or prostate-specific antigen, to release nitric oxide. Further systemic cytotoxic studies against PSA secreting tumor cell lines will be reported in due course.

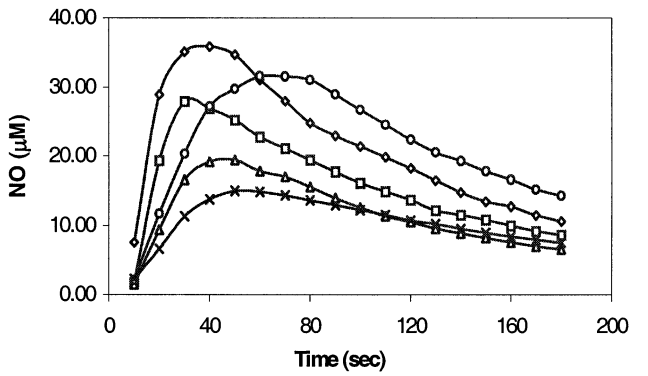


Figure 2. Enzymatic release of NO from prodrugs **5** (◇), **6** (□), **7** (×), **8** (△) and **9** (○) in the presence of α-chymotrypsin (substrate: 100 μM, α-chymotrypsin: 50 μg/mL).

Experimental

All reagents were purchased from commercial suppliers and were used without further purification. Melting points were measured on an Electrothermal IA 9100 digital apparatus without correction. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-300, a Mercury-400, or a Varian Unity-500 spectrometer. MS (ESI) and HRMS (EI) were performed at the Department of Chemistry, Wayne Sate University; HRMS (DCI or FAB) were performed at the Department of Chemistry, University of California, Riverside. Silica gel F_{254} plates (Merck) and silica gel 60 (70–230 mesh, Merck) were used in analytical thin-layer chromatography (TLC) and flash column chromatography, respectively.

Sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (1): Prepared by Keefer's method.[28] ^{1}H NMR (D_2O, 400 MHz): δ 2.99 (m, 4H), 1.68 (m, 4H); ^{13}C NMR (D_2O, 100 MHz): δ 51.58, 22.60.

***O*^{2}-Methylthiomethyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (2)**: A slurry of compound **1** (3.20 g, 20.9 mmol) and anhydrous sodium carbonate (2.22 g, 20.9 mmol) in 40 mL of dry THF was cooled with an ice-water bath, to which 2.28 mL (27.2 mmol) of chloromethyl methylsulfide was slowly added via a syringe, followed by 15 mL of DMF. After 5 min, the bath was removed and a catalytic amount of KI (200 mg) was added. The reaction mixture was stirred under nitrogen overnight. Then it was diluted with 200 mL of ether and washed with cold water. The separated aqueous layer was again extracted with ether. The combined ether solution was washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum. The residue was chromatographed on silica gel. Elution with 1:4 ethyl acetate/hexane provided 1.80 g (45%) of **2** as a colorless oil, which solidified when kept inside a refrigerator. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.09 (s, 2H), 3.44 (m, 4H), 2.15 (s, 3H), 1.83 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 78.06, 50.94, 23.02, 15.61; UV λ_{max} (MeOH, ε): 255 nm (10.0 mM^{-1} cm^{-1}); MS (ESI) 214 ($M+Na^+$), 405 ($2M+Na^+$); HRMS (EI) calcd for $C_6H_{13}N_3O_2S$ (M^+) 191.0728, found 191.0727.

***O*^{2}-Chloromethyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (3)**: A solution of compound **2** (91 mg, 0.48 mmol) in 10 mL of dry CH_2Cl_2 was cooled with an ice-water bath, to which 0.48 mL of sulfuryl chloride (1.0 M in CH_2Cl_2) was added dropwise. After 10 min, the ice-water bath was removed, and the mixture was stirred for another 30 min. Evaporation under vacuum gave **3** as a yellow oil, which was immediately used in the following reactions without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.80 (s, 2H), 3.60 (t, 4H), 1.95 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 79.86, 50.86, 23.32.

General procedure for the synthesis of peptide-diazeniumdiolate prodrugs: To a DMF solution of freshly prepared compound **3** was added amino acid or peptide (one equivalent) in one portion, followed by the addi-

tion of cesium carbonate (one equivalent). The mixture was stirred at room temperature until the disappearance of starting material as monitored by TLC. Then the mixture was diluted with water and extracted with CH_2Cl_2. The separated organic phase was combined, washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum to give the crude product, which was further purified by flash column chromatography.

***O*²-(*N*^α^-Acetyl L-alanine)methyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (4)**: Prepared from N^{α}-acetyl alanine and collected as a colorless oil (60%). ^{1}H NMR ($CDCl_3$, 400 MHz): 6.45 (d, 1H, $J=6.4$ Hz), 5.78 (d, 1H, $J=6.4$ Hz), 5.67 (d, 1H, $d=6.4$ Hz), 4.55 (m, 1H), 3.52 (m, 4H), 1.95 (s, 3H), 1.91 (m, 4H), 1.34 (d, 3H, $J=6.8$ Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.79, 169.78, 87.63, 50.48, 47.82, 28.81, 17.81; UV λ_{max} (MeOH, ε): 253 nm (6.5 mM^{-1} cm^{-1}); MS (ESI) found 297 ($M+Na^+$), 313 ($M+K^+$).

***O*²-(*N*^α^-Acetyl L-phenylalanine)methyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (5)**: Prepared from N^{α}-acetyl phenylalanine and collected as colorless needles (60%). Mp 91–92°C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.30–7.70 (m, 3H), 7.08 (d, 2H, $J=8.0$ Hz), 5.96 (d, 1H, $J=7.5$ Hz), 5.87 (d, 1H, $J=7.0$ Hz), 5.70 (d, 1H, $d=7.0$ Hz), 4.92 (m, 1H), 3.56 (m, 4H), 3.16 (dd, 1H, $J=14.0$, 6.0 Hz), 3.11 (dd, 1H, $J=14.0$, 5.5 Hz), 1.96 (m, 7H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.41, 169.62, 135.39, 129.26, 128.56, 127.11, 97.90, 52.84, 50.58, 37.33, 23.00, 22.93; UV λ_{max} (MeOH, ε): 252 nm (8.4 mM^{-1} cm^{-1}); MS (ESI) found 373 ($M+Na^+$), 723 ($2M+Na^+$); HRMS (DCI) calcd for $C_{16}H_{23}N_4O_5$ ($M+H^+$) 351.1668, found 351.1655.

***O*²-(*N*^α^-Acetyl L-tyrosine)methyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (6)**: Prepared from N^{α}-acetyl tyrosine and collected as a yellow solid (54%). Mp 97–102°C; ^{1}H NMR (CD_3OD, 500 MHz): δ 6.99 (d, 2H, $J=8.5$ Hz), 6.67 (d, 2H, $J=8.5$ Hz), 5.83 (d, 1H, $J=7.0$ Hz), 5.68 (d, 1H, $J=7.0$ Hz), 5.68 (d, 1H, $d=7.5$ Hz), 4.62 (m, 1H), 3.53 (m, 4H), 3.03 (dd, 1H, $J=14.5$, 6.0 Hz), 2.87 (dd, 1H, $J=14.5$, 6.0 Hz), 1.96 (m, 4H), 1.91 (s, 3H); ^{13}C NMR (CD_3OD, 125 MHz): δ 171.97, 170.74, 156.28, 130.07, 127.11, 115.04, 87.55, 54.30, 50.54, 36.25, 22.67, 21.01; UV λ_{max} (MeOH, ε): 253 nm (7.5 mM^{-1} cm^{-1}); MS (ESI) found 389 ($M+Na^+$), 755 ($2M+Na^+$); HRMS (FAB) calcd for $C_{16}H_{23}N_4O_6$ ($M+H^+$) 367.1618, found 367.1634.

***O*²-(*N*^α^-Acetyl L-serine-L-serine-L-tyrosine-L-tyrosine)-methyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (7)**: Prepared from the peptide Ac-SSYY-OH (71%). ^{1}H NMR (CD_3OD, 500 MHz): δ 7.00 (m, 4H), 6.67 (m, 4H), 5.77 (d, 1H, $J=7.5$ Hz), 5.69 (d, 1H, $J=7.0$ Hz), 4.60 (m, 1H), 4.51 (m, 1H), 4.44 (t, 1H, $J=6.0$ Hz), 4.40 (t, 1H, $J=5.5$ Hz), 3.83–3.68 (m, 4H), 3.50 (m, 4H), 3.04–2.89 (m, 4H), 2.01 (s, 3H), 1.93 (m, 4H); UV λ_{max} (MeOH, ε): 276 nm (4.3 mM^{-1} cm^{-1}); MS (ESI) found 726 ($M+Na^+$); HRMS (FAB) calcd for $C_{31}H_{41}N_7O_{12}Na$ ($M+Na^+$) 726.2711, found 726.2715.

***O*²-(*N*^α^-Acetyl L-serine-L-serine-L-phenylalanine-L-tyrosine)methyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (8)**: Prepared from the peptide Ac-SSFY-OH (41%). ^{1}H NMR (CD_3OD, 500 MHz): δ 7.26–7.16 (m, 5H), 7.00 (m, 2H), 6.69 (m, 2H), 5.78 (d, 1H, $J=7.5$ Hz), 5.69 (d, 1H, $J=7.0$ Hz), 4.60 (m, 2H), 4.42 (t, 1H, $J=6.0$ Hz), 4.36 (t, 1H, $J=5.5$ Hz), 3.80–3.67 (m, 4H), 3.50 (m, 4H), 3.12–2.83 (m, 4H), 2.01 (s, 3H), 1.92 (m, 4H); partial ^{13}C NMR (CD_3OD, 125 MHz): δ 130.21, 129.18, 129.11, 128.27, 127.05, 126.57, 115.12, 87.60, 61.82, 61.42, 55.90, 55.60, 54.97, 54.42, 50.50, 37.38, 36.24, 22.65, 21.34; UV λ_{max} (MeOH, ε): 254 nm (5.0 mM^{-1} cm^{-1}); MS (ESI) found 710 ($M+Na^+$).

***O*²-(*N*^α^-Acetyl L-glycine-L-isoleucine-L-serine-L-serine-L-phenylalanine-L-tyrosine)methyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (9)**: Prepared from the peptide Ac-GISSFY-OH (45%). ^{1}H NMR (CD_3OD, 500 MHz): δ 7.23–7.17 (m, 5H), 7.00 (m, 2H), 6.67 (m, 2H), 5.78 (d, 1H, $J=7.5$ Hz), 5.69 (d, 1H, $J=7.0$ Hz), 4.60 (m, 2H), 4.38 (t, 1H, $J=6.0$ Hz), 4.22 (m, 1H), 3.72–3.60 (m, 4H), 3.50 (m, 4H), 3.12–2.85 (m, 4H), 2.00 (s, 3H), 1.92 (m, 4H), 1.17 (m, 3H), 0.90 (m, 6H); partial ^{13}C NMR (CD_3OD, 125 MHz): δ 174.00, 173.65, 173.21, 171.91, 171.43, 157.42, 138.39, 131.37, 130.31, 129.45, 128.25, 127.73, 116.30, 88.78, 62.95, 59.54, 56.69, 56.00, 55.63, 51.69, 43.67, 38.59, 38.04, 37.40, 25.93, 23.83, 22.41, 16.99, 11.60; UV λ_{max} (MeOH, ε): 252 nm (7.1 mM^{-1} cm^{-1}); MS (ESI) found 880 ($M+Na^+$).

Acknowledgements

This work was supported by grants from the National Institutes of Health (GM 54074).

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