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Synthesis and DNA-Cleaving Activity of a Series of Substituted Arenediazonium Ions[1]

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Abstract—We investigated the reactions of substituted aryl radicals and aryl cations derived from arenediazonium ions and their ability to cause cleavage of supercoiled DNA and their tendency toward free radical or cation formation in the presence and absent of copper (I) chloride. It was found that the substituted arenediazonium salts can cleave supercoiled DNA to the open circular form II DNA and linear form III DNA. Results methodical studies indicate that both carbon-centered radicals and aryl cations participate in the cleavage pathways.

Keywords: Agaricus bisporus, dediazoniation, DNA cleavage, substituted aryl radicals, substituted aryl cations

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INTRODUCTION

Arendiazonium ions ($Ar–N_2^+$), such as those found in the common mushroom *Agaricus bisporous* were shown to be mutagenic and carcionogenic [1–12]. However, the mechanisms involved in their carcinogenicity remain unclear. Arenediazonium ions are chemically different from alkanediazonium ions: they are more stable. Their reactions include azo coupling, substitution of nitrogen by nucleophiles, or induction of free radical processes [13–14]. The degradation of arenediazonium ions, known as dediazoniation, may occur via two mechanisms, either heterolytic to form aryl cation or homolytic to form aryl radical. The latter requires the transfer of one electron from a reducing agent. Both processes can occur simultaneously; the heterolytic one can be activated thermally or photochemically, whereas the second one is favoured by strong reducers [15].

Stock et al. [16] found that the reaction of benzenediazonium ion with adenine, adenosine, or adenylic acid resulted in the formation of N^6 triazenes, while that with guanine and its derivatives, in the formation of C-8 azo coupled or C-8 arylated products and unstable N^2 triazenes [17]. The formation of a triazene adduct has also been considered to be an intermediate step in the arylation of adenine residues in DNA [18]. Stiborova et al. [19] determined that the reaction of benzenediazonium ion with guanine results in the formation of not only a C-8 azo-coupled product, 8-(phenylazo)guanine, but also in the formation of another product, 8-(biphenylazo)-guanine. The ability of arendiazonium ions to cleave DNA might result from the simultaneous action of arendiazonium ions and aryl radicals or aryl cation derived from them [7] (Scheme 1).

A direct attack on DNA by aryl radicals would explain the carcinogenic activity attributed to arenediazonium ions in the light of in vitro aryl cation caused by aryl radicals to both natural and synthetic nucleobases, nucleosides, and polynucleotides [20] and their ability to produce unspecific DNA cleavage [21–25]. It must be remembered that a number of reducers will increase the chance of aryl radical formation in the biological medium.

Some other pathways have been also suggested, for example the capacity of carbon-centered radicals derived from the *p*-methylbenzenediazonium ion to activate the AP-1 system and thus stimulate the phosphorylation of ERK1, ERK2, and p38 proteins in similar way to that observed for superoxide anion and hydroxyl radicals [26].

It is evident that the toxicity of arenediazonium ions derives from the appearance during the dediazoniation processes of very reactive compounds, the aryl radical and aryl cation, which are intermediates in the homolytic or heterolytic dediazoniation mechanisms, respectively [27]. It was suggested by many authors [28] that both mechanisms may operate simultaneously and competitively, depending on the experimental conditions under which dediazoniation occurs [29].

p-Hydroxybenzenediazonium is an arenediazonium ion present in the inedible mushroom *A. xanthodermus* [30]. It cleaves DNA [22] and is responsible for the formation of the C8-guanine adduct [31] and also causes tumors when repeatedly administered as a sulfate salt

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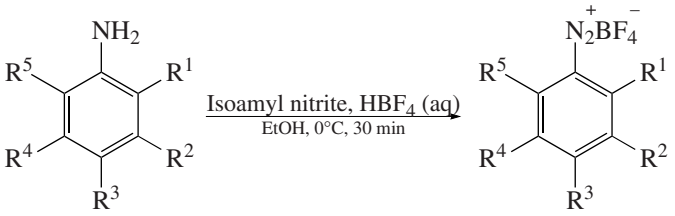


(1)–(10)

(1) $R^1 = R^2 = CH_3$, $R^3 = R^4 = R^5 = H$ **(2)** $R^1 = R^5 = CH_3$, $R^2 = R^3 = R^4 = H$ **(3)** $R^1 = R^5 = CH_2CH_3$, $R^2 = R^3 = R^4 = H$ **(4)** $R^1 = CH_2CH_3$, $R^2 = R^3 = R^4 = R^5 = H$

(5) $R^1 = R^3 = R^4 = R^5 = H$, $R^2 = CH_2CH_3$ **(6)** $R^1 = R^2 = R^4 = R^5 = H$, $R^3 = CH_2CH_3$ **(7)** $R^1 = Cl$, $R^3 = NO_2$, $R^2 = R^4 = R^5 = H$ **(8)** $R^1 = NO_2$, $R^2 = R^4 = R^5 = H$, $R^3 = Cl$

(9) $R^1 = CH_2CH_2OH$, $R^2 = R^3 = R^4 = R^5 = H$ **(10)** $R^1 = R^2 = R^4 = R^5 = H$, $R^3 = CH_2OH$

Scheme 1. Synthesis of arenediazonium salts (**1**)–(**10**).

by subcutaneous injection [32]. Moreover, it is known that the edible mushroom *A. bisporus* contains several arylhydrazides, arylhydrazines, and arenediazonium ions [33]. Arenediazonium ions occur naturally as the secondary metabolites. These ions can also be formed from other precursors, such as azo dyes [34–35] and aryl nitrosamines [13]. For example, 4-(hydroxymethyl)benzenediazonium (HMBD) induces sucutis and skin cancer in mice [36–37]. Hiramoto et al. [24] found that arenediazonium ions are reductively decomposed and form the carbon-centered aryl radicals.

Ganett et al. [38] have shown that arenediazonium ions react with cellular DNA, forming mainly C8-arylguanine adducts, including *p*-methyl, *p*-methoxymethyl, and *p*-hydoxymethyl derivatives. It was also suggested that the phenyl radicals and biradicals are generated in biological systems a intermediates of some potential drug candidates, whose target is DNA [39–41]. Radical attack on DNA is one of the main reasons for DNA damage [42–43]. It was found that aromatic σ,σ-biradicals play an important role in the action of some antitumor drugs, such as the enediyne type antitumor antibiotics. The key step in the DNA cleavage by such radicals is hydrogen atom abstraction from the sugar moiety in DNA [42–43]. In addition to aromatic biradical, many monoradicals formed upon metabolic conversion of organic compounds can also attack DNA by hydrogen atom abstraction and also by addition to a nucleobase. It was shown that OH and alkoxyl radicals can attack the C5=C6 double bond of the pyrimidine base moiety via addition or the sugar moiety via hydrogen abstraction in nucleosides, nucleotides, and DNA [42–44]. It was also reported that the thiyl radical can attack the C6 position of a pyrimidine nucleosides [45]. Furthermore, benzoyl peroxide can be metabolized to phenyl radicals, which can damage DNA [46].

However, the chemical behavior of phenyl radicals toward DNA and DNA components was not been extensively investigated [23]. A better understanding of the structural features and other factors that control the reactivity of phenyl radicals toward DNA and its components could greatly benefit the rational design of synthetic DNA cleavers and, hence, facilitate the development of more efficient and less toxic pharmaceuticals.

Obviously, the side reactions of radical intermediates with proteins, which may cause cytotoxicity and other unwanted side effects, are of great interest. Therefore, further studies of the ability of phenyl radical to damage DNA, amino acids, peptides, and eventually proteins, are clearly warranted. There are several aspects regarding arenediazonium ions chemistry and the mechanism of interaction with biological macromolecules, which remain partially unknown and become the subject of investigation nowadays. Among the aspects are the interaction between diazonium group and the rest of the molecule, the identity of the genotoxic agent, the exact site where the genotoxic agent induces the DNA damage and whether the damage is produced as a result of a direct attack of the genotoxic agent or occurs in a secondary process. It is also unknown, whether the DNA damages induced by arenediazonium ions really cause subsequent carcinogenic effects. The present study was devoted to explore the reactions of a series of substituted aryl radicals and aryl cations which are derived from arenediazonium salts, toward supercoiled DNA.

RESULTS

Synthesis of Arenediazonium Salts

We prepared various substituted arenediazonium tetrafluoroborates (**1**)–(**10**) in 22–89% yields by the reaction of the corresponding amine with isoamyl

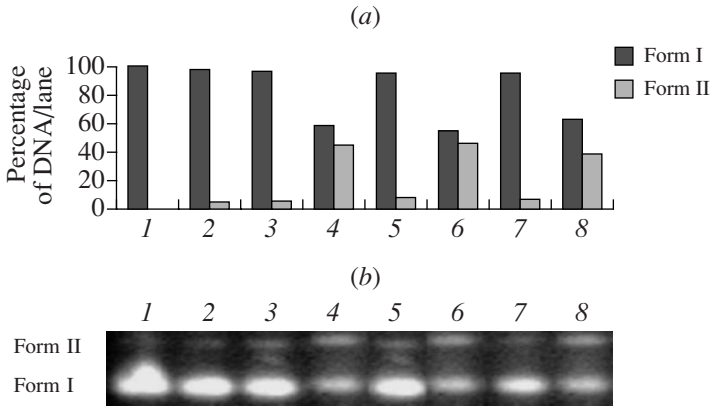


Fig. 1. *a* The quantified band intensity for the sc-DNA (form I), oc-DNA (form II), and l-DNA (form III) with Quantity One 4.5.2 version software. *b* Single-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA (form 1) to relaxed circular (form II) by arenediazonium salts (**1**)–(**3**). Lane *1*, control DNA; lane *2*, DNA + CuCl (3.1 mM); lane *3*, DNA + (**1**) (1.5 mM); lane *4*, DNA + (**1**) (1.5 mM) + CuCl (3.1 mM); lane 5, DNA + (**2**) (1.5 mM); lane 6, DNA + (**2**) (1.5 mM) + CuCl (3.1 mM); lane *7*, DNA + (**3**) (1.5 mM); lane *8*, DNA + 3 (1.5 mM) + CuCl (3.1 mM).

nitrite and aqueous fluoroboric acid in ethanol [46]. (Scheme 1).

DNA Cleavage

The DNA cleaving activity of arenediazonium salts (**1**)–(**10**) was evaluated by monitoring the conversion of circular supercoiled DNA (form I) to circular relaxed DNA (form II) or linear (form III) DNA.

pBluescript M13+ supercoiled DNA (Form I) was incubated with various arenediazonium salts in the presence and absence of copper(I) chloride at 37°C for 2 h in dark, and then the reaction mixtures were analysed by gel electrophoresis in 1% Agarose gel (Ethidium Bromide staining). Figure 1*b* shows the agarose gel picture of DNA cleavage induced by arenediazonium salts (**1**)–(**3**). The upper band represent the circular relaxed DNA (form II). The columns above the gel picture (Fig. 1*a*) correspond to the quantitified band intesity for the circular supercoiled DNA (form I) and the circular relaxed DNA (form II). Lane *1* is the control DNA; it represents the original structure of DNA with 98.42% of form I DNA and 1.58% of form II. Lane *2* represents a DNA incubation with an aqueous solution of copper(I) chloride to determine, whether copper(I) ions promotes the strand scission. Lane *3* represent the effect of arenediazonium salt (*1*) on DNA without copper(I) chloride. Cleavage of the DNA would indicate either the photolytic formation of aryl cation or direct alkylation of purines. DNA cleavage was not observed in this lane. Lane *4* represents an incubation of DNA with an aqueous solution of copper(I) chloride and aqueous solution of arenediazonium salt (**1**). This experiment was carried out to investigate the reduction of arenediazonium ion to aryl radical leading to the cleavage of the DNA strand. Lanes *2* and *3* show that neither copper(I) chloride nor arenediazonium salt (**1**) themselves can effect DNA cleavage.

The net DNA cleavage values in the presence of one-electron donor for the arenediazonium salts (**1**)–(**3**) with 2,3- and 2,6-dimethyl groups and 2,6-diethyl groups are 41.97%, 44.78% and 37.00%, respectively (table). This net DNA cleavage is due to DNA single-strand cleavage caused by (**1**)–(**3**) and copper(I) chloride. It is known that a nick or a single stand cleavage of the form I DNA converts it to the form II DNA. The absence of a band for the linear form III DNA suggests that the DNA cleavage is single rather than double-stranded. Otherwise, the linear DNA band would have appeared between the form I DNA and the form II DNA bands.

The cleaving efficiencies of (**1**)–(**3**) in TAE buffer in the presence of coper(I) chloride were found to be similar to each other, excect the arenediazonium salts (**1**) and (**2**) were more efficient for the induction of DNA relaxation than (**3**). The high potency of (**1** and (**2**) can be attributed to their methyl groups, which improve their ability to cleave DNA by facilitating the generation of aryl radical.

Our gel electrophoresis results indicate that the attachment of various electron-donating groups in *o*-, *m*-, and *p*-positions of benzene ring of an arenediazonium salt could enhance the DNA-cleaving activity, ethyl groups demonstrating the strogest effect among the groups. As shown in Fig. 2 and table, these are ethyl-containing arenediazonium salts (**4**), (**5**), and (**6**). Moreover, a direct comparision of net DNA cleavage allowed us to find that the net DNA cleavage was 80.03% for (**4**), 100% for (**5**), and 100% for (**6**). Thus, in the presence of copper(I) chloride, the corresponding ethyl-containing aryl radical generated in situ acted at the DNA cleavage more potently, than the aryl cation. During the investigation of a series of arenediazonium

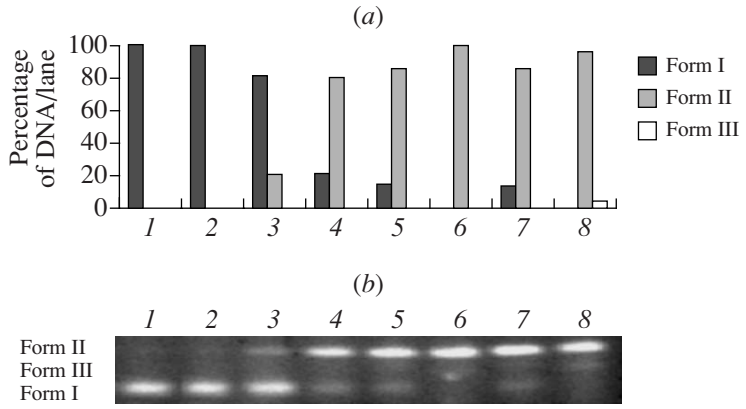


Fig. 2. *a* The quantified band intensity for the sc-DNA (form I), oc-DNA (form II), and l-DNA (form III) with Quantity One 4.5.2 version software. *b* Single- and double-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA (form 1) to relaxed circular (form II) and linear (form III) by arenediazonium salts (**4**)–(**6**). Lane *1*, control DNA; lane *2*, DNA + CuCl (3.1 mM); lane *3*, DNA + (**4**)(1.5 mM); lane *4*, DNA + (**4**)(1.5 mM)+ CuCl (3.1 mM); lane *5*, DNA + (**5**)(1.5 mM); lane *6*, DNA + (**5**)(1.5 mM) + CuCl (3.1 mM); lane *7*, DNA + (**6**) (1.5 mM); lane **8**, DNA + (*6*) (1.5 mM) + CuCl (3.1 mM).

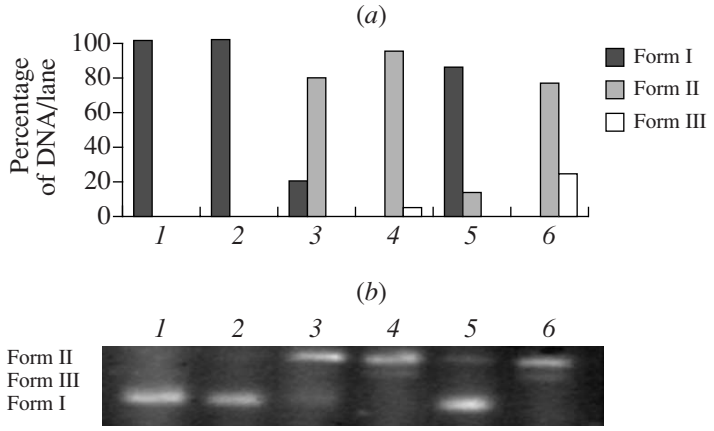


Fig. 3. *a* The quantified band intensity for the sc-DNA (form I), oc-DNA (form II), and l-DNA (form III) with Quantity One 4.5.2 version software. *b.* Single- and double-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA (Form 1) to relaxed circular (form II) and linear (form III) forms by arenediazonium salts (**7**) and (**8**). Lane *1*, control DNA; lane *2*, DNA + CuCl (3.1 mM); lane *3*, DNA + (**7**)(1.5 mM); lane *4*, DNA + (**7**)(1.5 mM) + CuCl (3.1 mM); lane *5*, DNA + (**8**) (1.5 mM); lane *6*, DNA + (**8**) (1.5 mM) + CuCl (3.1 mM).

salts with alkyl groups in benzene ring, it was noticed that compounds (**4**), (**5**), and (**6**) are unstable in daylight; these compounds showed a light dependent activity in plasmid relaxation assay for DNA cleavage. It is important to remind that we controlled all our experiments to protect all our samples from the exposure to light; in this way, we avoided the formation of carbocation from diazonium salts via a photochemical pathway. To avoid the effects of photoexcitation of samples, all the reactions were carried out in Eppendrof tubes protected from light by means of foil covering. Nevertheless, DNA cleavage was still observed for (**4**), (**5**), and (**6**) in the absence of copper(I) chloride (Fig. 2, lanes *3*, *5*, and *7*, and table). This confirms that the plasmid relaxation does not require an added electron donor (mediating homolytic dediazoniation of diazonium group) as shown for other diazonium reagents [20]. In that study, the authors observed DNA cleavage and the reaction was shown to be light-dependent.

Aqueous solutions of arenediazonium salts are known to be converted very easily on illumination into aryl cations, which are good alkylating agents. Alkylation of purine bases in DNA is widely used to trigger DNA cleavage. In this study, an enhanced DNA cleavage by (**4**), (**5**), and (**6**) (relative to the absence of copper(I) chloride) was observed (Fig. 2, cf. lanes *4*, *6* and *8* with lanes *3, 5* and *7*).

We carried out a comparative study of dediazoniation of *o*- nitro- and *p*-nitrochloroarenediazonium ions on DNA cleavage. The electron-withdrawing and donating properties of NO_2- and Cl groups strongly determine the reactivity of both compounds, thus exerting different influences upon dediazoniation reaction

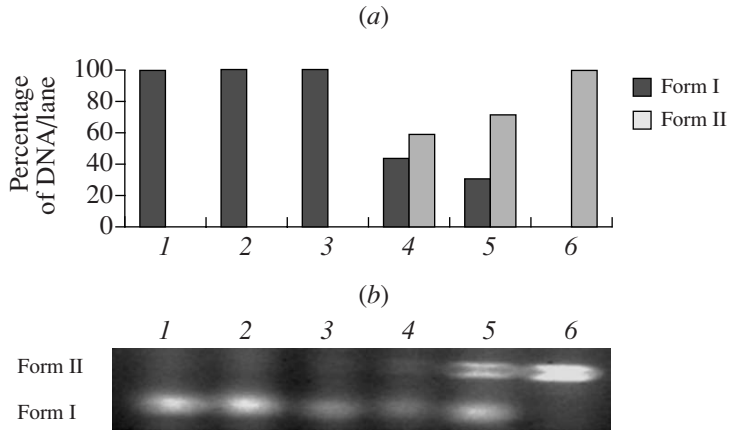


Fig. 4. a The quantified band intensity for the sc-DNA (form I), oc-DNA (form II), and l-DNA (form III) with Quantity One 4.5.2 version software. b Single-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA (form 1) to relaxed circular (form II) by arenediazonium salts (**9**) and (**10**). Lane *1*, control DNA; lane *2*, DNA + CuCl (3.1 mM); lane *3*, DNA + (**9**) (1.5 mM); lane *4*, DNA + (**9**) (1.5 mM) + CuCl (3.1 mM); lane *5*, DNA + (**10**) (1.5 mM); lane *6*, DNA + (**10**) (1.5 mM) + CuCl (3.1 mM).

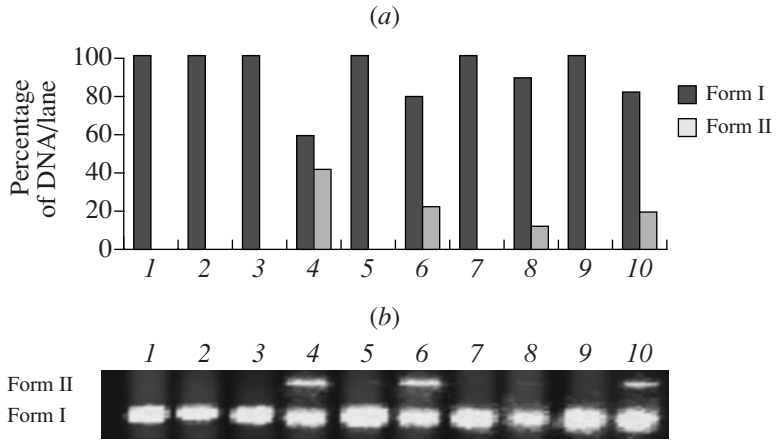
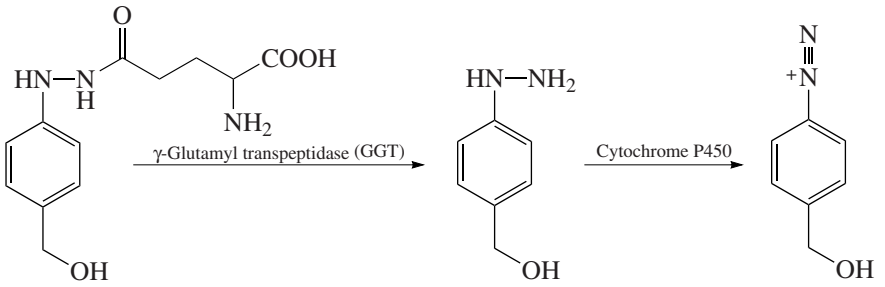


Fig. 5. *a* The quantified band intensity for the sc-DNA (form I), oc-DNA (form II), and l-DNA (form III) with Quantity One 4.5.2 version software. b Effects of ethanol, cysteine, and β-mercaptoethanol on the single-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA induced by arenediazonium salt. Lane *1*, control DNA; lane *2*, DNA + CuCl (3.1 mM); lane *3*, DNA + (**1**) (1.5 mM); lane *4*, DNA + (**1**) (1.5 mM) + CuCl (3.1 mM); lane 5, DNA + ethanol (2.5 mg/ml); lane *6*, DNA + (**1**) (1.5 mM) + ethanol (2.5 mg/ml); lane *7*, DNA + cystein (2.5 mg/ml); lane *8*, DNA + (**1**) (1.5 mM) + cysteine (2.5 mg/ml); lane *9*, DNA + β-mercaptoethanol (2.5 mg/ml); lane *10*, DNA + (**1**)(1.5 mM) + β-mercaptoethanol (2.5 mg/ml).

[46]. Figure 3 shows the DNA cleavage activity of compounds (**7**) and (**8**). The net DNA cleavage activity for (**7**) was found to be 100% and 79.69% in the presence and absence of copper(I) chloride, respectively. The net DNA cleavage activity for (**8**) was found to be 100% and 14.00% in the presence and the absence of copper(I) chloride, respectively (table). A chloro substituent is a strong π donor and a very strong σ electron acceptor, both π- and σ effects are important for stabilization of triplet aryl cations. Electron donor substituents always increase the electron density within the ring, thus stabilising the cation; of these the best are π electron donors in the order *para* and *ortho* > *meta* [47].

The most extensively consumed species of mushroom *A. bisporous* contains a number of aromatic hydrazines, among which the most abundant is agaritine, β-*N*-(γ-*L*(+)-glutamyl-4-(hydroxymethyl)phenylhydrazine. It occurs at concentrations as high as 1.7 mg/g of raw mushroom [48]. The postulated ultimate carcinogenic metabolite of agaritine is believed to be the highly reactive and mutagenic 4-(hydroxymethyl)benzenediazonium ion (HMBD) [49]. For this intermediate to be formed, the glutamyl moiety must be removed to release the free hydrazine that can presumably be oxidised to form a diazonium ion (Scheme 2).



Agaritine | 4-(Hydroxymethyl)phenylhydrazine | 4-(Hydroxymethyl)benzenediazonium ion

Scheme 2. Putative pathways of the agaritine bioactivation.

It was demonstarted that the 4-(hydroxymethyl)benzenediazonium ion found in edible mushroom *A. bisporous* [33] and *p*-hydroxybenzenediazonium ion in the inedible mushroom *A. xanthodermus* [31] cleave DNA and are responsible for the sucutis and skin cancer in mice [36]. Thus, a detailed knowledge of the mechanisms associated with the degradation process of arenediazonium ions would appear to be important for understanding of the genotoxic properties of this kind of compounds, particularly when the hemolytic pathway may compete with the heterolytic one for the producing potentially genotoxic agents. In addition, *p*-hydroxybenzenediazonium ion has a good electron-releasing group in *p*-position and an electron-attracting diazonium group, so that an effective electron transfer from the donor group to the acceptor might be expected. It must be remembered that arenediazonium compounds are genearlly very unstable and that they become more stable according to the nature of the substituents, especially if these are electron donors [50]. Hydroxy substitution contributes to the strength of the Ar–N_2 bond by means of a resonance mechanism, particularly, if the OH group is deprotonated.

The DNA cleavage ability of 2-(hydroxyethyl)benzenediazonium ion (**9**) and 4-(hydroxymethyl)benzenediazonium ion (**10**) was investigated. DNA cleavage was observed for (**9**) only in the presence of copper(I) chloride (Fig. 4, lane 4); thus, in the absence of copper(I) chloride, no DNA cleavage was observed for (**9**) (Fig. 4, lane *3*). The net DNA cleavage value for (**9**) was found to be 57.34% in the presence of copper(I) chloride (table). On the other hand, DNA cleavage was observed for 10 both in the presence and absence of copper (I) chloride (Fig. 4, lanes *5* and *6*). The net DNA cleavage value for (**10**) was found to be 100% and 69.88 % in the presence and absence of copper(I) chloride, respectively (table).

The potential involvement of carbon-centered radicals into DNA cleavage by arenediazonium salt (**1**) was investigated by trapping experiments using ethanol, cystein, and β-mercapto ethanol (Fig. 5, lanes 6, 8, and 10). It was found that DNA cleavage by arenediazonium salt (**1**) 1 was inhibited by ethanol, cystein, and β-mercapto ethanol. Inhibition of DNA cleavage by ethanol may be due to its scavenging activity toward the carbon-centered radical [24], but the mechanism of inhibition of cystein and β-mercapto ethanol may be different. We think that cystein and β mercapto ethanol reacted with arenediazonium salt (**1**) to form an unstable adduct, which may prevent the generation of the carbon-centered radical [51]. At 2.5-mg/ml concentration, cystein, a general radical trap, inhibited 72.13% of DNA cleavage. Similarly, ethanol and β-mercapto ethanol inhibited 49.57% and 57.33% of DNA cleavage, respectively (Fig. 6). The trapping studies are consisitent with the participation of carbon-centered radicals in the cleavage process by arenediazonium ion (**1**).

Effects of ethanol, cystein, and β-mercaptoethanol on the single-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA induced by arenediazonium salt (**1**). Percent of inhibition of the single-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA was calculated as follows [51]: inhibition (%) =

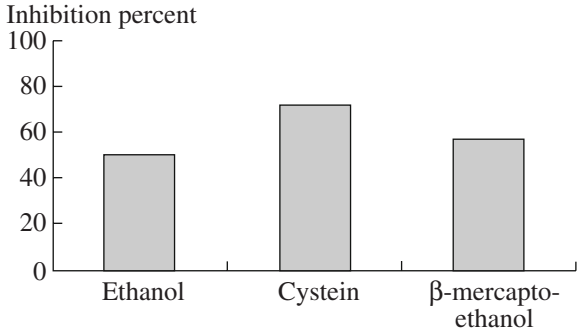


Fig. 6. Effects of ethanol, cystein, and β-mercaptoethanol on the single-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA induced by arenediazonium salt (**1**). Percent of inhibition of the single-strand cleavage of pBluescript M13+ (3.2 kb) supercoiled DNA was calculated as follows [51]: inhibition (%) = 100–[(Sm+a – Sc/(Sm–Sc)] where Sm+a is % remaining supercoiled after treatment with mix without agent, Sc is percent of supercoiled DNA remaining in control untreated plasmid, and Sm is percent of remaining supercoiled DNA in mixture without agent.

Net DNA cleavage by arenediazonium salts (**1**)–(**10**) in the presence and absence of one-electron donor (CuCl)

Donor	(**1**)	(**2**)	(**3**)	(**4**)	(**5**)	(**6**)	(**7**)	(**8**)	(**9**)	(**10**)
–	2.61	4.00	5.17	18.66	18.05	86.57	79.69	14.00	0	69.88
+	41.97	44.78	37.00	80.03	100	100	100	100	57.34	100

Note: The percentage of net DNA cleavage was calculated by the following equation: $\{[(\text{Form II})_s + (\text{Form III})_s] - [(\text{Form II})_c + (\text{Form III})_c]/[(\text{Form II})_s + (\text{Form III})_s]\} \times 100\%$. The subscripts 's' and 'c' refer to the samples and controls, respectively.

1−[(Sm+a – Sc/(Sm–Sc)] where Sm+a is % remaining supercoiled after treatment with mix without agent, Sc is percent of supercoiled DNA remaining in control untreated plasmid, and Sm is percent of remaining supercoiled DNA in mixture without agent.

The net DNA cleavage by arenediazonium salt (**6**) was found to be 86.57 % in the absence of one electron donor, copper(I) chloride. This value was the highest among the all tested arenediazonium salts in the absence of copper(I) chloride (table). Therefore, to access the potential role of carbon-centered radicals in the reaction that lead to DNA cleavage by (**6**), radical trapping experiments using ethanol, cystein, and β-mercaptoethanol were carried out. The DNA cleavage was inhibeted by none of these radical scavengers. This suggests that carbon-centered cation may be involved in the pathway(s) leading to the strand scission (data not shown). These results suggest that DNA damage induced by arenediazonium ions may arise not only due to aryl radicals, but also aryl cation production.

Among the arenediazonium salts (**1**)–(**10**), only salts (**6**)–(**8**) were the molecules that performed the double-strand scission in the presence of copper(I) chloride (Fig. 2, lane *8*, Fig. 3, lanes *4*, *6*). In comparision with single-strand cleavers (**1**)–(**5**) and (**9**), (**10**), stronger potency for compounds (**6**), (**7**), and (**8**) could be due to the great reactivity associated with the resulting radicals.

Arenediazonium salts

Net DNA cleavage in the absence of one electron donor (%)

Net DNA cleavage in the presence of one electron donor (%)

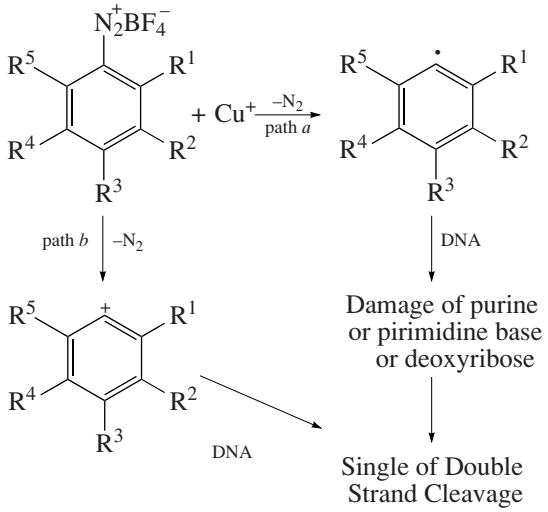
DISCUSSION

We prepared a series of substituted arenediazonium salts in order to explore the role of substituted aryl radical and aryl cation on the DNA-cleavage efficiency. Consequently, we used in investigation arenediazonium salts containing an electron-donating (+M) group, such as methyl and ethyl, and also both electron-donating (+M) group and electron-withdrawing (–M) group, such as NO_2 and Cl, in the same molecule. We have also investigated the DNA cleavage ability of 4-(hydroxymethyl)benzenediazonium ion [36] (**9**) and *o*-(hydroxyethyl)benzenediazonium ion (**10**). Our results in table and table show that the efficiency, indicated by net DNA cleavage, depending upon the substitutents attached to the aryl unit. The electrical effects [52] resulting from substituents attached to the aromatic rings of arenediazonium salts could play important roles in the DNA cleaving process.

Aryl substitution does affect the reactivity and rate of deprotonation by aryl radicals [53]. A carboxylic acid or carboxylate substitution was calculated to result in a bond dissociation energy of the *p*-hydrogen of 113.8 kcal/mol and 112.3 kcal/mol, respectively [54], which is comparable to the experimental bond dissociation energy value of 110.6 ± 3.4 kcal/mol for the *p*-hydrogen of phenyl radical [55] in gas phase. The influence of the environment on the reaction of the thermal dediazotization in acidic methanol solutions was investigated by Bunnett et al. [56]. In nitrogen atmosphere, the dediazotization is homolytical, whereas, under oxygen, the reaction proceeds heterolytically via the formation of an arene cation. The presence of strong electron acceptors in the phenyl ring favors the homolytical type of the reaction via the intermediate formation of phenyl radical. Due to the presence of diazo group, arenediazonium salts are highly reactive and widely used in the chemical synthesis of azodyes [57], in preparative synthesis [58], and in analytical methods [59]. Arenediazonium ions can undergo either thermal or photochemical heterolytic dediazoniation, yielding the aryl cation [60]. These ions can also be dediazoniated in a homolytic process via one-electron reduction, thus generating aryl radicals.

Although most arenediazonium ions are attributed to oxidants, such structural characteristics as the electron-donating and withdrawing properties of substituents in the aromatic ring, together with interference from solvent interactions, other reducing and oxidising compounds, light, or the conditions of the reaction medium may substantially modify their reactivity, giving rise to different patterns of decomposition and resulting in different biological effects. In this context, heterolytic dediazoniation has been reported to occur with *p*-methylbenzenediazonium and *p*-nitrobenzenediazonium ions in an aqueous medium [61–62], while other authors interpreted their results as indicating heterolytic and homolytic processes during the thermal and photochemical dediazoniation of several arenediazonium ions in trifluoroethanol and ethanol [63, 64]. Furthermore, in certain cases, the reducing capacity of water was sufficient to reduce arenediazonium to the aryl radical [65]. It is therefore obvious that the reaction

conditions need to be chosen carefully when trying to establish the mechanisms involved in the decomposition of such reactive compounds as arenediazonium ions.



Scheme 3. The possible reaction of arenediazonium salts with DNA through generation of aryl radicals (path *a*) or aryl cation (path *b*).

The possible mechanism for DNA strand–breaking activity of arenediazonium salts are shown in Scheme 3. The degradation of arenediazonium ions, known as dediazoniation, may ocur via two mechanisms, either heterolytic (Scheme 3, path b) or homolytic (Scheme 3, path *a*). The latter requires the transfer of electron from a reducing agent. In the absence of catalyst, with weakly basic nucleophiles, in the presence of O_2, in aqueous acid, and in the dark, the dediazoniation reactions are believed to proceed via rate-determining loss of N_2, generating a highly reactive phenyl cation, which reacts rapidly and with a very low selectivity with available nucleophiles [66]. The DNA cleavage by aryl radical involves a deprotonation from deoxyribose sugar, wheraes DNA cleavage by aryl cations, which are capable of behaving as alkylating agents, includes alkylation of purine bases. The alkylation of purine bases in DNA triggers the DNA cleavage.

CONCLUSIONS

The ability of substituted arenediazonium ion to cleave DNA was studied. It is suggested that the toxicity of the arenediazonium salts may be due to generation of reactive aryl radicals damaging biomolecules. All the arenediazonium salts tested can cause a certain amount of DNA cleavage, although their cleaving potency is different from each other. Among various substituents in the benzene ring of an arenediazonium salt, our results on gel electrophoresis indicate that attachment of an electron-donating (+M) group, such as ethyl and also both electron-donating (+M) group and electron-withdrawing (–M) group such as NO_2 and Cl in the same molecule exhibit an appealing potency. Moreover, it is possible that the damage of DNA may be genotoxic, in addition to acute toxicity. There are very little data available for the toxicity of the substituted arenediazonium salts. Our results indicate that the carbon-centered aryl radical and aryl cations, generated by arenediazonium salts, are active species for DNA scission. Application of the process to specific DNA sequences promises to lead to new possibilities in the design of anticancer and chemical nucleases.

EXPERIMENTAL

Caution: Arenediazonium ions are tumorigenous and should be handled with care. The completly dry arenediazonium ions are shock sensitive any may explode.

Melting points were measured on a Gallenkamp Model apparatus in open capillaries. IR spectra were recorded in KBr pellets using a FTIR Unicam spectrophotometer. ^{1}H- and ^{13}C NMR spectra were measured in CD_3COCD_3 at 400/100 MHz on a Bruker NMR spectrometer; chemical shifts are reported in δ scale (ppm). A DNA Agarose Gel Electrophoresis was performed on a Bio Rad Gel Doc Electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA United States). Quantitation of bands in the gels were accomplished with Quantity one 4.5.2 version software.

General procedure for synthesis of arenediazonium salts (1–10). The diazonium tetrafluoroborates were synthesised according to the procedure described in [46] and stored at –18°C in darkness before use. The corresponding to (**1**)–(**10**) amine (2.0 mmol) was dissolved in ethanol (1 ml) and aqueous fluoroboric acid (48% solution, 6.0 mmol) was added to the solution; the mixture was then cooled to –5°C. Isoamyl nitrite (2.50 mmol) was added dropwise to the reaction mixture, and stirring continued for 0.5 h. Dilution of the mixture with diethyl ether (30 ml) led to precipitation. The solid was collected by filtration under nitrogen and washed with cold diethyl ether. The crude products were recrystallised from acetone–diethyl ether to give a pure sample. The physical and spectra data of compounds (**1**)–(**10**) are as follows:

2,3-Dimethylbenzenediazonium tetrafluoborate (1); colorless crystals (0.34 g, 74%); mp 69–71°C; 69–71°C; ν_{max} (cm^{-1}): 3043, 2932, 2264, 1609, 1562, 1094, and 812; ^{1}H NMR (400 MHz, CD_3COCD_3): 7.12 (1 H, dd, *J* 8.2, 1.1 Hz, Ar*H*), 7.05 (1 H, ddd, *J* 8.0, 1.1 Hz, Ar*H*), 6.9 (1 H, dd, *J* 8.2, 1.1 Hz, ArH), 2.28 (3 H, s, CH_3), 2.16 (3 H, s, C*H*$_3$); ^{13}C NMR (100 MHz, CD_3COCD_3): 9.7 (q), 18.6 (q), 112.2 (d), 122.9 (d), 125.2 (d), 139.1 (s), 159.9 (s), 162.3 (s).

2,6-Dimethylbenzenediazonium tetrafluoborate (2); colorless crystals (0.10 g, 22%). mp 86–87°C; ν_{max} (cm^{-1}): 3063, 2932, 2257, 1610, 1584, 1080, 822; ^{1}H NMR (400 MHz, CD_3COCD_3): 7.06 (1 H, ddd, *J* 8.0, 8.0, 1.2 Hz, Ar*H*), 6.6 (2 H, dd, *J* 8.3, 1.5, ArH), 2.9

(6 H, s, C*H*$_3$); ^{13}C NMR (100 MHz, CD_3COCD_3): 14.6 (q), 119.4 (d), 123.6 (d), 128.2 (s), 129.5 (s).

2,6-Diethylbenzenediazonium tetrafluoborate (3); colorless crystals (0.26 g, 51%); mp 99–101°C; ν_{max} (cm^{-1}): 3063, 2932, 2251, 1630, 1576, 1086, 816; ^{1}H NMR (400 MHz, CD_3COCD_3): 7.06 (1 H, ddd, *J* 8.0, 8.0, 1.2 Hz, Ar*H*), 6.9 (2 H, dd, *J* 8.3, 1.5, Ar*H*), 2.9 (4 H, m, C*H*$_2$), 2.6 (4 H, m, C*H*$_3$); ^{13}C NMR (100 MHz, CD_3COCD_3): 13.5 (q), 15.6 (q), 119.4 (d), 123.6 (d), 128.2 (s) 128.9 (s).

2-Ethylbenzenediazonium tetrafluoborate (4); light blue crystals (0.30 g, 65%). mp 73–74°C; ν_{max} (cm^{-1}): 2978, 2937, 2264, 1641, 1562, 1094, 776; ^{1}H NMR (400 MHz, CD_3COCD_3) 9.5 (1 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 9.3 (1 H, ddd, *J* 8.1, 8.1, 1.6 Hz, Ar*H*), 9.2 (1 H, dd, *J* 8.2, 1.1 Hz, Ar*H*), 9.0 (1 H, ddd, *J* 8.0, 8.0, 1.2 Hz, Ar*H*), 3.4 (2 H, m, C*H*$_2$CH$_3$) 1.6 (3H, t, *J* 4.8 CH$_2$C*H*$_3$); ^{13}C NMR (100 MHz, CD_3COCD_3): 13.7 (q), 22.9 (t), 114.7 (d), 119.5 (d), 126.6 (d), 129.1 (d), 130.2 (s), 209.0 (s).

3-Ethylbenzenediazonium tetrafluoborate (5); pink crystals (0.25 g, 55 %). mp 68–70°C; ν_{max} (cm^{-1}): 2970, 2932, 2270, 1642, 1470, 1080, 803; ^{1}H NMR (400 MHz, CD_3COCD_3): 8.8 (1 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 8.7 (1 H, ddd, *J* 8.1, 8.1, 1.6 Hz, Ar*H*), 7.2 (1 H, dd, *J* 8.2, 1.1 Hz, Ar*H*), 6.9 (1 H, ddd, *J* 8.0, 8.0, 1.2 Hz, Ar*H*), 2.9 (1 H, s, *J* 7.5, ArH) 2.6 (2 H, m, C*H*$_2$CH$_3$), 1.2 (3 H, t, *J* 4.8 CH$_2$C*H*$_3$); ^{13}C NMR (100 MHz, CD_3COCD_3): 14.0 (q), 112.4 (d), 114.6 (d), 186.9 (d), 129.2 (d), 145.7 (s), 157.3 (s).

4-Ethylbenzenediazonium tetrafluoborate (6); yellow crystals (0.20 g, 44%); mp 87–88°C; ν_{max} (cm^{-1}): 2978, 2932, 2264, 1641, 1584, 1086, 849; ^{1}H NMR (400 MHz, CD_3COCD_3): 7.8 (2 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 7.5 (2 H, dd, *J* 8.1, 1.5 Hz, Ar*H*), 3.0 (2 H, m, C*H*$_2$CH$_3$) 1.3 (3 H, t, *J* 4.8, CH$_2$C*H*$_3$); ^{13}C NMR (100 MHz, CD_3COCD_3): 13.9 (q),105.0 (t), 111.7 (s) 131. (d), 133.0 (d), 160.7 (s).

2-Chloro-4-nitrobenzenediazonium tetrafluoborate (7); colorless crystals (0.15 g, 60%); mp 162–163°C; ν_{max} (cm^{-1}): 3063, 2991, 2276, 1641, 1562, 1080, 843; ^{1}H NMR (400 MHz, CD_3COCD_3): 9.27 (1 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 9.01 (1 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 8.82 (1 H, s, Ar*H*); ^{13}C NMR (100 MHz, CD_3COCD_3): 121.78 (s), 124.88 (s), 127.33 (d), 136.88 (d), 138.52 (d), 154.48 (s).

4-Chloro-2-nitrobenzenediazonium tetrafluoborate (8); colorless crystals (0.17 g, 68%). mp 125–127°C; ν_{max} (cm^{-1}): 3063, 2967, 2284, 1649, 1562, 1344, 1173, 849; ^{1}H NMR (400 MHz, CD_3COCD_3): 9.2 (1 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 8.7 (1 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 7.4 (1 H, s, Ar*H*); ^{13}C NMR (100 MHz, CD_3COCD_3): 118.6 (s), 124.9 (s), 129.01 (d), 136.88 (d), 137.96 (d), 149.64 (s).

2-(Hydroxyethyl)benzenediazonium tetrafluoborate (9); yellow crystals (0.30 g, 62%); mp 118–120°C; ν_{max} (cm^{-1}): 2924, 2507, 2264, 1603, 1497, 1067, 756; ^{1}H NMR (400 MHz, CD_3COCD_3): 7.9 (1 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 7.7 (1 H, ddd, *J* 8.1, 8.1, 1.6 Hz, Ar*H*), 7.6 (1 H, dd, *J* 8.2, 1.1 Hz, Ar*H*), 7.5 (1 H, ddd, *J* 8.0, 8.0, 1.2 Hz, Ar*H*), 3.9 (2 H, t, *J* 4.8 Hz, CH$_2$C*H*$_2$OH), 3.48 (2 H, m, C*H*$_2$CH$_2$OH), 1.13 (1 H, t, CH$_2$O*H*); ^{13}C NMR (100 MHz, CD_3COCD_3): 54.86 (t), 55.9 (t), 57.1 (s), 60.9 (d), 123.25 (d), 130.5 (d), 134.95 (d), 141.02 (s).

4-(Hydroxymethyl)benzenediazonium tetrafluoborate (10) [46]; mustard colour crystals (0.40 g, 89%). mp119–121°C; ν_{max} (cm^{-1}): 2932, 2594, 2270, 1589, 1516, 1067, 830; ^{1}H NMR (400 MHz, CD_3COCD_3): 8.7 (1 H, dd, *J* 8.3, 1.5 Hz, Ar*H*), 7.9 (1 H, dd, *J* 8.1, 8.1, 1.6 Hz, Ar*H*), 7.6 (1 H, dd, *J* 8.2, 1.1 Hz, Ar*H*), 7.5 (1 H, dd, *J* 8.0, 8.0, 1.2 Hz, Ar*H*), 4.9 (2 H, d, *J* 7.5, C*H*$_2$OH), 1.2 (1 H, t, CH$_2$O*H*); ^{13}C NMR (100 MHz, CD_3COCD_3): 62.7 (t), 112.3 (s), 128.5 (d), 132.9 (d), 158.67 (s).

Purification of plasmid DNA. The plasmid pBluescript M13+ DNA was prepared and isolated according to standard protocols using Qiagene plasmid mini preparation kit (Qiagene) [67]. The purity of pBluescript M13+ was confirmed via both Agarose Gel Electrophoresis and UV spectroscopy by determining the ratio of absorbances at 260 and 280 nm [68]. The concentration of DNA was determined from the absorbance at 260 nm (A_{260} = 1.0 for 50 μg/ml).

Densitometric analysis of treated and control pBluescript M13+ plasmid DNA Gel was scanned by a Gel documentation system (Gel-Doc-XR, BioRad, Hercules, CA, United States). Bands in the gel were quantified using discovery series Quantity One programme (version 4.5.2, BioRad Co.).

DNA cleaving activity. The ability of compounds (**1**)–(**10**) to cleave DNA was investigated using a plasmid relaxation assay to monitor the conversion of circular supercoiled pBluescript M13+ DNA (form I) into the relaxed circular (form II) and linear DNA (form III). Plasmid relaxation require an added electron donor (mediating homolytic dediazoniation of diazonium group) as shown for other diazonium reagents. Like in similar to previous studies [21, 40], we examined inorganic and organic one-electron donors such as copper(I) chloride, sodium iodide, and ascorbic acid in order to reduce the arenediazonium salts to aryl radicals and to optimize conditions for efficient DNA cleavage. It was found that copper(I) chloride works the best [21] (data not shown).

To investigate their DNA-cleaving potency, supercoiled pBluescript M13+ DNA (200 ng) was placed in 0.5-ml Eppendorf tubes protected from light by means of a foil cover and used in dark room. The DNA (200 ng) was treated with a solution of diazonium salt (1.5 mM) in dark in the presence of copper(I) chloride (1.5 mM), which acted as a one-electron donor. The DNA was also exposed to control solutions of the diazonium salt with none of these additives and to each of the additives separately with no diazonium salt added. After incubation for 2 h, 5 μl of a gel-loading dye solu-

tion [48] (containing 0.1% Bromophenol Blue, 150 mM EDTA, 1% SDS in 2 mM Tris, 1 mM acetate, pH 8) was added into each well before loading (in dark) onto 1% Agarose gel containing an aqueous Ethidium Bromide solution (10 mg/ml). The gel was electrophoresed for 2 h at 120 V in 1 × TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.2) buffer. Incubations with arenediazonium salts were carried out for 2 h using the following final concentrations: 31.53 nM DNA, 0.375 mM arenediazonium salt, and 0.775 mM copper chloride. DNA in gel was analyzed with BioRad Gel Doc XR Gel-Documentation system. The amount of supercoiled form I DNA (sc-DNA), the relaxed open circular form II DNA (oc-DNA), and linear form III DNA (l-DNA) were quantified by the intensity of the bands with Quantity One programme (version 4.5.2, BioRad Co.).

The percentage of net DNA cleavage was calculated by the following equation: $[(\text{Form II})_s + (\text{Form III})_s] - [(\text{Form II})_c + (\text{Form III})_c]$. The subscripts 's' and 'c' refer to as the samples and controls, respectively [51].

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