Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 13 (2005) 1615–1622
Bioorganic & Medicinal Chemistry

Thio-heterocylic naphthalimides with aminoalkyl side chains: novel alternative tools for photodegradation of genomic DNA without impairment on bioactivities of proteins

Qing Yang,[a,*] Xuhong Qian,[b,*] Jianqiang Xu,[a] Yuanshe Sun[a] and Yonggang Li[b]

[a]*Department of Bioscience and Biotechnology and State Key Laboratory of Fine Chemistry, Dalian University of Technology, No 2 Lingong Road, Dalian 116024, PR China*

[b]*Shanghai Key Laboratory of Chemical Biology, Institute of Pesticides and Pharmaceuticals, East China University of Science and Technology, No 130 Meilong Road, Shanghai 200237, PR China*

Received 27 October 2004; revised 8 December 2004; accepted 8 December 2004
Available online 12 January 2005

Abstract—Thio-heterocylic naphthalimides (**R1**–**R5**) were designed, synthesized and evaluated as nonmetallic and long-wavelength photocleavers. Some of them showed highly efficient abilities in the degradation of plasmid and genomic DNA under the mild conditions without obvious impairment on the proteins' bioactivities, when compared with frequently applied nucleic acids removal reagents or precipitants. Their differences in photodegradation selectivity to DNA rather than proteins were dependent on their photodamage mechanisms and binding modes with bio-macromolecules. When maize genomic DNA was used as substrate, 2.38×10^{-4} M of **R5** exhibited the nuclease activity of 8 Unit DNase I, **R5** has some characteristic as a typical catalyst as no consumption after two cycles of the photodegradation for DNA. The experiments of enzymatic activity assay and immunology activity analysis showed that **R5** was safe to proteins, suggesting its potential in the removal of transgenic material during the preparation of bioactive proteins or enzyme preparations.

1. Introduction

The removal of nucleic acids from protein or enzyme preparations, especially the removal of transgenic materials from biochemical preparations[1] is of biotechnological and commercial importance due to the big issue of transgenic safety among societies. Normally, nucleic acids were removed by precipitation, or degraded by the addition of exogenous nucleases.

Ammonium sulfate precipitation can be effective in removing nucleic acids from the mixture of proteins, however, it will remove some proteins at the same time. Nucleic acids bind efficiently to anion exchangers (DEAE- and Q-) but sometimes traces of nucleic acids can escape and be mixed with the protein of interest. Various more specific precipitants have been used (e.g., polyethyleneimine,[2] cetyltrimethylammonium bromide,[3] streptomycin sulfate,[4] and protamine sulfate.[5]). All of these are expensive and possibly toxic, and also they may complex undesirably with certain enzymes resulting in a loss of purity. Therefore, it is very important that these reagents are not harmful to the proteins, at least without obvious impairment on proteins' bioactivities and that they can easily be removed in the purification procedure.

Nucleic acid degrading enzymes are the often choice early in the purification process. However, besides expensive, majority of natural enzymes show an obligate requirement of metal ions for their activities, which prevent their uses in the presence of metal chelators or chelating buffers.[6–8] Therefore, novel alternatives for nucleic acids degradation are of great potential.

Chemical or photochemical reaction reagents as artificial nucleases have received considerable attentions for their potential applications[9–11] in molecular biology, since they can be easily designed and synthesized. However, less attention was paid to their applications for the separation of biomolecules in biotechnology. The reasons may be that they could not avoid (1) the obligate

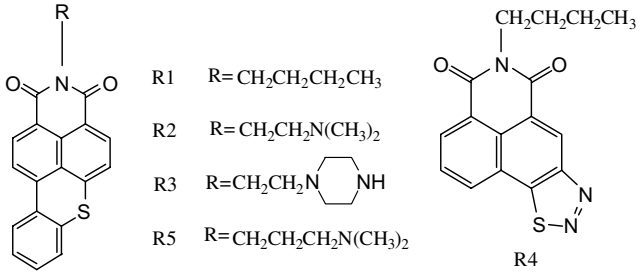
Keywords: Enzyme; Nucleic acids; Photocleaver.
* Corresponding authors. Tel.: +86 411 3673466; fax: +86 411 3673488; e-mail addresses: qingyang@dlut.edu.cn; xhqian@ecust.edu.cn

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.12.012

requirement of metal ions,[12–17] and (2) the impairment on proteins.[14] To the best of our knowledge, the latter reason should be the most prominent limitation of their applications. Kumar and co-workers ever applied organometallic chemicals as proteases for photocleavage of hen egg lysozyme and bovine serum albumin[18,19] through cation radical and free-radical mechanism, indicating photoactivated compounds easily caused damage to proteins. Similarly, we ever observed that H_2O_2 and other organic peroxides could form hydroxyl free radical to efficiently degrade genomic DNA in the presence of Fe^{2+}, while they damaged proteins at the same time if prolonging exposure time. The object for this paper is to explore a synthetic DNA photocleaver as a kind of efficient but inexpensive nuclease alternative for the degradation of genomic DNA, which should exhibit nuclease-similar activities and does not have obvious impairment on proteins' bioactivities under physiological conditions and in the absence of metal ions.

Many naphthalimide derivatives were well known for their wide uses as anticancer drugs or photonucleases. We ever previously reported that the presence of thio-moiety on chromophore would obviously promote the photocleavage.[20] Meanwhile, *N*-alkyl substituted amino-alkyl side chain, which commonly appeared in anticancer drugs,[21] could possibly enhance the affinity interactions of the parent structure with DNA. Thus, the design, synthesis and evaluation of thio-heterocyclic naphthalimides **R1**–**R5** with *N*-alkyl substituted amino-alkyl side chain (Scheme 1) became our target.

Their structures were synthesized and confirmed by IR, ^{1}H NMR, MS, and elemental analysis. Under physiological conditions, **R2**, **R4**, and **R5**, photoactivated at 400 nm, showed high cleavage activities for plasmid and genomic DNA in the absence of metal ions. **R5**, 2-(3-dimethylamino-propyl)-6-thia-2-aza-benzo[*def*]chrysene-1,3-dione, showed the best results in highly-efficient photocleavage for genomic DNA, and its impairment effect on proteins' bioactivities was not observed by compared with frequently applied nucleic acids removal reagents or precipitants.



Scheme 1. Structural features of DNA photocleavers.

2. Results

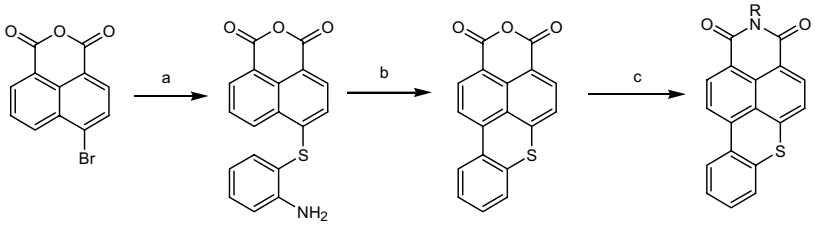
2.1. Syntheses and spectra of compounds

The 2-substituted-6-thia-2-aza-benzo[*def*]chrysene-1,3-diones (**R1**–**R3** and **R5**) were synthesized according to the literature[20b] from 4-bromonaphthalic anhydride (Scheme 2), while 5-butyl-10-thia-5,8,9-triaza-cyclopenta[*a*]phenalene-4,6-dione (**R4**) was synthesized according to the literature[20c] from 4-bromo-3-nitronaphthalic anhydride (Scheme 3). Separation and purification of products were performed through a silica gel column chromatography. All the structural details were confirmed by IR, ^{1}H NMR, MS, and elemental analysis.

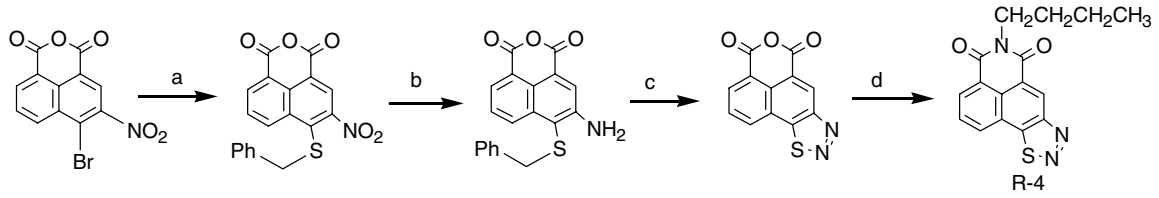
The comparisons of compounds' spectral properties suggested that there might be some differences in their photochemical or photophysical behaviors. 2-Substituted-6-thia-2-aza-benzo[*def*]chrysene-1,3-diones (**R1**–**R3** and **R5**) showing the emission at 520 nm with strong fluorescence and absorptions around 464 nm with the similar intensities, were composed of naphthalimide chromophore and electron-donating benzenethioxyl group, which implied that the electron transfer from them might easily occur during DNA photocleavage.[20b] 5- Butyl-10-thia-5,8,9-triaza-cyclopenta[*a*]phenalene-4,6-dione (**R4**), composed of naphthalimide chromophore and thiadiazole, which would produce triplet state or several active radical intermediate photochemically,[20c] had absorption at 362 nm, its weak fluorescence ($\Phi < 0.0003$) implied that their excitation energy might be transferred from the excited singlet state to triplet state to show some characters of radicals in DNA photocleavage (Table 1).[20c]

2.2. Photocleavage activity of plasmid DNA

For artificial photonuclease or photoprotease, light at wavelengths longer than 300 nm is a highly selective cofactor and the number of side reactions is limited because nucleic acids and most proteins will be transparent under the light except the chromophore of the photocleaver.[9,18] The investigation showed the above compounds



Scheme 2. Syntheses of **R1**, **R2**, **R3**, and **R5**. Reagents and conditions: (a) *ortho*-aminobenzenethiol, K_2CO_3, DMF, reflux, 30 min; (b) H_2O, HCl, HOAc, $NaNO_2$, 0–5 °C, 20 min, $CuSO_4$, H_2O, HOAc, reflux, 1.5 h; (c) amine, ethanol, reflux, 2 h.



Scheme 3. Synthesis of compound **R4**. Reagents and conditions: (a) benzyl mercaptan, K_2CO_3, DMF, Ar, 80 °C, 8 h; (b) $SnCl_2{\cdot}2H_2O$, HCl, 90 °C, 2 h; (c) HOAc, H_2O, HCl, $NaNO_2$, 0 °C, 50 min; (d) butylamine, EtOH, reflux, 2 h.

Table 1. Spectra data of compounds **R1**–**R5**[a,b]

Compound	UV λ_{max}/nm (lg ε)	FL λ_{max}/nm (ϕ)
R1	462 (4.45)	519 (0.89)
R2	462 (4.42)	521 (0.51)
R3	461 (4.20)	521 (0.47)
R4	362 (3.87)	470.4 (<0.0003)
R5	462 (4.37)	521 (0.63)

[a] In absolute ethanol.
[b] With fluorescein as standard (ϕ = 0.90).

could photocleaved plasmid DNA under the irradiation of light at long-wavelength more than 400 nm, which is safe for the operator and control of side reaction.

R1–**R5** (5 μM) dissolved in DMSO could converse supercoiled pBR322 DNA (form I) buffered in 20 mM Tris–HCl, pH 7.5, into relaxed circular DNA (form II) at different conversions and followed to linear DNA (form III) with a transluminator (400 nm) at a distance of 20 cm at 25 °C for 2 h photoactivation (Fig. 1). **R4** and **R5** showed high photocleaving efficiencies.

In our case, all the cleavage was observed without using piperidine, although ones expected that the excited naphthalimide might be able to directly oxidize the guanine in DNA and the oxidation would be detected as strand cleavage only after piperidine treatment.[9] For **R1**–**R3**, and **R5** (10 μM), mechanistic experiment was performed with the addition of histidine (6 mM), dithiothreitol (DTT, 30 mM), superoxide dismutase (SOD, 100 μg/mL), and ethanol (1.7 M). It was found that histidine (singlet oxygen quencher) had no effect on cleavage, ethanol (free radical quencher) strangely and slightly accelerated the reaction somewhat. However, DTT (superoxide anion radical scavenger) retarded

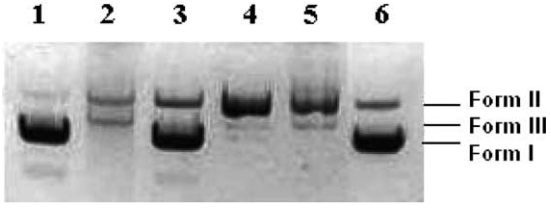


Figure 1. 1% Agarose gel eletrophoresis assay of plasmid pBR322 DNA cleaved by artificial compounds. Lane 1, plasmid pBR322 DNA; lane 2-5, pBR322 DNA photocleaved by compound **R2**, **R3**, **R4**, **R5**, respectively; lane 6, **R1**.

the reaction very efficiently. It was also observed that SOD (superoxide anion radical killer) accelerated the rate of DNA-cleaving reaction, because the hydrogen peroxide produced by SOD from superoxide anion radical would decompose to produce more active hydroxyl radicals in the presence of UV light or trace of metal ions. Thus, we presumed that electron transfer involved the formation of superoxide anion radical played an important role in the photodamage,[20b] that is, under the irradiation of light, the thio-heterocyclic aromatic rings of **R1**–**R3**, and **R5** took an electron from DNA and their imide moieties also transfer an electron to oxygen for the formation superoxide anion radical, which at last damaged DNA.[20b] As to **R4** (10 μM), the investigation showed that ethanol inhibited its reactivity a little while DTT did a lot. Thus, we presumed that free radicals mechanism (partly) as well as the similar electron transfer mechanism (mainly) played roles in the photodamage,[20c] the triplet state of triazole was possibly involved for this free radical mechanism.[20c]

2.3. Photodegradation activity of genomic DNA

So far, the evaluations for most of the known DNA photocleavers were localized to plasmid DNA or synthesized oligonucleotides, which could not reflect the real features of genomic DNA. Therefore, we evaluated the photodegradation activities of the above compounds for genomic DNA.

R1–**R5** (0.01 M) dissolved in DMSO for 40 min photoactivation resulted in 100% degradation efficiency when the natural substrate, genomic DNA of maize in 20 mM Tris–HCl, pH 7.5, was applied (Fig. 2a). It showed that the size of degraded genomic DNA fragments could be even smaller (<300 bp) if prolong photoactivation. According to Kunitz,[22] the cleavage of polymerized DNA would result in an increase in absorbance at 260 nm. Therefore, the slope of the curves in Figure 3 represented the increased rate of the absorbance at 260 nm. The slope of 1 Unit DNase I was used as standard. The order of their photocleavages to genomic DNA is **R5** > **R4** > **R2**. Under the same conditions, 2.38×10^{-4} M of **R5** exhibited the bioactivity of 8 Unit DNase I, suggesting it was an efficient agent for the degradation of DNA (Fig. 2b). In fact, few synthetic and nonmetallic compounds were reported to degrade a genomic DNA, though there were many reports about their cleavage activities to plasmid supercoiled DNA and some oligonucleic acids.

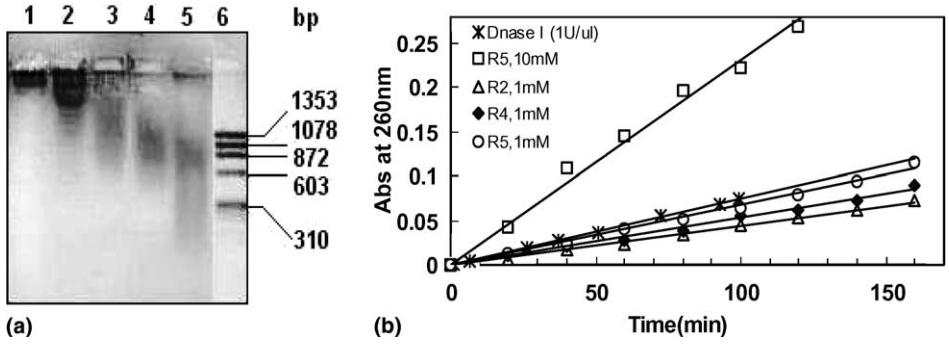


Figure 2. (a) 2.5% Agarose gel electrophoresis assay for the photodegradation of genomic DNA in 20 mM Tris–HCl, pH 7.5, treated by 0.001 M of **R5** dissolved in DMSO at different time intervals. Sample (1 μL) was applied. Lane 1–5, 0, 20, 40, 60, 80 min; lane 6, marker ΦX174; (b) nuclease activity comparison of **R2**, **R4**, **R5**, and DNase I. 5 μL 10 mM of the compound in DMSO into a reaction mixture containing 500 ng freshly prepared maize genomic DNA in 15 μL 20 mM of Tris–HCl, pH 7.5. The reaction initiated with light at 400 nm and stopped at different time points. Nuclease activity determined at 260 nm. When 3 μL DNase I (70 U/μL) from bovine pancreas applied as standard, the reaction buffer was changed to 20 mM of Tris–HCl, pH 7.5, containing 4 mM of $MgCl_2$ and 1 mM of dithiothreitol.

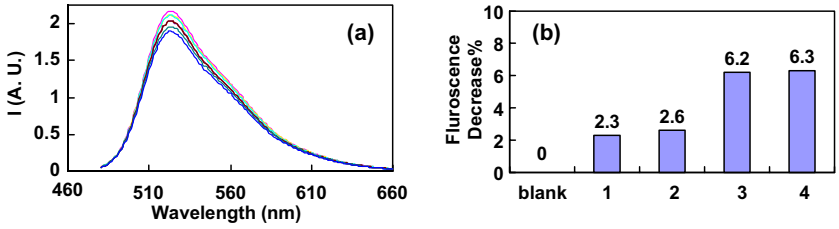


Figure 3. (a) Fluorescence spectral analysis of **R5** (0.01 mM) in 20 mM Tris–HCl, pH 7.5, before and after photoactivation in the presence of maize genomic DNA at various concentrations. Emission at 470 nm and adsorption at 522 nm; (b) decrease rate of fluorescence intensity of the compound before and after photoactivation in the presence of different concentration of maize genomic DNA. Legends: (1) with addition of 500 ng/μL DNA, no light; (2) with addition of 500 ng/μL, with photoactivation; (3) with another addition of 500 ng/μL DNA, no light; (4) with another addition of 500 ng/μL, with photoactivation.

2.4. Stability analysis of the compound during DNA degradation

Only few of the known DNA photoactivated agents actually were treated as 'photonucleases', which in an electronically excited state react directly and catalytically with the nucleic acid chain to result in an immediate scission, and almost not consumed in the process.

To evaluate the **R5** as a DNA cleavage catalyst, we designed an experiment by tracking the changes of its fluorescence intensity during the catalytic reactions, which was described in Experimental section. Agarose (1%) gel electrophoresis showed the same DNA cleavage efficiency of the compound after two cycles of DNA degradation (data not shown). The spectral experiments showed a decrease extend of the fluorescence intensity of **R5** in the process of DNA cleavage (Fig. 3a), and the decreased rate became 6.29% (Fig. 3b) after the second addition of DNA. However, the analysis indicated the fluorescent quenching of **R5** might mainly be due to its binding with DNA rather than itself real consumption during the photocleavage, in fact, the fluorescence quenching was also observed when **R5** was mixed with calf thymus DNA in dark, for example, 0%, 25%, 35%, and 47% fluorescent quenching for **R5** during addition of calf thymus DNA (0, 50, 100, 200 μM) in DMSO–10 mM Tris–HCl (pH 7.5) solution (1:4, v/v). Therefore, the decreasings for **R5**'s fluorescence really corresponding to the consumption during the first and second photocleavage reaction, was only 0.363% and 0.06%, respectively. Therefore, we concluded that **R5** was almost not consumed during two cycles cleavage reactions. The same results were obtained for **R4** and **R2**.

2.5. Effect on protein's immunology activity

The photoeffects of **R2**, **R4**, and **R5** on a recombinant protein fused with a 6His-Tag, was analyzed by the method of Western blotting. The recombinant protein was produced by *Escherichia coli*, which carried the gene of a thrombin-like enzyme, gloshedobin, from the snake venom of *Gloydius shedaoensis*.[23] Western blotting analysis using anti-His-Tag serum showed positive combination of antibody and the enzyme of interest, suggesting almost no effect on the immunology activity by using the compounds (Fig. 4). Of course, in the control experiments of the photoeffects of the compounds on amino acids HPLC analysis suggested that the degradation of tyrosine happened, although it was much complicated to know how less these compounds to modify the structure of the protein.

2.6. Effect on protein's enzymatic activity

The effects of **R2**, **R4**, and **R5** on enzymatic activity of trypsin, which is a frequently used serine protease, was

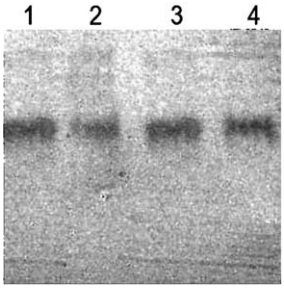


Figure 4. Western blotting analysis for the effect on recombinant gloshedobin mixed with the compounds after phototreatment under 400 nm for 120 min. Lane 1, without compounds; lane 2, with **R2**, **R4**, and **R5**, respectively.

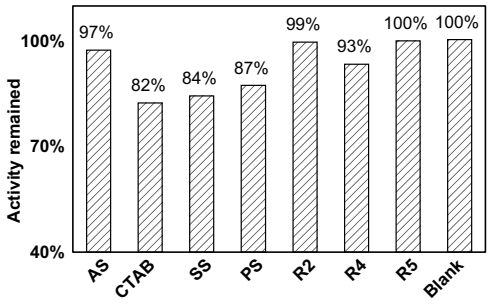


Figure 5. Effects on specific activity of trypsin by specific precipitants and the compounds. The enzyme (2 mg/mL) in 20 mM Tris–HCl, pH 7.5, mixed at 25 °C with 0.2 g/L specific precipitant, ammonium sulfate (AS), cetyltrimethyl ammonium bromide (CTAB), streptomycin sulfate (SS), and protamine sulfate (PS), respectively. Sixty minutes later, the mixture centrifuged at 13,000*g* for 30 min. The enzymatic activity assay performed by adding 100 μL supernatant into 2.9 mL buffer, 20 mM Tris–HCl, pH 7.5, containing 1 mM substrate, *N*α-benzoyl-L-arginine ethyl ester. The liberation of products monitored at 253 nm. For compounds, the enzyme was incubated with 0.01 M artificial compounds under 400 nm for 60 min before assay.

assayed by using *N*α-benzoyl-L-arginine ethyl ester as substrate. Figure 5 shows that **R2** and **R5** had almost no photodamage to the enzymatic reactivity (99% or 100% activity remained, respectively), while **R4** showed slight effect that caused 7% activity loss.

Under the identical conditions, several frequently applied nucleic acids removal reagents involving ammonium sulfate (AS), cetyltrimethyl ammonium bromide (CTAB), streptomycin sulfate (SS), and protamine sulfate (PS) were added to the enzyme solution. As shown in Figure 5, the addition of CTAB, SS, and PS resulted in the loss of enzymatic activities ranging from 13% to 18%, which was much higher than for the compounds. Special attention is given to the precipitants, such as AS, which can be used not only as nucleic acids precipitants but also as protein precipitants. Therefore, it is not surprising that it can be effective in removing nucleic acids and removing proteins at the same time. In this research, we found that only 2 g/L ammonium sulfate will cause 3% activity loss (Fig. 5). However, in most cases, the efficient nucleic acids removal required at least 70 g/L ammonium sulfate, which will cause much loss of activity.

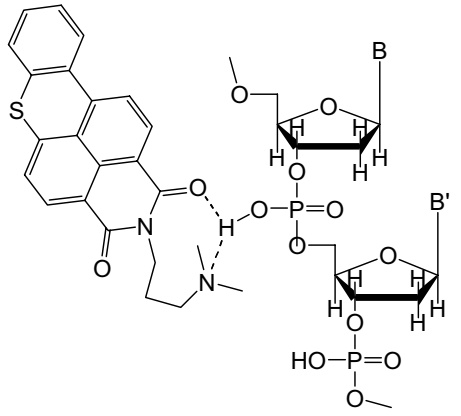
3. Discussion

Although **R2**, **R4**, and **R5** could be used as efficient tools for the degradation of genomic DNA rather than proteins, their selectivities and mechanisms were different. **R5** gave the best result: high degradation ability for genomic DNA and no damage to protein's activity; **R2** showed almost no damage to protein's activity but moderate degradation ability for genomic DNA; **R4** damaged both to protein's activity and to genomic DNA. These differences in the selectivity might be caused by their different damage mechanisms. **R2** and **R5** photodegraded DNA through electron transfer mechanism involved superoxide anion radical like co-sensitization.[20b,9] For **R4**, the degradation was partly through free radical mechanism involved the triplet state of tridiazole.[20c,9] The photobioactivities of compounds were mainly dependent on their photochemical behaviors, the electron transfer process was usually orientation to some targets and free radicals were disperse around reaction site, therefore, the compounds in electron transfer mechanism usually had high selectivity in photocleavage by comparison with the poor selectivity of ones in free radical mechanism.[20,9]

Their selectivities might be also related with their binding modes with DNA. Although there was some possibility that the ring structures of **R2**, **R5**, and **R4** interacted with DNA bases like intercalation, the flexible side chain of *N*-alkyl substituted aminoalkyl group as acceptor at imide moieties of **R5** and **R2** might easily form hydrogen bonding with the proton[24] at the phosphorous hydroxyl groups of DNA duplex–helix (Scheme 3), and **R4** cannot bind with DNA in the same way. The binding mode of hydrogen bonding for **R2** and **R5** made them close to DNA more easily to mainly increase their selectivities, as well as efficiencies partly. In addition, enzymes bury their catalytic amino acid residues in a bag via self-folding hinder the approximation of nonsubstrate molecules, therefore, this binding mode would not affect the bioactivity of a protein.

Although **R5**, **R1**–**R3** could photocleave both plasmid and genomic DNA via electron transfer mechanism, **R3** showed the weakest bioactivity due to the possible steric hindrance for hydrogen bonding with DNA by piperazino moiety at its side chain. **R2**, **R5** with a proper-length side chain to facilitate hydrogen bonding, show higher activity. Consequently, it is not surprising that **R1** photocleave DNA weakly because of its disability of hydrogen bonding. Moreover, the reason of **R4** having stronger bioactivity, possibly be owing to its partly free radical mechanism for photocleavage (Scheme 4).

Although we could not know how less these compounds to modify the structure of the protein, the comparison of impairment to an enzymatic activity by commercially available precipitants and the synthetic compounds, suggested that **R2**, **R4**, and **R5** were good nucleic acids removal reagents alternative. Under physiological conditions, 2.38×10^{-4} M of **R5** exhibited the nuclease activity of 8 Unit DNase I in the degradation of maize genomic DNA.



Scheme 4. The binding and photocleavage to DNA by compound **R5**.

4. Conclusion

We present novel genomic DNA photonuclease of thioheterocyclic naphthalimides with *N*-alkyl substituted aminoalkyl side chain, which showed highly efficient abilities in the degradation of plasmid and genomic DNA under the mild conditions without obvious impairment on the proteins' bioactivities. Their differences in photodegradation selectivity to DNA rather than proteins were depended on their photodamage mechanisms and binding modes with bio-macromolecules. **R5** almost have no consumption after two cycles of the photodegradation, and showed the best results in the selectivity and efficiency of photodamage. The structural characteristics accounting for these properties will invoke chemists to design new types of chemical nucleases and biologist to apply these compounds. Also, the application potential will attract attentions of bioengineers who would like to seek inexpensive and efficient nuclease to the removal of nucleic acids from biochemical and pharmaceutical preparations.

5. Experimental

5.1. Syntheses of compounds

Melting points were taken on a digital melting point apparatus WRS-1 (Shanghai, China) and it was uncorrected. Infrared spectra were recorded on a Nicolet FT IR-20SX, mass spectra on a Hitachi M80, ^{1}H NMR on a Bruker AM-300 or AM-500 using TMS as an internal standard. Combustion analysis for elemental composition was done on Italy MOD.1106 analyzer. Absorption spectra were measured on Shimadzu UV-265, fluorescence spectra on a Hitachi 850.

The syntheses of **R1**–**R3**, and **R5**: (a) 4-Bromo-1,8-naphthalic anhydride (4.155 g), *o*-aminobenzenethiol (2.12 g), and K_2CO_3 (1.035 g) were mixed and refluxed in DMF (42 mL) for 30 min, then the mixture was poured into water (100 mL), acidified, and filtered to afford green solid (3.71 g, mp 198–203 °C, 77% yield) of 4-(2-amino-phenylthioxy)-1,8-naphthalic anhydride; (b) to the mixture of this intermediate (3.71 g), water (4.6 mL), HCl (2.9 mL), and acetic acid (28 mL) a solution of sodium nitrate (8.35 g) in water (11 mL) was added dropwise during 20 min at 0–5 °C. The reaction mixture was added to a solution of $CuSO_4$ (8.17 g) in acetic acid (7 mL) mixed with water (120 mL) at boiling temperature for 1.5 h to afford orange solid (3.5 g, mp >300 °C, 99% yield) of benzothioxanthene; (c) 0.3 g of benzothioxanthene was refluxed with appropriate amine (0.17 mL) in ethanol (20 mL) for 2 h to afford the product. **R1**: Separated on silica gel chromatography (ethyl acetate–petroleum = 2:5, v/v), mp 185–186 °C, 75% yield. δ_H (500 MHz, $CDCl_3$, Me_4Si) 0.98 (3H, t, *J* 7.37, CH_3), 1.43–1.50 (2H, m, (Me)–CH_2), 1.68–1.78 (2H, m, (C_2H_5)–CH_2), 4.19 (2H, t, *J* 7.60, $CONCH_2$), 7.38–7.43 (3H, m, 8-H, 9-H, 10-H), 7.52 (1H, d, *J* 7.98, 7-H), 8.20–8.27 (2H, m, 1-H, 6-H), 8.44 (1H, d, *J* 7.98, 2-H), 8.64 (1H, d, *J* 8.13, 5-H). $C_{22}H_{17}NO_2S$ requires: C, 73.51; H, 4.77; N, 3.90. Found C, 73.23; H, 4.69; N, 3.88. **R2**: Separated on silica gel chromatography (chloroform–acetone = 1:1, v/v), 212–213 °C, 85% yield. δ_H (500 MHz, $CDCl_3$, Me_4Si) 1.20–1.34 (8H, m, NCH_3, NCH_2), 4.42 (2H, t, *J* 6.78 and 6.65, $CONCH_2$), 7.34–7.42 (m, 3H, 8-H, 9-H, 10-H), 7.48 (1H, d, *J* 7.99, 7-H), 8.14–8.20 (2H, m, 1-H, 6-H), 8.40 (1H, d, *J* 7.98, 2-H), 8.59 (1H, d, *J* 8.12, 5-H); EI-MS: *m*/*z* 374 (M^+, 1.62), 304 (23.30), 303 (29.64), 71 (29.58), 58 (100), 56 (9.81), 43 (12.49); ν_{max} (KBr)/cm^{-1} 2950, 2870, 1695, 1660, 1560, and 1380. $C_{22}H_{18}N_2O_2S$ requires: C, 70.57; H, 4.85; N, 7.48. Found: C, 70.46; H, 4.98; N, 7.35. **R3**: Separated on silica gel chromatography (chloroform–methanol–acetate = 9:2:1.5, v/v), 286–287 °C, 81% yield. δ_H (500 MHz, $CDCl_3$, Me_4Si) 2.80 (2H, t, *J* 5.96 and 6.00, NCH_2), 2.89 (4H, s, $NHCH_2$ (*cyclo*)), 3.14 (4H, s, NCH_2 (*cyclo*)), 4.32 (2H, t, *J* 6.08 and 5.88, $CONCH_2$), 7.38–7.46 (3H, m, 8-H, 9-H, 10-H), 7.54 (1H, d, *J* 8.06, 7-H), 8.19–8.25 (2H, m, 1-H, 6-H), 8.42 (1H, d, *J* 8.01, 2-H), 8.62 (1H, d, *J* 8.18, 5-H); EI-MS: *m*/*z* 415(M^+, 5.45), 373 (39.42), 330 (35.98), 303 (27.95), 99 (100.0), 70 (27.95), 56 (42.09), 42 (21.07); ν_{max} (KBr)/cm^{-1} 3410, 2970, 2840, 1690, 1640, and 1330. $C_{24}H_{21}N_3O_2S$ requires: C, 69.38; H, 5.09; N, 10.11. Found C, 69.56; H, 5.23; N, 9.96. **R5**: Separated on silica gel chromatography (chloroform–ethanol = 5:1, v/v), 202–203 °C, 78% yield. δ_H (500 MHz, $CDCl_3$, Me_4Si) 2.46 (8H, s, NCH_3, NCH_2), 2.70 (2H, t, *J* 7.44, CH_2), 4.25 (2H, t, *J* 7.24, $CONCH_2$), 7.40 (3H, m, 8-H, 9-H, 10-H), 7.49 (1H, d, *J* 7.98, 7-H), 8.20 (2H, m, 1-H, 6-H), 8.39 (1H, d, *J* 7.98, 2-H), 8.59 (1H, d, *J* 8.10, 5-H). $C_{23}H_{20}N_2O_2S$ requires: C, 71.11; H, 5.19; N, 7.21. Found: C, 71.23; H, 4.92; N, 7.14.

The synthesis of **R4**: (a) 2.32 g of 4-bromo-3-nitro-1,8-naphthalic anhydride was reacted with benzyl mercaptan (0.88 mL) in the presence of K_2CO_3 (0.52 g) at 80 °C for 8 h under Ar gas protection. The reaction mixture was poured into salted-ice water to afford yellow solid (2.47 g, mp 188–193 °C, 94% yield) of 4-(benzylthioxy)-3-nitro-1,8-naphthalic anhydride; (b) this intermediate was reduced by $SnCl_2{\cdot}2H_2O$ (7.6 g) in HCl (31 mL) at 90 °C for 2 h to give yellow solid (2.85 g, mp 171–185 °C); (c) to the mixture of the amino intermediate (1.46 g), acetic acid (57 mL), water (7 mL), and HCl (8.5 mL), a solution of $NaNO_2$ (0.31 g) in water (10 mL)

was added dropwise during 20 min at 0 °C and the reaction mixture was stirred for 30 min at 0 °C and for 7 h at 15–20 °C, yellowish green solid (0.97 g, mp 162–167 °C, 86% yield) of 5-oxa-10-thia-8,9-diaza-cyclopenta[*a*]-phenalene-4,6-dione was obtained; (d) then this intermediate (0.38 g) was reacted with butylamine (0.30 mL) in absolute ethanol (20 mL) at reflux for 2 h to afford yellow solid in 72% yield of **R4**, which was purified through silica gel chromatography (chloroform–acetate = 200:1, v/v). **R4**: mp 177–179 °C. ^{1}H NMR (500 MHz, DMSO-d_6, Me_4Si): δ_H (ppm) 0.94 (t, J_1 7.36, 3H, CH_3), 1.38 (m, 2H, $(CH_3)CH_2$), 1.64 (m, 2H, $(C_2H_5)CH_2$), 4.05 (t, J 7.49, 2H, $CONCH_2$), 8.03 (t, J 7.77, 1H, 2-H), 8.62 (d, J 7.11, 1H, 1-H), 8.82 (t, J 7.97, 1H, 3-H), 9.32 (s, 1H, 7-H). HR-MS: $C_{16}H_{13}N_3O_2S$ require: 311.0728. Found: 311.0728. EI-MS: *m/z* (%): 311.0728 (M^+, 17.00), 283.0705 (100.00), 266.0659 (27.86), 241.0200 (28.75), 227.0069 (89.84), 157.0128 (23.66). IR (KBr): 2950, 2870, 1710, 1660, 1300 cm^{-1}. $C_{16}H_{13}N_3O_2S$ require: C 61.72, H 4.21, N 13.50. Found: C, 61.59; H, 4.41; N, 13.62.

5.2. Total DNA isolation and purification from the leaf of maize

The extraction and purification of total DNA was performed by the method of CTAB. The CTAB isolation buffer (4 M NaCl, 100 mM Tris–HCl, pH 8.0, 20 mM EDTA, 20 g/L ethyltrimethylammonium bromide, 2 mL/L 2-mercaptoethanol) was preheated at 60 °C. Fresh leaf tissue (2 g) of maize was ground to a powder in liquid nitrogen in a chilled mortar and pestle. Then the powder was scraped into a chilled 50 mL tube. CTAB (5 mL) buffer per gram tissue was added into the tube and then incubated the sample at 60 °C for 30 min with occasional gentle swirling. The same volume of phenol–chloroform (1:1, v/v) was added to the sample with gently swirling. The precipitates were discarded after centrifugation at 16,000*g* at 25 °C for 10 min, while the yellow, aqueous phase with a wide-bore pipette was transferred to a new 50 mL tube. Two-thirds volume of cold 100% isopropanol was added to the tube and mixed gently to precipitate the nucleic acids. The precipitate was collected and then incubated for a minimum of 20 min with 75% ethanol. The DNA precipitate was obtained again by centrifugation at 16,000*g* for 10 min and then was left to dry in the air. The DNA pellet was resuspended in 2–3 mL TE buffer (1 M Tris–HCl, 0.5 M EDTA, pH 8.0) and then RNase A was added to the mixture at a final concentration of 10 μg/mL to remove RNA for 30 min at 37 °C. Finally, the RNase A was removed by adding an equal volume of phenol–chloroform (1:1, v/v) into the mixture followed with centrifugation at 16,000*g* at 25 °C for 10 min. The upper, aqueous phase was collected and then 7.5 M ammonium acetate, pH 7.7 to a final concentration of 2.5 M and 2.5 volume of ice-cold 100% ethanol was added into the supernatant to precipitate the DNA. Centrifuged at 10,000*g* for 10 min at 4 °C, the DNA pellet was obtained and then was washed with 75% ethanol followed with centrifugation at 10,000*g* for 10 min at 4 °C. The DNA pellet was resuspended in 50 μL of TE buffer (1 M Tris–HCl, 0.5 M EDTA, pH 8.0) for future usage.

5.3. Nuclease activity assay

Photosensitive compound (5 μL, 10 mM) dissolved in DMSO was added into a reaction mixture containing 500 ng freshly prepared maize genomic DNA in 15 μL 20 mM Tris–HCl, pH 7.5. The reaction was initiated with light activation under 400 nm and stopped by the removal of the light at different time points. Nuclease activity was determined by monitoring the increase in the absorbance at 260 nm. When 3 μL of bovine pancreas DNase I (70 U/μL, 20.4 mg/mL, Mw 3.1×10^4 Da) (Takara, Japan) was applied as standard, the reaction buffer was changed to 210 μL 20 mM Tris–HCl, pH 7.5, containing 4 mM $MgCl_2$ and 1 mM dithiothreitol. One unit was defined as the amount of nuclease that causes an increase in absorbance at 260 nm of 0.001 per minute per milliliter at 25 °C, pH 7.5, with highly polymerized DNA as the substrate.[18]

5.4. Fluorescence detection assay

Photosensitive compound (5 μL, 10 mM) dissolved in DMSO was added into a reaction mixture containing 500 ng freshly prepared maize genomic DNA in 15 μL 20 mM Tris–HCl, pH 7.5. The reaction was initiated with light activation under 400 nm and stopped by the removal of the light after 20 min. The reaction mixture was diluted until the concentration of the compound at 1×10^{-5} M for fluorescence detection assay. The emission wavelength is at 470 nm and the absorption at 522 nm. For consumption assay of the compound during the cleavage reaction, this procedure was repeated with another addition of 500 ng DNA into the reaction mixture. The other operation conditions remained the same.

5.5. Enzymatic activity assay

Enzymatic activity of trypsin (Ameresco, USA) was assayed using the substrate, *N*α-benzoyl-L-arginine ethyl ester (BAEE) (Sigma). In brief, trypsin (2 mg/mL, 500 U) in 20 mM Tris–HCl, pH 7.5, was mixed at 25 °C with 0.2 g/L specific precipitant, ammonium sulfate (AS), cetyltrimethyl ammonium bromide (CTAB), streptomycin sulfate (SS), and protamine sulfate (PS), respectively. Sixty minutes later, the mixture was centrifuged at 13,000*g* for 30 min. The enzymatic activity assay was performed by adding 100 μL of supernatant into 2.9 mL of buffer, 20 mM Tris–HCl, pH 7.5, containing 1 mM BAEE. The liberation of products was monitored at 253 nm, and the enzyme was incubated with 0.01 M artificial compounds under 400 nm for 60 min before assay.

5.6. Western blotting assay

The total cellular proteins after being treated with the compounds were analyzed by 12.5% SDS-PAGE. The protein bands were electrophoretically transferred onto nitrocellulose membrane at 0.65 mA/cm^2 for 2 h. The membrane was blocked with 20 g/L fat-free milk and incubated with horse anti-His-Tag serum (Invitrogen,

USA) diluted 1:300, followed by rabbit anti-horse IgG-HRP conjugate (Invitrogen, USA) diluted 1:500. The bound antibody was detected by using 3,3′-diaminobenzidine and hydrogen peroxide.

Acknowledgements

This study was under the auspices of the National Key Project for Basic Research and 863 Project for High-Tech, the Ministry of Science and Technology of China (2003CB114405 and 2003AA2Z3520).

References and notes

1. Klein, J.; Altenbuchner, J.; Mattes, R. *J. Biotechnol.* **1998**, *60*, 145–153.
2. Żylicz-Stachula, A.; Harasimowicz-Słowińska, R. I.; Sobolewski, I.; Skowron, P. M. *Nucleic Acids Res.* **2002**, *30*, 733.
3. Jiang, C. Z.; Yee, J.; Mitchell, D. L.; Britt, A. B. *Proc Natl. Acad. Sci. U.S.A.* **1997**, *94*, 7441–7445.
4. Bellamy, S. R. W.; Baldwin, G. S. *Nucleic Acids Res.* **2001**, *29*, 3857–3863.
5. Goulas, E.; Dily, F. L.; Simon, J. C.; Ourry, A. *J. Exp. Botany* **2002**, *53*, 1941–1948.
6. Kramer, R. *Coord. Chem. Rev.* **1999**, *182*, 243–261.
7. Franke, I.; Meiss, G.; Blecher, D.; Gimadutdinow, O.; Urbanke, C.; Pingoud, A. *FEBS Lett.* **1998**, *425*, 517–522.
8. Rangarajan, S.; Shankar, V. *Biochim. Biophys. Acta* **1999**, *1473*, 293–304.
9. Armitage, B. *Chem. Rev.* **1998**, *98*, 1171–1200.
10. Franklin, S. J. *Curr. Opin. Chem. Biol.* **2001**, *5*, 201–208.
11. Cowan, J. A. *Curr. Opin. Chem. Biol.* **2001**, *5*, 634–642.
12. Wan, R.; Zhao, G.; Chen, J.; Zhao, Y. *Chin. Sci. Bull.* **2000**, *45*, 2017–2028.
13. Wilcox, D. E. *Chem. Rev.* **1996**, *96*, 2435–2458.
14. Sigman, D. S.; Mazumder, A.; Perrin, D. M. *Chem. Rev.* **1993**, *93*, 2295–2316.
15. Branum, M. E.; Tipton, A. K.; Zhu, S.; Que, L. J. *J. Am. Chem. Soc.* **2001**, *123*, 1898–1904.
16. Sissi, C.; Rossi, P.; Felluga, F.; Formaggio, F.; Palumbo, M.; Tecilla, P.; Toniolo, C.; Scrimin, P. *J. Am. Chem. Soc.* **2001**, *123*, 3169–3170.
17. Neves, A.; Terenzi, H.; Horner, R.; Horn, A. J.; Szpoganicz, B.; Sugai, J. *Inorg. Chem. Commun.* **2001**, *4*, 388–391.
18. Kumar, C. V.; Buranaprapuk, A.; Opiteck, G. J.; Moyer, M. B.; Jockusch, S.; Turro, N. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10361–10366.
19. Kumar, C. V.; Buranaprapuk, A.; Thota, J. *Proc. Indian Acad. Sci. (Chem. Sci.)* **2002**, *114*, 579–592.
20. (a) Qian, X.; Huang, T., et al. *J. Chem. Soc., Perkin Trans. 2* **2000**, 715–718; (b) Qian, X.; Li, Y.; Xu, Y.; Liu, Y.; Qu, B. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2665–2668; (c) Li, Y.; Xu, Y.; Qian, X.; Qu, B. *Bioorg. Med. Chem. Lett.* **2003**, *13*(20), 3513–3515.
21. Brana, M.; Ramos, A. *Curr. Med. Chem.: Anti-cancer Agents* **2001**, *1*, 237–255.
22. Kunitz, M. *J. Gen. Physiol.* **1950**, *33*, 349–355.
23. Yang, Q.; Xu, J. Q.; Li, M.; Lei, X. Y.; An, L. J. *Biotechnol. Lett.* **2003**, *25*, 607–610.
24. DeSilva, A. P.; Gunaratne, H. Q. N.; Habib-Jiwan, J.-L.; McCoy, C. P.; Rice, T. E.; Soumiliion, J.-P. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1728.