8-Nitroguanosines as chemical probes of the protein *S*-guanylation†

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Azido- and fluoro- derivatives of 8-nitroguanosine were developed, and will contribute to the exploration of protein *S*-guanylation by endogenous nitrated nucleosides.

Nitric oxide (NO) has diverse physiological functions, such as vascular and neuronal signal transduction, host defense, and cell death regulation. NO activates soluble guanylate cyclase (sGC), which leads to the production of guanosine 3′,5′-cyclic monophosphate (cGMP).[1] Moreover, reactive nitrogen species are generated from nitric oxide (NO), and work as potent nitrating agents for proteins, nucleic acids, and unsaturated membrane lipids.[2–4]

We recently identified 8-nitro-cGMP **1**, the first endogenous cGMP derivative, in various cell lines.[5] This compound mediates a novel post-translational modification by reacting with proteinous thiol in cells (Fig. 1). A specific antibody that recognizes the SH modifications was prepared, and has already been used for detection of the "protein *S*-guanylation" in cells.[5] Our previous efforts to uncover the role of the *S*-guanylations, led to the identification of Keap1 (Kelch-like ECH-associated protein 1),[6] a redox sensor, as one of the target proteins of **1**.[5] Keap1 (*ca.* 70 kDa) includes 25 Cys residues, and senses oxidative stresses by their thiol modifications. Keap1 has also been shown to be cytoprotective *via* HO-1 (heme oxygenase 1) upregulation. These findings suggest that 8-nitro-cGMP **1** mediates a unique NO signaling pathway independent of cGMP.

Development of the 8-nitroguanosine based-probes should be a promising approach to answer further biological questions regarding *S*-guanylation at the molecular level. We describe here the synthesis, activity, and reactions of these chemical probes.

We initiated our study by exploring an efficient synthesis of 8-nitroguanosine **2**, which should constitute a basis for chemical studies of protein *S*-guanylation. Direct nitration of guanosine by peroxynitrate was reported to give a poor yield

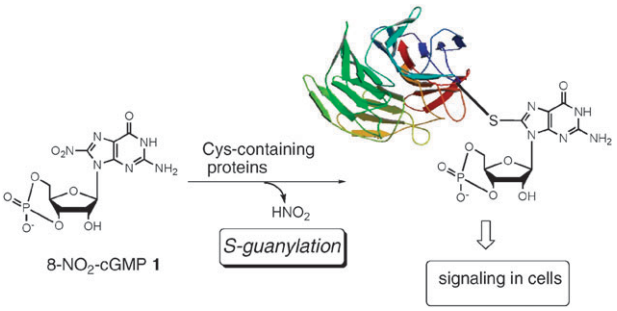
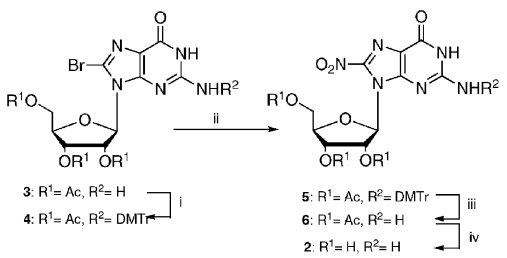


Fig. 1 The schematic reaction of an endogenous cGMP derivative **1** with proteinous thiols (*S*-guanylation), which plays important roles in NO-mediated signaling pathways.

(<<1% isolated yield),[7] and thus the substitution of 8-bromoguanosine by nitrite in DMSO has been used for preparative purposes.[8,9] A drawback of this protocol is that HPLC must be used to isolate the products from the high boiling-point solvent and from the unreacted starting material. The isolated yield of **2** remained moderate (10–20%),[8] which was in part due to the instability of **2** under the reaction conditions. And indeed, a nitration of the guanine moiety substantially destabilized the glycosyl bonds of guanosines, and the half lives at pH 7 and 37 °C were reported to be 5 h for 8-nitroguanosine, and < 3 min for the 2′-deoxy counterpart.[7]

We envisaged that use of proper protecting groups would minimize the undesired depurination of nitrated guanosines in polar solvents during the reactions and purifications. Thus, the 2-amino group of 8-bromo-2′,3′,5′-tri-*O*-acetyl-guanosine **3** was protected as a 4, 4′-dimethoxytrityl ether (DMTr) in 99% yield (Scheme 1). Nitration of **4** was then investigated under the conditions summarized in Table 1. Acetonitrile was first selected as solvent (entry 1), and the desired nitroguanosine **5** was obtained in 49% yield. The cleavage of the labile glycoside bond seemed to be slow under these conditions. *N*,*N*-Dimethylformamide was also investigated as a solvent



Scheme 1 *Reagents and conditions*: (i) DMTr-Cl, pyridine, room temp., 10 h (99%); (ii) see Table 1; (iii) *p*-TsOH, $CHCl_3$, room temp., 25 min (quant.); (iv) $MeNH_2$, MeOH, room temp., 12 h (83%).

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† Electronic supplementary information (ESI) available: Preparative procedures for compounds, stability experiments of compounds **7** and **8**, crystal structure of **7**, procedures for the reaction of **8** with cell lysate, and for detection of heme oxygenase-1 induction by compounds **1**, **2**, **7** and **8** in cells. CCDC 692937. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/b810771h

Table 1 Nitration of 8-bromoguanosine derivative **4**

Entry	Conditions[a]	Solvent	Reaction period/h	Yield (%)	Recovery (%)
1	A	CH_3CN	72	49	—
2	B	DMF	6	60	23
3	B	DMF	7	44	10
4	B	DMF	10	34	3
5	B	DMF	25	27	3

[a] Condition A: KNO_2 (6.5 eq.), 18-crown-6 (6.5 eq.), reflux; condition B: KNO_2 (10 eq.), 18-crown-6 (10 eq.), 100 °C.

with varied reaction periods (entries 2–5). In this case, the optimum isolated yield (60%, 78% based on recovered starting material) was obtained for the reaction period of 6 h (entry 2). Extension of the reaction period reduced the yield of **5** and the recovery of **4** (entries 3–5). The DMTr ether was cleaved by exposure of **5** to *p*-toluenesulfonic acid in chloroform (quant.). Triacetates of **6** were then removed cleanly by a methanolic methylamine solution at room temperature to give crude **2**. Since the only impurity was a trace amount of *N*-methylacetamide, the obtained **2** could be used for most purposes. The crude **2** was purified by ODS chromatography at 4 °C (83%, the overall yield was 49% from **3** in 4 steps). The described synthesis avoided the use of HPLC for purification and would be easily scaleable to obtain larger amounts of the sample for biological studies.

Considering the instability of **2** in water (half life: 5 h), we next explored novel analogues suitable for biochemical research. Similar synthetic schemes (Fig. S1 and S2 of ESI) were applied for 2′-deoxy-2′-fluoro-8-nitroguanosine **7** and 5′-azido-5′-deoxy-8-nitroguanosine **8** (Fig. 2). The half-lives of these analogues in 0.05 M sodium phosphate buffer were examined (pH 7.4, 37 °C). The half-lives were 72 d for **7** and 4.5 d for **8** (Fig. S3 and S4†). The stability of these analogues is an important advantage for their use as chemical probes in biological studies. Moreover, this characteristic allowed X-ray crystallographic analysis of the stabilized 2′-F-derivative **7**. A single-crystal of **7** was obtained from its water–ethyl acetate solution.

Because this is the first crystallographic experiment conducted among 8-nitroguanosine derivatives, important features of the obtained structure should be mentioned here (Fig. 3). (i) The bond length of the glycosidic C–N bond (1.523 Å) was remarkably longer than those of guanosine (1.454 Å)[10] and 8-Br-guanosine (1.473 Å).[11] These results would reflect a significant destabilization of the glycosidic bond by an electron-withdrawing nitro group at the adjacent carbon. (ii) The oxygen atoms of the nitro group were involved in a hydrogen-bonding with NH_2 of another molecule in a crystal lattice (Fig. S5 of ESI). This fact might suggest the

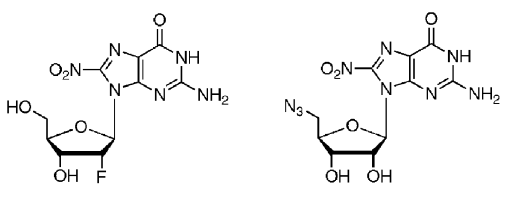


Fig. 2 Fluoro- and azido-analogues of 8-nitroguanosine.

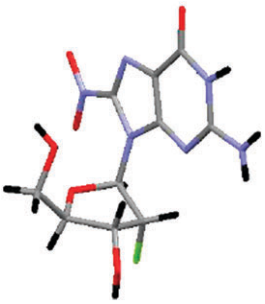


Fig. 3 Molecular structure of **7** in crystal. Atom labels: hydrogen = black, carbon = gray, nitrogen = purple, fluorine = green.

possibility of novel base-pair recognition patterns of nitroguanosines, although the guanine nitrations in nucleic acids have not been fully uncovered in cells.

Having synthesized 8-nitroguanosines, we then turned our attention to the biological activities of these compounds. Induction of heme oxygenase-1 (HO-1) expression has been associated with adaptive cytoprotection against a wide array of cellular stresses such as nutrient starvation or hypoxia. It has been suggested that carbon monoxide (CO), one of the products of the HO-1 reaction, may play a role in this protective activity.[12] Our previous study[5] suggested that *S*-guanylation of the sensor protein Keap1 by endogenous 8-nitro-cGMP **1** upregulates HO-1 through a signaling pathway.[13] An important question here is whether the cGMP substructure of **1** is indispensable for the upregulation.

Indeed, 8-nitroguanosine **2** and the 2′-fluoro analogue **7**, which lack cyclic phosphate moieties could enhance the induction of HO-1 in HepG2 cells, and protected the cells from glucose deprivation-induced cytotoxicity[14] (Fig. 4). The 5′-azido analogue **8** also exhibited upregulation of HO-1 (Fig. S7 of ESI†). A more detailed interpretation of these results will have to wait until both the full biological potential of nitroguanosines and an complete structure-activity relationships have been clarified. However, these findings do reveal that, in principle, 8-nitroguanosine derivatives, which lack a cyclic phosphate are useful as chemical probes for study of the protein *S*-guanylations.

So far, only Keap1 has been identified to be a physiological target protein of *S*-guanylation.[5] However, there may be other important sensors or enzymes that are regulated by the

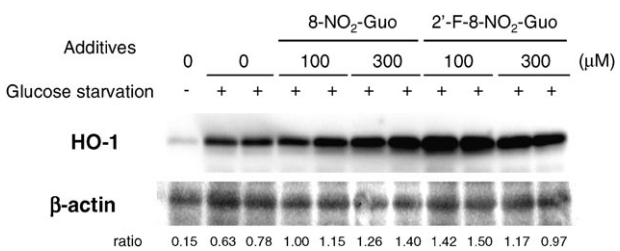
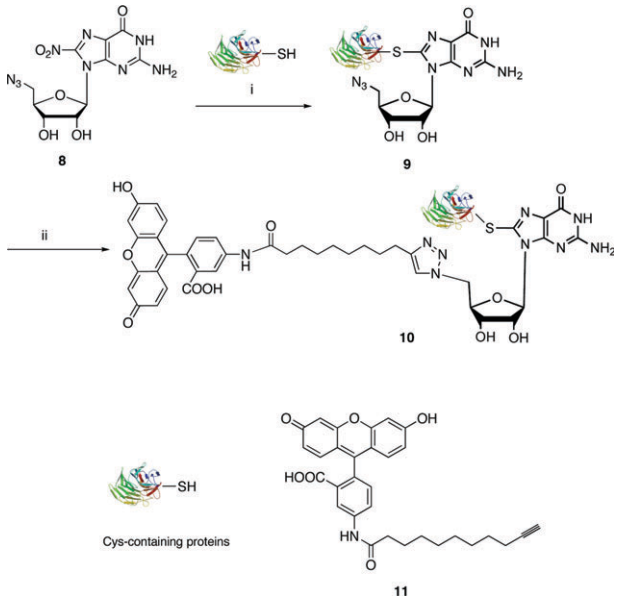


Fig. 4 Heme oxygenase-1 induction by 8-nitroguanosine **2** and its fluorinated derivative **7**: HepG2 cells were treated with indicated concentrations of **2** or **7** under glucose-starved condition for 8 h, and then were analyzed by Western blotting. As a control, expression of β-actin is shown. The ratio represents relative intensity of HO-1 and β-actin.



Scheme 2 Reaction of **8** with a cell lysate, and fluorescent labeling by Huisgen reaction: *Reagents and conditions*: (i) cell lysate (C6 cell), 0.6 M Na_2HPO_4aq., room temp., 5 h; (ii) CuBr, **11**, tris[(1-benzyl-1*H*-1,2,3-triazole-4-yl)methyl]amine, 0.5 M sodium phosphate buffer, pH 8, room temp., overnight.

guanylation. In this context, fluorecent labelings by 8-nitroguanosine-based probes could become powerful tools for the rapid and comprehensive identification of potential target proteins. Thus, we investigated the potential of the azide derivative **8** as a labeling probe. Treatment of **8** with glutathione (GSH: data not shown) or bovine serum albumin (BSA: data not shown) were first examined to confirm its reactivity to thiols. Whole rat glioma C6 cell lysate was then treated with compound **8** (Scheme 2). The progress of the reaction could be monitored by the release of NO_2^- ion by the Griess method.[15]

The *S*-guanylated proteins **9** in the lysate were then labeled by a fluorescent tag *via* a Huisgen reaction.[16] Fluorescent scanning of a SDS-PAGE gel (Fig. 5) confirmed the presence of several labeled proteins. We are currently investigating the guanylated proteins in a series of cell lines under defined culture conditions using the protocols introduced above. Enzymatic digestion and sequence analysis of the labeled bands is also in progress. The results should provide an insight into the possible physiological targets of protein *S*-guanylation, and the results will be described elsewhere.

In conclusion, 8-nitroguanosine and its stable analogues were synthesized. The cyclic phosphate moiety of 8-nitro-cGMP was proven unnecessary for cytoprotective HO-1 up-regulation, because these analogues also induced the HO-1 in cells. Successful fluorescent labeling of the cell lysate was also described. Thus, the derivatives could become useful chemical probes for biological or proteomic investigations into protein-*S*-guanylations.

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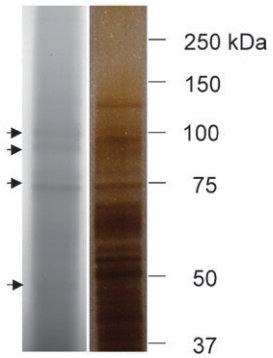

Fig. 5 Fluorescence scan (left) and silver stain (right) of the SDS-PAGE gel of the labeled C6 cell lysate **10** (Scheme 2). The fluorescent labeled bands are indicated by arrows.

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