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Metal-free artificial nucleases based on simple oxime and hydroxylamine scaffolds

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ABSTRACT

Hydrolysis of DNA is of increasing importance in biotechnology and medicine. In this Letter, we present the DNA-cleavage potential of metal-free hydroxylamines and oximes as new members of nucleic acid cleavage agents.

Small molecules that interact with DNA through recognition, binding, modifying, cleaving, or cross-linking have attracted great interest in the research fields of chemistry, biology, biotechnology, and medicine. Like natural enzymes, artificial nucleases can hydrolyze DNA, and therefore these cleavage agents have found extensive application in DNA manipulations and as potential chemotherapeutic agents.[1]

Several organometallic complexes have been developed as artificial nucleases for DNA-cleavage.[2] Synthetic metallonucleases require ligands which effectively deliver the metal ion to the vicinity of the DNA strand. Recent examples include transition-metal[3–5] as well as lanthanide[6] complexes that promote fast cleavage of DNA or model phosphodiesters under physiological conditions.[7]

In spite of the successful results involving artificial metallonucleases, studies devoted to metal-free organic molecules as nucleic acid cleavage agents are not as frequent.[8,9] In fact, the cleavage of DNA by metal-free compounds can be of great importance for the development of biotechnology and gene therapy, including the use of photo-induced processes[10] for treatment of cancer.

Hydroxylamines, oximes, and analogues are nucleophilic molecules that have found broad application as biological tools. Oxime derivatives promote re-activation of acetylcholinesterase after being inhibited by organophosphorus agents,[11] and are also potent inhibitors of the fms-like tyrosine kinase 3.[12] Alkyl and acyl hydroxylamines have been explored as potent antitumor agents[13] as well as mediators of neurodegeneration[14] caused by oxidative stress of 3-aminopropanal (a product of polyamine oxidase metabolism of spermine and spermidine). Particularly, it has been found that simple alkyl hydroxylamines are potent cleaving agents of phosphodiesters and related model compounds.[15]

Therefore, we envisaged that oximes and hydroxylamines might be interesting candidates for cleaving phosphate bonds in nucleic acids. Herein, we present our results featuring a family of

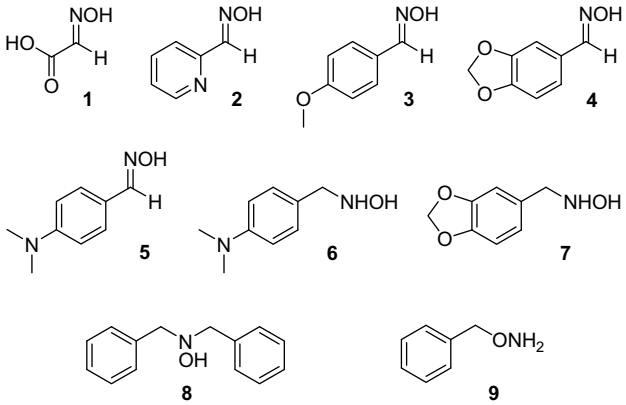

Figure 1. Oximes and hydroxylamines tested as DNA-cleavage agents.

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oximes and hydroxylamines (Fig. 1) as metal-free synthetic nucleases.

Oximes **1**–**5** containing tethered functional groups were prepared in good yields by condensation of the corresponding aldehyde with hydroxylamine hydrochloride in mild basic medium[16] (route a in Scheme 1). Preparation of N-mono and N,N-disubstituted hydroxylamines was achieved by a pH-controlled reduction of oximes with $NaBH_3CN$ (routes b and c in Scheme 1).[17,18] Reduction must be conducted at strictly controlled pH range (2.0–2.1), otherwise a competitive over-reduction under higher pH ultimately leads to the corresponding N,N-disubstituted hydroxylamines as the major product. We found that hydroxylamines were also cleanly obtained in moderate yields by a one-pot process involving condensation of an aldehyde with $NH_2OH{\cdot}HCl$ at pH 6, followed by in situ reduction of oxime at pH 1.5 (route d in Scheme 1).

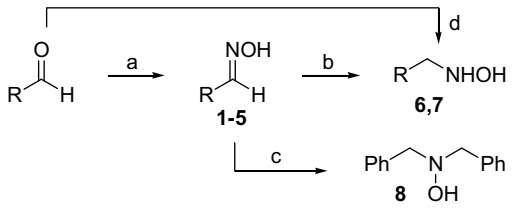
Initial screenings against plasmid DNA (pBSK-II, Stratagene)[19–21] did not show any catalytic activity for oximes **1**–**4**. However, 4-(*N*,*N*-dimethylamino)benzaldoxime (**5**) was able to cleave DNA to some extent (Fig. 2), and its catalytic efficiency as a DNA-cleavage agent was quantified using a Michaelis–Menten formalism (Table 1). Noteworthy, cleavage mediated by oxime **5** was not significantly altered in the presence of distamycin, a minor groove DNA-binding molecule (Table 2). Inhibition of DNA cleavage was also small in the presence of DMSO, a hydroxyl-radical scavenger.

We then focused our attention on functionalized hydroxylamines due to their highly nucleophilic character.[15] Indeed, DNA-cleavage assays conducted with hydroxylamines **6**–**8** demonstrated their superior ability for cleaving the tested DNA strands (Fig. 3). By comparing the experimental rate constants obtained for hydroxylamines **6**–**8** (Table 1), it is clear that a high catalytic efficiency (k_{cat}/K_M) was achieved by *N*-[3,4-(methylenedioxy)benzyl]hydroxylamine (**7**) (and, to some extent, also by N,N-disubstituted derivative **8**), while the amino analogue **6** was less efficient than oxime **5**.

In order to gain insightful information associated with the mechanism of DNA cleavage mediated by hydroxylamines **6**–**8**, a set of experiments was performed in the presence of distamycin as a DNA minor-groove binder (Fig. 4). The main results, summarized in Table 2, indicate that suppression of DNA cleavage by distamycin is not relevant for oxime **5**, while this effect becomes more important for N-substituted hydroxylamines **6**–**8**. For instance, the catalytic efficiency of hydroxylamine **7** as a DNA-cleavage agent was moderately reduced (up to 35%) in the presence of distamycin.

Mechanistic profiles of DNA cleavage by hydroxylamines **6**–**8** were also evaluated in the presence of DMSO as a radical suppressor. The small-to-moderate inhibition observed for compounds **6**–**8** (up to 24%, Table 2) is a plausible indication that mechanisms based on hydrolytic, rather than oxidative processes, are dominant but not exclusive. Therefore, the intermediacy of free-radical species may also operate in alternative pathways or in photo-induced transformations.[10]

Encouraged by the positive results obtained for the N-substituted hydroxylamines **6**–**8**, we became interested in evaluating



Scheme 1. Reagents and conditions: (a) $NH_2OH.HCl$, NaOAc, MeOH, 25 °C (70–90%); (b) $NaBH_3CN$, MeOH, pH 2.0–2.1, 25 °C (80–90%); (c) $NaBH_3CN$, MeOH, pH 4, 25 °C (65–75%); (d) i—$NH_2OH{\cdot}HCl$, MeOH, KOH, pH 6; ii—$NaBH_3CN$, HCl, pH 1.5, 25 °C (30–40%).

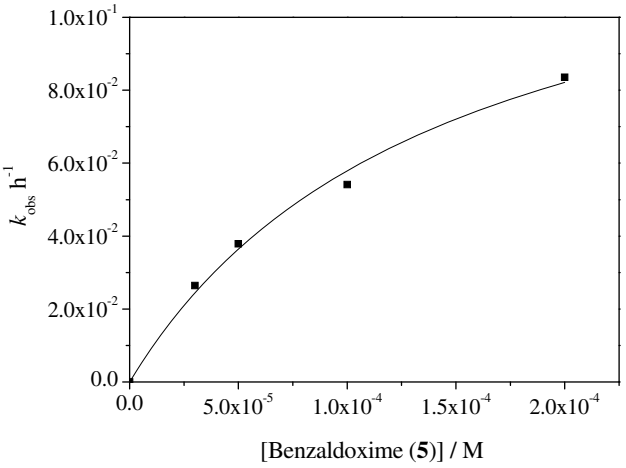


Figure 2. Plot of k_{obs} (h^{-1}) versus concentration [M] of 4-(*N*,*N*-dimethylamino)benzaldoxime (**5**), at pH 7.0 (PIPES buffer 25 mM) and 50 °C.

Table 1
Kinetic data for DNA cleavage

Compound	k_{cat} ($\times 10^{-3}$ h^{-1})	K_M ($\times 10^{-5}$ M)	k_{cat}/K_M (M^{-1} h^{-1})
5	141	14	1008
6	79	14	570
7	187	6	3127
8	157	11	1433
9	424	7	6065

Table 2
Relative inhibition of DNA-cleavage by distamycin, DMSO, and glycerol

Compound	Distamycin (%)	DMSO (%)	Glycerol (%)
5	2–16	8–12	—
6	14–19	12–23	—
7	20–35	16–18	—
8	10–23	14–24	—
9	61–77	73–97	71–85

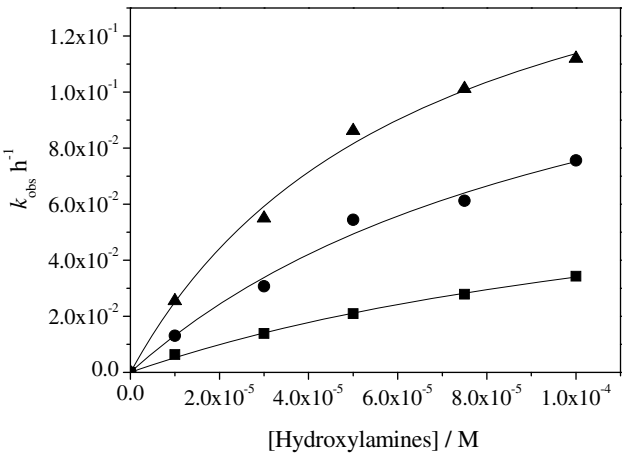


Figure 3. Plot of k_{obs} (h^{-1}) versus concentration [M] of *N*-[4-(dimethylamino)benzyl]hydroxylamine (**6**) (■), *N*-[3,4-(methylenedioxy)benzyl]hydroxylamine (**7**) (▲), and *N*,*N*-dibenzylhydroxylamine (**8**) (●) at pH 8.0 (HEPES buffer 25 mM) and 50 °C.

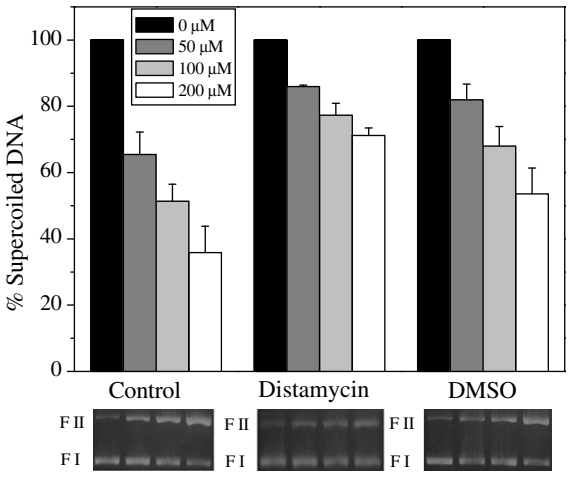


Figure 4. Inhibition of plasmid DNA interaction with *N*-[3,4-(methylenedioxy)benzyl]hydroxylamine (**7**) by distamycin and DMSO, incubated in 25 mM HEPES, pH 8.0 in a final volume of 20 μL, for 8 h with pBSK-II at different concentration (0, 50.0, 100.0 and 200.0 μM) control (left panel), distamycin (50 μM) (center) and DMSO (10%) (right panel). (F I) Supercoiled and (F II) Circular pBSK-II DNA.

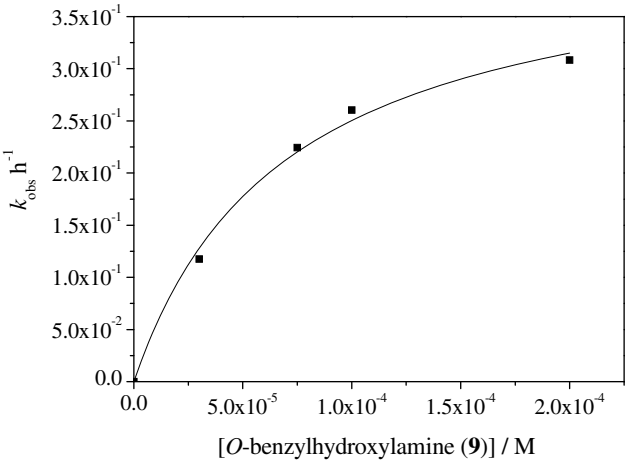


Figure 5. Plots of variation of k_{obs} (h^{-1}) *versus* concentration [M] of the *O*-benzylhydroxylamine (**9**) at pH 9.0 (CHES buffer 25 mM) and 50 °C for 8 h.

whether an O-substituted hydroxylamine analogue[18] such as **9** would be capable of cleaving DNA. Surprisingly, the kinetic profile of *O*-benzylhydroxylamine (**9**) revealed higher catalytic efficiency than any other tested hydroxylamine or oxime (Table 1 and Fig. 5). Efficient DNA cleavage was accomplished even at low concentration (50 μM) of **9** (Fig. 6).

Contrary to the results observed for N-substituted hydroxylamines **6**–**8**, the presence of distamycin promoted a sensible suppression in the catalytic efficiency of *O*-benzylhydroxylamine (**9**) as DNA-cleavage agent (Table 2 and Fig. 6). These results strongly suggest that N-substituted hydroxylamines **6**–**8** prefer cleaving toward the DNA major groove (not inhibited by distamycin), while O-substituted hydroxylamine **9** selectively nicks the minor groove and, therefore, competes with distamycin for the same binding region.

Experiments carried out in the presence of DMSO led to a more dramatic inhibition of DNA cleavage (Table 2 and Fig. 6). The cleavage was almost entirely suppressed under a variety of concentrations of **9**. Notably, a similar pronounced inhibition was also achieved when glycerol replaced DMSO as a radical scavenger. These results clearly differentiate the mechanism of

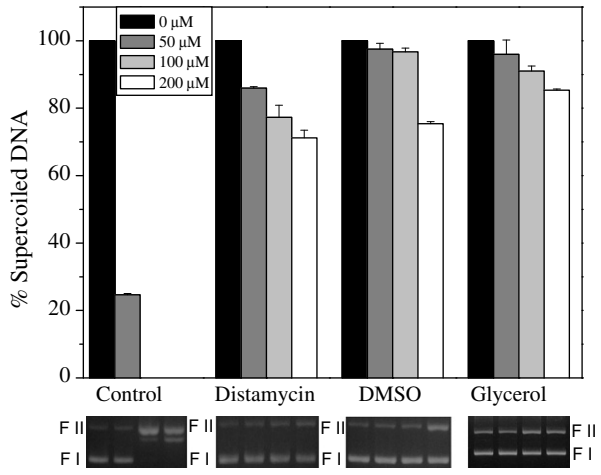


Figure 6. Inhibition of plasmid DNA interaction with *O*-benzylhydroxylamine (**9**) by distamycin (50 μM), DMSO (10%), and glycerol (10%), incubated in 25 mM CHES, pH 9.0 in a final volume of 20 μL, for 8 h with pBSK-II at different concentrations (0, 50.0, 100.0, and 200.0 μM). (F I) Supercoiled and (F II) Circular pBSK-II DNA.

DNA cleavage by O-substituted hydroxylamine **9** (which is predominantly oxidative) in relation to that observed for N-substituted hydroxylamines **6**–**8**, where the reaction shows more hydrolytic character.

It is important to note that the catalytic activity of *O*-benzylhydroxylamine (**9**) is of comparable magnitude to the values reported in the literature for organometallic complexes. For example, the DNA-cleavage activity of a gadolinium complex tested by our group[22] presents a rate constant of 0.47 h^{-1}, while that for compound **9** is, approximately, 0.42 h^{-1}.

The higher catalytic efficiency (k_{cat}/K_M) of the *O*-benzylhydroxylamine (**9**, Table 1), when compared with the simple oximes and *N*-alkylhydroxylamines (compounds **5**–**8**), is not consistent with the results observed in aqueous solutions. In general, *N*-alkyl hydroxylamines are substantially more nucleophilic than the *O*-alkyl derivatives for the hydrolysis of either an artificial substrate (2,4-BDNPP)[15b] or organophosphorous anticholinesterases such as DFP and Sarin.[15d] The results suggest that the mechanism of DNA cleavage is hydrolytic for the *N*-alkylhydroxylamines and probably oxidative when *O*-benzylhydroxylamine (**9**) is considered. Indeed, despite the fact that hydroxylamine **9** is a good nucleophile against artificial substrates, such as 2,4-BDNPP, the DNA cleavage in the presence of **9** depends critically on the oxygen availability (Fig. S16).

Based on the kinetic experiments involving representative oximes and hydroxylamines **1**–**9**, we conclude that oximes and, mainly, hydroxylamines show reasonable activity as DNA-cleavage agents. The N- versus O-substitution directs the catalytic efficiency as well as the mechanism pathways involved in the cleavage. Due to the simplicity of the molecules presented in this study, it can be anticipated that proper functionalization of hydroxylamines might lead to metal-free synthetic nucleases with enhanced activities. For example, these compounds can be coupled to peptides or other molecules to promote targeting to specific DNA sequences, since they are rather soluble in polar solvents and are small molecules with at least two reactive groups, one for covalent attachment and the other for nucleic acid cleavage.

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Supplementary data

General information, experimental procedures, compound characterization data (including ^{1}H and/or ^{13}C NMR spectra for compounds **5**–**9**), and full kinetic data for the DNA cleavage (including inhibition experiments with distamycin, DMSO, and glycerol).

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.052.

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19. *Preparation of plasmid DNA and screenings:* Plasmid DNA pBSK-II (Stratagene) was produced in *Escherichia coli* DH5-α and extracted following protocols described in the literature.[20] Plasmid DNA (pBSK-II BlueScript, Stratagene) was obtained and purified according to standard techniques (Qiagen Plasmid Maxi Kit).[21] Spectrophotometric DNA quantification was carried out assuming an absorptivity at 260 nm. DNA-cleavage screenings were carried out by incubating in a 20.0 μL final volume: pBSK-II plasmid DNA 330 ng/μl, corresponding to a final concentration of 25.0 μM bp (nucleotide base pairs), in the presence of different concentrations of compounds (50.0 and 100.0 μM) in different buffers and temperatures (PIPES, pH 7.0, 37 and 50 °C; HEPES, pH 8.0, 37 and 50 °C and CHES, pH 9.0, 37 and 50 °C) for 16 h (other conditions are depicted in the figure legends). Reactions were stopped by chilling tubes on ice and adding 5.0 μL of ice-cold loading-buffer (50% glycerol, 0.25% bromophenol blue, 25.0 mM EDTA pH 8.0). All tests were performed in duplicate. Reaction products were observed by agarose gel (0.8%) electrophoresis and stained with ethidium bromide. The resulting gels were scanned with a photodocumentation system (UVP, Inc.) and analyzed with the software LabWorks™ Software v4.0 (UVP, Inc.). The kinetic parameters were calculated from the non-linear fit of the pseudo Michaelis-Menten equation $k_{obs} = (k_{cat} \times [compound])/([compound] + K_M)$.
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