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Efficient Covalent Capture of 8-Nitroguanosine *via* a Multiple Hydrogen-Bonded Complex

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Supporting Information

ABSTRACT: A novel 1,3-diazaphenoxazine nucleoside derivative (nitroG-Grasp) bearing a thiol group with a urea linker forms multiple hydrogen-bonded complexes with 8-nitroguanosine and efficiently displaces the nitro group; thus, it is the first molecule that can covalently capture 8-nitroguanosine.

Reactive oxygen species (ROS) and reactive nitrogen species (RNOS) are representative chemical sources of oxidative stress for cells and cause nucleic acid damage, such as 8-oxoguanine and 8-nitroguanine derivatives.[1−3] They are highly mutagenic and are regarded as biomarkers of oxidative stress.[4] 8-Nitroguanosine (8-nitroG) is generated frequently in inflammatory or infected tissues.[5] Despite its conventional classification as a genotoxic material, 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) has recently attracted significant interest due to its unique biological properties.[6−9] For instance, 8-nitro-cGMP is produced in response to iNOS activity and reacts with sulfhydryl groups of specific proteins (protein *S*-guanylation).[10] 8-Nitro-cGMP also reacts with highly nucleophilic H_2S/HS^- to form 8-SH-cGMP,[11] but its reactivity is low for glutathione, an abundant thiol in cells.[10] These findings have led to a proposal for a new metabolic cycle or signaling pathway mediated by 8-nitro-cGMP.[10,12] Thus, selective molecules for 8-nitroG derivatives are needed for further understanding their biological functions. However, except for antibodies,[13] there is no recognition compound for 8-nitroguanosine derivatives. This paper reports for the first time new 1,3-diazaphenoxazine nucleoside derivatives bearing a thiol arm that exhibits efficient covalent capture of 8-nitroguanosine *via* a multiple hydrogen-bonded complex.

Our early studies demonstrated that oxoG-clamp (**1**) is a selective fluorescent binder of 8-oxo-dG and that a carbamate group of **1** is responsible for the selective recognition of 8-oxo-dG (Figure 1A, K_s: 8-oxo-dG/dG = 10).[14−16] ^{1}H NMR and the structure-binding relationship of **1** have indicated that the complex is formed through multiple hydrogen bonds as shown in Figure 1.[14,17] Compound **2** became selective for dG because of the substitution of a urea for the carbamate in **1** (Figure 1B, R = H, K_s: 8-oxo-dG/dG = 0.04).[17] A preliminary investigation revealed that 8-nitroG formed more stable complexes with **2** than with **1** (K_s in $CHCl_3$, **1**: 0.9×10^6 M^{-1} vs **2**: 3.6×10^6 M^{-1} (Figure S1). Thus, we designed new recognition molecules for 8-nitroG such that the thiol group attached to a urea linker could reach the 8-position to displace the nitro group (**3a−c**). The compound was named nitroG-grasp based on its reaction scheme, in which access of the thiol group is possible due to the fixed conformation of the urea-alkyl linker in the multiple hydrogen-bonded complex through which the compound undergoes *S*-guanylation (Figure 2).

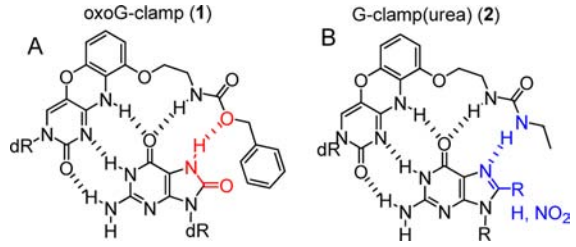

Figure 1. Selective complex formation with 8-Oxo-dG (**A**) and G or 8-nitroG (**B**), in which single hydrogen bonding is responsible for selectivity.

In this study, 8-nitro-2′,3′,5′-tri-*O*-acetylguanosine (triAc-8-nitroG) was used as a target molecule and synthesized according to previous literature.[8] The nitroG-Grasp derivatives (**3a−c**) were synthesized through a reaction between the amino group of the 3′,5′-*O*-diTBDMS-G-clamp unit as a common intermediate and the *S*-trityl protected mercaptoalkylamido-

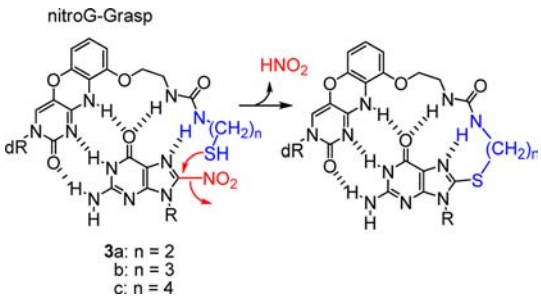

Figure 2. Design of a new reactive molecule (NitroG-Grasp) for the covalent capture of 8-NitroG.

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imidazole, followed by the deprotection of the *S*-trityl group by TFA in the presence of triethylsilane (Scheme S1). As a control compound, the carbamate derivative **4** was synthesized using the 3′,5′-*O*-diTBDMS-G-clamp unit and *S*-trityl-protected mercaptopropyloxycarbonylimidazole. The control compound **4** lacks the hydrogen-bonding site for the N^7 atom of nitroguanosine.

The reaction of the nitroG-Grasp derivatives with triAc-8-nitroG was performed in MeCN and monitored by HPLC. An equimolar mixture of C3-nitroG-Grasp (**3b**) and triAc-8-nitroG formed an adduct quantitatively as a single product within 20 min at 30 °C in MeCN containing Et_3N (Figure 3). This

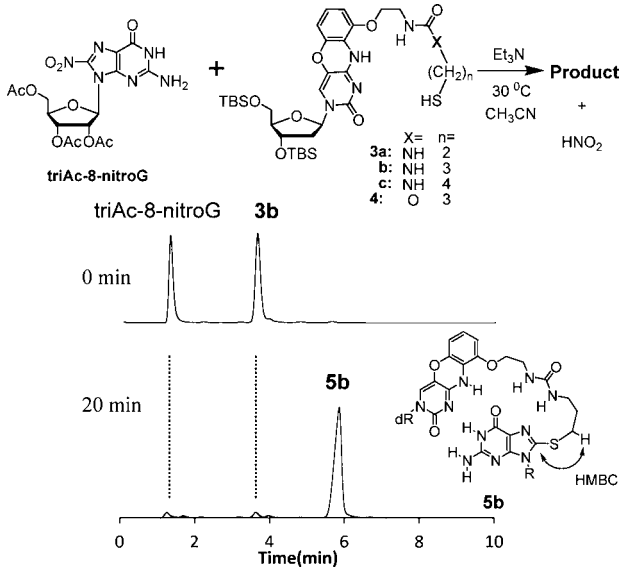

Figure 3. Reaction of C3-nitroG-Grasp (**3b**) with triAc-8-nitroG monitored by HPLC and the structure of the guanylated-product (**5b**). The reaction was performed using 1 mM each of **3b** and 8-nitroG in the presence of 10 mM Et_3N in CH_3CN at 30 °C. HPLC conditions: column: Xbridge C8 3.5 μm, 3.0 mm × 100 mm; solvents: (A) 0.1 M TEAA buffer and (B) CH_3CN, A/B = 20:80; flow rate: 0.5 mL/min; monitored by UV at 254 nm.

product was isolated, and its structure was confirmed to be that depicted in **5b** by ^{1}H NMR, ESI-MS, and HMBC. The chemical shifts of the methylene protons adjacent to the sulfur atom were shifted from 2.5 to 3.2 ppm (Supporting Information). The HMBC spectrum revealed the correlation between the 8-carbon atom of the 8-nitroguanine unit and the methylene protons next to the sulfur atom, as depicted in **5b** in Figure 3. The released HNO_2 was confirmed by the formation of the azo dye compound using Bratton−Marshall reagents (Figure S2).[18]

The time courses of the reaction between the nitroG-clamp derivatives (**3a−c**, **4**) and triAc-8-nitroG are compared in Figure 4. C3-nitroG-Grasp (**3b**) exhibited the most efficient reactivity; moderate reactivity was observed with C2-nitroG-Grasp (**3a**), and relatively slow reactions were observed with C4-nitroG-Grasp (**3c**). The reaction by **3b** proceeded efficiently at concentrations as low as 1 μM (Figure S3). In contrast, the control compound **4**, which lacks a hydrogen-bonding site for the N^7 of 8-nitroguanine due to its carbamate linker, exhibited much slower reactivity (Figure 4). *N*-Cbz-aminopropanethiol ($ZNH(CH_2)_3SH$), used as a control without a binding site for 8-nitroguanosine, did not yield any

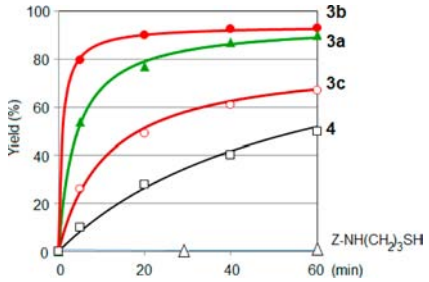

Figure 4. Comparison of the time courses of the reactions with nitroG-Grasp **3a−c** or the control compounds (**4** and *Z*-NH-$(CH_2)_3$SH). Product yields were obtained at the indicated time points by HPLC analysis, as described in the footnote to Figure 3.

adduct under the same conditions. The guanylation of **3b** occurred in aqueous media (CH_3CN−H_2O) buffered with TEA and AcOH at pH 7.0, which was not largely inhibited by glutathione (Figure S4). These results exemplify the contribution of the complexation of **3b** for efficient reactivity.

To clarify the origin of the differences in reactivity observed for **3a−c**, we next evaluated the reaction kinetics by monitoring the reaction by UV−vis spectroscopy. The absorbance at 368 nm due to the 8-nitroguanine base[19] gradually decreased with time (Figure 5). Simultaneously, the absorbance at 260 nm due

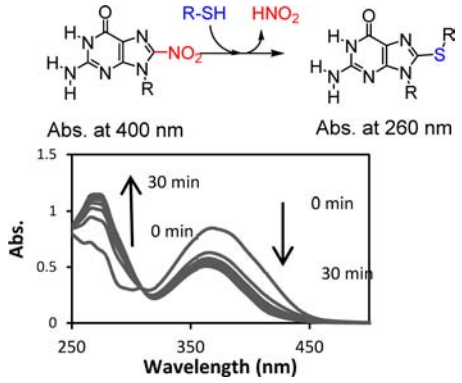

Figure 5. UV−vis spectral changes due to the desorption of the nitro group of triAc-8-nitroG. Example spectra were measured at the indicated time intervals using **3b** (50 μM) and triAc-8-nitroG (50 μM) in CH_3CN containing Et_3N (500 μM).

to the 8-thioguanine base[20] increased with time. These changes agree with the time course obtained by HPLC (Figure 4). Thus, the absorbance at 400 nm was monitored in the presence of excess nitroG-Grasp derivatives to obtain the kinetic parameters of the pseudo-first-order reaction. The obtained k_{obs} values were in the order **3b** (0.0336 s^{-1}) > **3a** (0.0256 s^{-1}) > **3c** (0.0064 s^{-1}) > **4** (0.0008 s^{-1}), agreeing with the results determined using HPLC (Figure 4A).

The k_{obs} values increased proportionally with the concentrations of the nitroG-Grasp derivatives without exhibiting saturation phenomena (Figure S5), suggesting that the formation of the hydrogen-bonded complex is not a rate-determining step under these conditions. The reaction was performed at different temperatures, and kinetic parameters were obtained by Arrhenius plots (Table 1, Figure S6). The reaction of **3b** exhibited the lowest values of $\Delta G^{‡}$ and $\Delta H^{‡}$. Although its $\Delta S^{‡}$ term was in the average range, the highly favorable $\Delta H^{‡}$ term produced the lowest $\Delta G^{‡}$ value. Interestingly, the $\Delta H^{‡}$ value of **3c** is relatively low, but the

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Table 1. Kinetic Parameters for the Guanylation Reaction[a]

R−SH	k_{obs} (s^{-1})[b]	E_a (kJ/mol)	$\Delta G^{\ddagger}$ (kJ)	$\Delta H^{\ddagger}$ (kJ)	$\Delta S^{\ddagger}$ (J/K)
3a	0.0256	21.6	85.5	19.1	−224
3b	0.0336	16.2	81.8	13.7	−231
3c	0.0064	17.8	87.3	15.4	−244
4	0.0008	12.0	91.4	9.5	−272

[a]The pseudo-first-order rate constants (k_{obs}) were obtained by monitoring the absorbance at 400 nm using R−SH (200 μM) and triAc-8-nitroG (10 μM). [b]The rate constants at 30 °C.

unfavorable $\Delta S^{\ddagger}$ term leads to a high $\Delta G^{\ddagger}$ value. In addition, although the $\Delta H^{\ddagger}$ value of **4** is the lowest, it possessed the most unfavorable $\Delta S^{\ddagger}$ term, making its $\Delta G^{\ddagger}$ value the highest.

As the intrinsic nucleophilicity of thiolate is several orders of magnitude greater than that of the corresponding thiol, the $\Delta H^{\ddagger}$ values of **3a−c** may reflect the ionization of their thiol group. The pK_a values of the thiol of **3a−c** and **4** were simulated using quantum mechanical calculations and plotted against the corresponding $\Delta H^{\ddagger}$ values. A clear linear relationship was obtained, as depicted in Figure 6A, suggesting that the

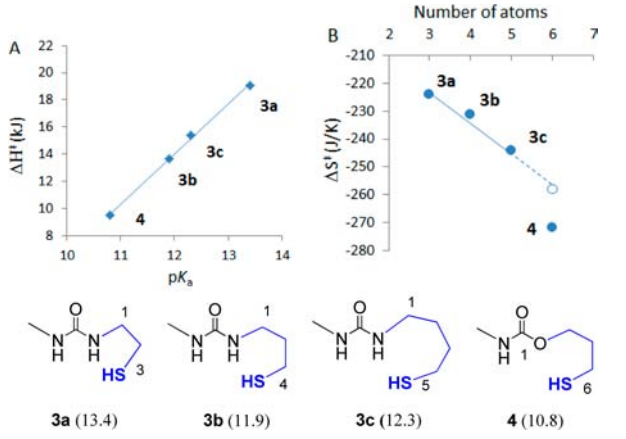

Figure 6. (A) Plot of $\Delta H^{\ddagger}$ against the calculated pK_a. (B) Plot of $\Delta S^{\ddagger}$ against the number of atoms between the NH and SH groups. (C) The numerals in parentheses represent the pK_a values predicted by the quantum mechanical calculation (pK_a calculation protocol installed in Jaguar) for the partial structure of the thiol units corresponding to **3a−c** and **4**. The numerals marked on the structures represent the number of atoms from the NH to the SH group.

$\Delta H^{\ddagger}$ values include the process of thiolate formation.[21] Acceleration by TEA also supports that the thiolate is a nucleophile (Figure S7). As **4** showed the lowest reactivity despite the lowest $\Delta H^{\ddagger}$ value, the $\Delta H^{\ddagger}$ value is not the determinant of the selectivity. In other words, the selectivity of **3b** is not due to the acidity of the thiol. When the $\Delta S^{\ddagger}$ values were plotted against the number of atoms from the NH to the SH group, a linear relationship was obtained for **3a−c** (Figure 6B). Assuming that the two NH groups of the urea unit have limited flexibility in the hydrogen-bonded complex in the transition state, as shown in Figure 2, it is reasonable that the $\Delta S^{\ddagger}$ value reflects the freedom of the *N*-alkyl chain. The $\Delta S^{\ddagger}$ value of **4** with the carbamate linker was more unfavorable (closed circle) than that expected based on the number of linker atoms (open circle), as depicted in Figure 6B. It is likely that the strength of one hydrogen bond is insufficient, increasing the flexibility of the entire linker, including the NH group. In other words, the thiol group of the carbamate linker of **4** has the highest reactivity in terms of its nucleophilicity, but its displacement reactivity is diminished because of its limited access to the reactive site. Thus, we concluded that the highest displacement reactivity of **3b** toward 8-nitroG is due to both the limited flexibility of the urea linker in the multiple hydrogen-bonded complex and the relatively facile formation of the thiolate.

In this study, we designed new thiol-containing recognition molecules **3a−c** for the covalent capture of 8-nitroguanosine based on the G-clamp skeleton by introducing thioalkylurea linker units of varying length. Compounds **3a−c** efficiently displaced the nitro group of 8-nitroG to form a sulfide bond with the guanine residue (guanylation). Among them, **3b** exhibited the most efficient guanylation even at low concentrations, supporting the selective complex formation for efficient reactivity for 8-nitroG. In the control experiments, $ZNH(CH_2)_3SH$ did not show reactivity; **4** with the carbamate linker displayed only low reactivity. The design principles, in which binding selectivity can be controlled by one of multiple hydrogen bonds and efficient reactivity can be achieved due to the close proximity in the complex, have been validated through the efficient reactivity of **3b**, and may be applied to develop recognition molecules for other 8-substituted guanine derivatives such as 8-chloro- or 8-thioguanosine, etc. Because interest in 8-nitroguanosine derivatives is increasing due to their biological roles in signal transduction pathways, the nitroG-Grasp molecule is expected to be a potential platform structure to develop specific molecules for 8-nitroG. In our group, systematic studies are now ongoing to develop nitroG-Grasp derivatives conjugated with a variety of phosphates binding moiety,[22] aiming to attain selective affinity to 8-nitro-cGMP in aqueous media, which will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details include the synthesis, HNO_2 detection, NMR spectra of the nitroG-Grasp derivatives, reaction monitoring, and kinetic evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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