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Synthesis of the Pyridine Hydrazones as Metal-free Artificial Nucleases

Zhifen Li, Jun Qiao, Zhifang Jia, and Shuangming Meng*
School of Chemical and Environmental Engineering, Datong University, Xingyun Street, Shanxi 037009, P. R. China

(E-mail: 200931150019@mail.bnu.edu.cn)

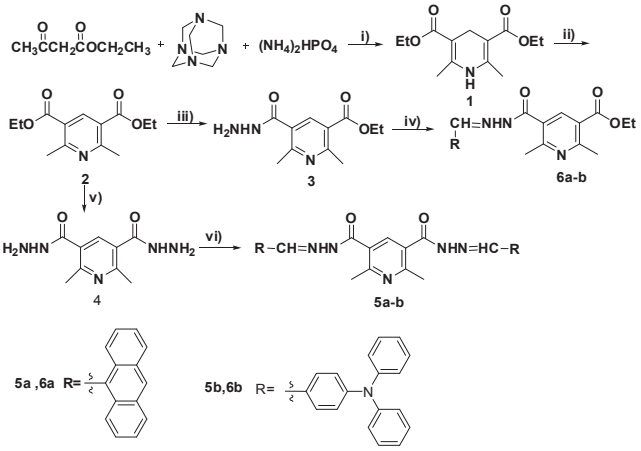
In this report, four pyridine hydrazones containing anthracene and triphenylamine as a new type of metal-free nucleases were synthesized. Results indicate that the conjugates can cleave the plasmid DNA to Form II or Form III under physiological conditions via hydrolytic pathway.

Intensive research has been focused on the development of artificial nucleases. These studies are beneficial for shedding light on cellular and molecular biology and have been applied in gene therapeutic agents.[1–7] With the consideration that many natural nucleases have been constructed with amino acids and two or more metal ions in the active sites, many studies have been devoted to mimic the active sites of natural nucleases.[8–10] So most of cleaving agents have been focused on the metal complexes, including hydrazone complexes,[11–13] which need to be triggered to induce oxidative DNA cleavage by a reducing agent.[14–16] Considering the metal lability and toxicity of metal complexes in the potential clinical application, the absence of metal ions in the cleavage reagents were considered safer for therapy.[17–20] Amino acids or various peptide derivatives were first considered as metal-free artificial nucleases.[21–23] Recently, other metal-free artificial nucleases have also attracted significant interest; guanidinium derivatives,[24,25] macrocyclic polyamines,[26–30] cyclodextrin derivatives,[31] and hydroxylamine[32] have been used as metal-free DNA nucleases, but hydrazones are rarely reported as metal-free artificial nuclease. Here, we synthesized the pyridine hydrazones containing anthracene and triphenylamine and reported the hydrolytic cleavage of supercoiled plasmid DNA by these compounds.

In this study, we combined 2,6-dimethylpyridine with 9-anthracenecarboxaldehyde and 4-(diphenylamino)benzaldehyde together through the –CONHN=CH– linkage and investigated their feasibility in the DNA cleavage.

Firstly, ethyl acetoacetate was reacted with hexamethylenetetramine and ammonium phosphate in ethanol and water at 80 °C to afford the desired product **1**, which was then oxidized with ammonium persulfate in acetone and water at room temperature for 3 h to give **2** (Scheme 1).[33] The monocarbohydrazide **3** and dicarbohydrazide **4** were obtained by the reaction between **2** and hydrazine hydrate in different solvents.[34] **4** and 9-anthracenecarboxaldehyde or 4-(diphenylamino)benzaldehyde were mixed in ethanol and water to obtain **5a** and **5b**. **3** and 9-anthracenecarboxaldehyde or 4-(diphenylamino)benzaldehyde were mixed in ethanol and water to obtain **6a** and **6b**. The yield of each step is acceptable. The detailed data were listed in the Supporting Information.

The DNA catalytic activities of **5a**, **5b**, **6a**, and **6b** on the cleavage of DNA were studied using supercoiled pUC18 DNA plasmid DNA as a substrate in HEPES (pH 7.2, 37 °C). Agarose gel electrophoresis was used to assess the conversion of supercoiled plasmid DNA (Form I) to nicked circular DNA



Scheme 1. i) C_2H_5OH, H_2O, 80 °C; ii) ammonium persulfate, CH_3COCH_3, H_2O; iii) $NH_2NH_2{\cdot}H_2O$, DMSO; iv) 9-anthracenecarboxaldehyde or 4-(diphenylamino)benzaldehyde, H_2O and C_2H_5OH; v) $NH_2NH_2{\cdot}H_2O$, C_2H_5OH; vi) 9-anthracenecarboxaldehyde or 4-(diphenylamino)benzaldehyde, H_2O and C_2H_5OH.

Figure 1. Effect of **5a**, **5b**, **6a**, and **6b** (100–400 μM) on the cleavage reactions of pUC 18 DNA (9 μg mL^{-1}) in a HEPES buffer (20 mM, pH 7.2) at 37 °C for 6 h. (a) Agarose gel electrophoresis diagram: Lane 1, DNA control; Lane 2–5 **5a** (100, 200, 300, and 400 μM), Lane 6–9 **5b** (100, 200, 300, and 400 μM), Lane 10–13 **6a** (100, 200, 300, and 400 μM), Lane 14–17 **6b** (100, 200, 300, and 400 μM).[35]

(Form II) or the linear DNA (Form III) in the absence of any external agents or metal ions. Figure 1 shows the agarose gel of concentration-dependence assays, which indicates that the supercoiled DNA (Form I) relaxes to form a nicked circular DNA (Form II) in the presence of **5a**, **5b**, **6a**, and **6b** at different concentrations. The results indicated that the four conjugates cleaved the plasmid DNA with almost the same activities and the increase of concentration appeared to be insignificant. Thus, further investigation of the cleaving ability has been carried out through the increase of time. Figure 2 shows the agarose gel of supercoiled plasmid DNA cleavage promoted by **5a**, **5b**, **6a**, and **6b** in HEPES buffer (pH 7.2) at 37 °C for 12 h. It was obvious that all of the plasmid DNA was converted to nicked (Form II) and linear forms (Form III). Hind III (endonuclease) that was used to clarify the new band (Figure S3), the result clearly indicated that the new band produced by **6a** was Form II and Form III.

To verify the catalytic activity of DNA cleavage promoted by **5a**, **5b**, **6a**, and **6b**, several control experiments were carried

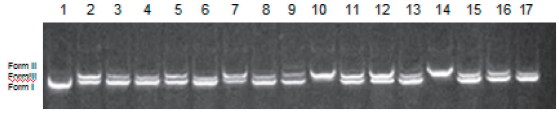


Figure 2. Effect of **5a**, **5b**, **6a**, and **6b** (100–400 μM) on the cleavage reactions of pUC 18 DNA (9 μg mL^{-1}) in a HEPES buffer (20 mM, pH 7.2) at 37 °C for 12 h. (a) Agarose gel electrophoresis diagram: Lane 1, DNA control; Lane 2–5 **5a** (100, 200, 300, and 400 μM), Lane 6–9 **5b** (100, 200, 300, and 400 μM), Lane 10–13 **6a** (100, 200, 300, and 400 μM), Lane 14–17 **6b** (100, 200, 300, and 400 μM).[36]

out. DNA cleavages in the presence of compounds **2**, **3**, and **4** were measured (Figure S4). It was found that no obvious DNA cleavage was observed at the concentration of 200 μM of **2**, **3**, and **4**. Thus the pyridine hydrazones result in new reactivity.

In order to avoid any effect of trace metal ions involved in DNA cleavage, a potent metal-chelating agent EDTA (1 mM) was introduced into the experiment (Figure S5). The results indicated that the conjugates could cleave DNA without the participation of any metals. To confirm the cleavage mechanism, reactive oxygen species (ROS) scavengers were added to DNA cleavage promoted by compound **6a**. As shown in Figure S6, the addition of hydroxyl radical scavengers (DMSO, *t*-BuOH), singlet oxygen scavenger (NaN_3), and superoxide scavenger (KI) did not inhibit the cleavage of DNA with compounds **6a**. The results excluded the involvement of cleavage by ROS and indicated that a hydrolytic mechanism is involved in the cleavage process with the hydrazones.

In conclusion, we designed and prepared four hydrazone derivatives, which were firstly studied as artificial nucleases, and the results demonstrated that efficient cleavage of DNA can be achieved at low concentrations and in short times. Further studies to explore specific DNA cleavage and to investigate the relationship between the structure and activity are in progress in our laboratory.

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Supporting Information is available electronically on J-STAGE.

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35 Quantitation of % plasmid relaxation (Form II%) relative to plasmid DNA per lane was showed in Figure S1.
36 Quantitation of % plasmid relaxation (Form II% and Form III%) relative to plasmid DNA per lane was showed in Figure S2.