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Photoinduced DNA cleavage by fullerene–lysine conjugate

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ABSTRACT

A novel water-soluble [60] fullerene-substituted lysine derivative **3** has been synthesized and characterized by elemental analysis, ^{1}H NMR, ^{13}C NMR and FAB-MS. The synthetic procedure involved condensation of Boc-protected lysine with terephthaldehyde followed by 1,3-dipolar cycloaddition reaction with C_{60} in the presence of sarcosine and finally deprotection of the amino group using trifluoroacetic acid. The synthesized compound **3** exhibited high DNA cleavage efficiency upon visible light irradiation in the presence of NADH.

Recent years have seen the potential utility of fullerenes as pharmaceuticals with a wide variety of applications such as antibacterials,[1] anti-HIV agents,[2] anti-inflammatory[3] and antiapoptosis agents.[4] We have recently reported some of the novel derivatives of fullerene with potential application as antibacterials[5] and anti-TB agents.[6] Of all the reported activities of C_{60}, the DNA-cleaving activity and lipid peroxidation, in particular, have attracted considerable attention.[7] Photoirradiation of C_{60} results in the formation of the singlet excited state $^{1}C_{60}^{*}$, which undergoes efficient intersystem crossing (ISC) to give the triplet excited state $^{3}C_{60}^{*}$.[8] There are several possible pathways for the DNA-cleaving process involving $^{3}C_{60}^{*}$ to occur (a) via superoxide anion, (b) via singlet oxygen or (c) direct oxidation of DNA.[9] Two reasons for the interest in the incorporation of fullerene into molecular structures of biological importance are the highly hydrophobic nature and unusual physico-chemical properties of fullerenes, making them ideal candidates as interesting pharmacophores. Unfortunately, the direct use of fullerenes in biological applications is limited by their poor solubility in aqueous media.[10] To overcome this obstacle, two different approaches have been adopted to increase the solubility. The first strategy involves non-covalently encapsulating fullerene molecules into soluble polymeric or host molecules.[11] The second strategy relies on covalent functionalization of fullerene by the introduction of hydrophilic groups by chemical modification.[12] The latter approach is attracting more interest as it can not only alter the physical and chemical properties of fullerene to readily achieve the desired properties, but also provide useful building blocks for further molecular constructions. It is this latter approach that has piqued our interests due to the potential of using a fullerene-based amino acid derivative for the systematic creation of nano bio-conjugates with the application as DNA-photocleaving agent. Amino acids are the most basic and essential building units for living organisms at all levels. The incorporation of fullerene-based amino acids into proteins, peptides or antibodies could lead to new applications in medicinal chemistry. To date, several approaches have been taken towards synthesizing fullerene-based amino acids.[13]

In parallel L-lysine derivatives possessing a chromophore have been found to induce efficient and highly selective cleavage of double stranded DNA upon photoexcitation.[14] Previous efforts to develop intercalator-based probes utilizing the diverse chemistry of amino acids for novel DNA-binding reagents have yielded compounds with nuclease activity. However, in most of the systems described to date, the intercalating moiety functions solely to deliver the peptides with low intrinsic binding affinity to DNA and does not contribute to chemical reactivity. Hence if we attach fullerene to lysine, DNA cleavage activity from both the photoexcited C_{60} and appended amino acids can be conceived. To the best of our knowledge, no examination of DNA cleavage by fullerene amino acid conjugate has been performed. With these points in view we have synthesized a novel fullerene lysine conjugate **3** by a simple and modular strategy. Moreover, the positive charge that compound **3** carries can help in better interaction with the negatively charged DNA.

The synthetic procedure for fullerene–lysine conjugate **3**[15] involved the condensation of Boc-protected lysine with terephthalaldehyde followed by prato's 1,3-dipolar cycloaddition reaction with C_{60} to form fulleropyrrolidine **2** and finally deprotection of amino group by treating with trifluoroacetic acid to get fulleropyrrolidine

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3 (Scheme 1). The synthesized fulleropyrrolidine **3** showed good solubility in water and also fluorescence quantum yield that are comparable to C_{60}. Fulleropyrrolidines **2** and **3** were characterized by elemental analysis, FTIR, ^{1}H NMR, ^{13}C NMR and FAB-MS.

FTIR spectra of both fulleropyrrolidines **2** and **3** showed peaks corresponding to fullerene moiety, azomethine linkage and carbonyl group. A single peak corresponding to N–H stretching was observed for **2** while two broad peaks corresponding to N–H stretching were obtained for **3** indicating the deprotection of amino group in **3**.

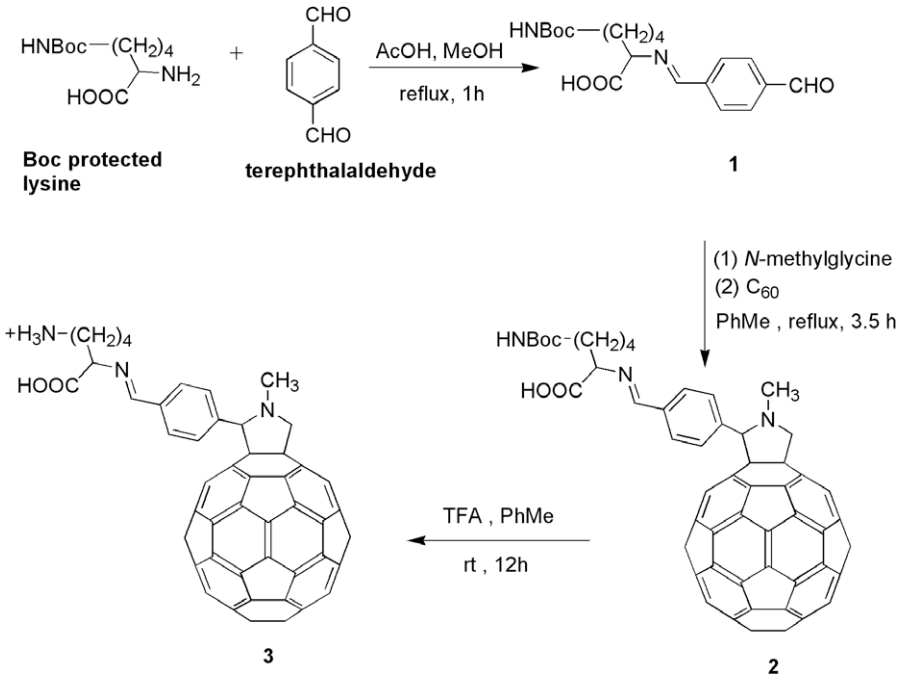
^{1}H NMR spectra of **3** (Fig. 1) showed singlets at δ 12.1, 8.23 and 7.80 corresponding to the carboxylic proton (H1), proton of azomethine linkage (H2) and protons of NH_3^+ (H3), respectively. Other peaks such as doublets for aromatic protons and protons of pyrrolidine ring were also observed. All these peaks were also observed in compound **2** except the peak of NH_3^+.

In ^{13}C NMR spectra of **3** (Fig. 2), apart from the peaks corresponding to C of carboxylic and azomethine groups, 29 signals were observed in the range of δ 150 to 130 corresponding to sp^2 C's of C_{60}. Two peaks at δ 75.2 and 68.4 were also observed for the sp^3 C's of C_{60}.

The DNA cleavage activity of **3** was investigated using pBR322 supercoiled plasmid DNA (form 1) in a **3**-NADH system under visible light irradiation.[16] As shown in Figure 3, buffer solution (pH 8.0) of **3**-NaDH system was found to cleave pBR322 supercoiled DNA into nicked DNA (form 2) after 3 h visible light irradiation at 298 K (Lane 3). Under dark condition no DNA cleavage was observed (Lane 5). When the same experiment was carried out with the intermediate lysine-terephthalaldehyde conjugate **1**, no DNA cleavage was observed either on photoirradiation or in dark (Lanes 6, 7 and 8). In control experiments, it was found that no DNA cleavage was found to occur in dark or under photoirradiation in the absence of either **3** or NADH (Lane 1, 2, 4), showing the importance of both NADH and compound **3** in the cleavage activity. Effect of visible light irradiation time on DNA cleavage was analyzed by changing the irradiation time from 1 to 6 h. The amount of nicked DNA was found to increase with the prolongation of irradiation time. After 6 h of irradiation the percentage of nicked DNA almost reaches saturation (see ESI). The concentration of **3** and NADH has been investigated to obtain a better understanding of the DNA cleavage by the synthesized fullerene–lysine conjugate **3**. The yield of nicked DNA improved drastically with the increase in the concentration of **3**. At a low concentration (1×10^{-6} M), only a small amount of supercoiled plasmid DNA is converted to nicked DNA, while the conversion ratio of supercoiled plasmid DNA is improved dramatically as the concentration of **3** is increased to 1.0×10^{-5} M which is much lower than that reported for γ-cyclodextrin-bicapped C_{60} (CD/C_{60}) by Wang et al.[7a] The conversion ratio of supercoiled plasmid DNA is quite small at a low NADH concentration (1.0×10^{-5} M–1.0×10^{-4} M), while the proportion of nicked DNA was substantially increased at a high NADH concentration (1 mM), consistent with the previous results that NADH is an important coagent for the photoinduced cleavage of DNA by fullerenes. In order to analyze the role of positive charge on compound **3** in cleavage activity, the same experiments were also carried out with intermediate compound **2**. Because of low solubility, a suspension of compound **2** in water was prepared for biological studies. Under the same experimental conditions, that is, with photoirradiation and in the presence of NADH, compound **2** was also found to cleave DNA but only at concentration higher than 1 mM, which is about 100 times more than that required for compound **3**. This difference in activity clearly indicates the better interaction of positively charged compound **3** with negatively charged DNA.

The effect of pH on DNA cleavage was also analyzed by varying the pH from 7 to 9. The DNA-cleaving efficiency was found to increase from pH 7 to 8, reached its maximum at pH 8 and then declined at pH 8–9. Therefore pH 8 is the optimum pH for photoinduced DNA cleavage by **3**-NADH system.

The photoinduced DNA cleavage depends greatly on the generation of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\cdot-}$) and hydroxyl radical (**.**OH), which are proposed to be the actual active species for the photoinduced DNA cleavage. The triplet excited state C_{60} ($^3C_{60}^*$) generated via the intersystem crossing from the singlet excited state of C_{60}, which is produced upon light irradiation, is a key intermediate



Scheme 1. Synthetic route of fullerene–lysine conjugate **3**.

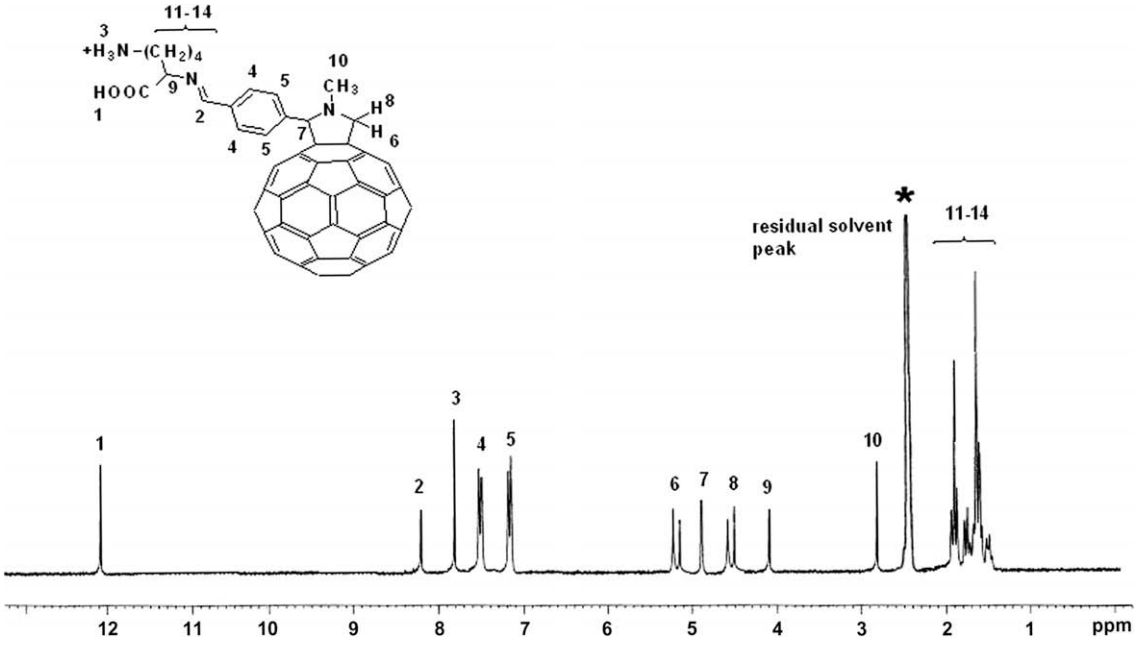


Figure 1. ^{1}H NMR spectra of **3** in DMSO-d_6 at 25 °C.

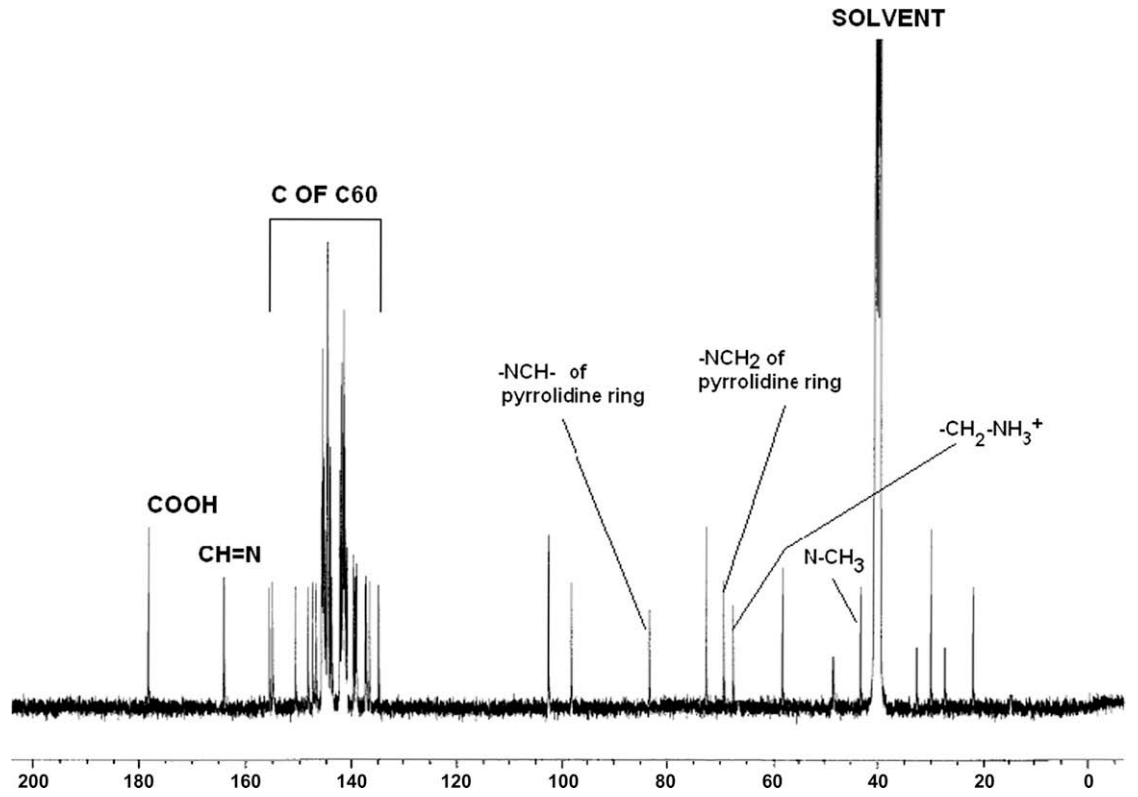


Figure 2. ^{13}C NMR spectra of **3** in DMSO-d_6 at 25 °C.

for the generation of ROS. Either the energy transfer process can occur, where the energy is transferred from $^{3}C_{60}^{*}$ to the oxygen molecule to generate $^{1}O_2$, or the electron transfer process can occur in the presence of NADH, where $^{3}C_{60}^{*}$ is reduced to produce C_{60} anion radical ($C_{60}^{\cdot-}$), followed by an electron transfer from $C_{60}^{\cdot-}$ to the oxygen molecule to give rise to $O_2^{\cdot-}$ or ·OH in a further step. However, the aggregation of C_{60} has been shown to significantly accelerate the decay of $^{3}C_{60}^{*}$, resulting in less interaction time between $^{3}C_{60}^{*}$ and oxygen molecule, thus reducing the generation of $^{1}O_2$. Zhang et al. have shown that larger aggregation destabilizes C_{60} anion radical suggesting that the C_{60} radical anion generated by reducing $^{3}C_{60}^{*}$ with NADH would be less stable in the more aggregated C_{60} solution, thus impairing the generation of $O_2^{\cdot-}$ and **.**OH. Water-soluble fullerenes are found to be easily aggregated in aqueous solution, where the hydrophobic force between fullerene surfaces is proposed to be the driving force for such aggregation. Wang et al.[7a] have shown that although the hydrophobic force that drives the aggregation of C_{60} molecules can also enhance

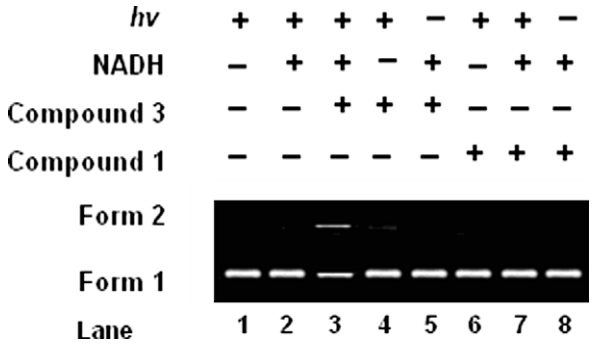


Figure 3. Photocleavage of pBR322 supercoiled DNA by fullerene–lysine conjugate **3** analyzed by agarose gel electrophoresis. [**3**] = 1×10^{-5} M, [**1**] = 1×10^{-5} M, [NADH] = 1×10^{-3} M.

the interactions between C_{60} and DNA since DNA bases are hydrophobic, smaller the size of aggregated particles more is the efficiency in photoinduced DNA cleavage. Hence dynamic light scattering (DLS) experiment was carried out to analyze the size of the aggregated particles of **3** if any. The concentration for DLS analysis was taken as 1.0×10^{-5} M, the same that was used for DNA photocleavage. The average size of aggregated particles were found to be 20 nm which is much smaller as compared to the aggregates of γ-cyclodextrin-bicapped C_{60} (CD/C_{60}) reported by Wang et al.[7a] In order to understand the mode of DNA cleavage, the same experiments of gel electrophoresis were carried out in the presence of sodium azide and L-histidine, singlet oxygen scavengers. No change in DNA cleavage was observed in the presence of either of these two singlet oxygen scavengers. However the cleaving activity was clearly inhibited by the addition of superoxide dismutase (SOD), which quenches $O_2^{\cdot -}$. This result suggested that $O_2^{\cdot -}$ is a key intermediate for DNA-cleaving activity of **3** in the presence of NADH.

In conclusion a novel fullerene–lysine conjugate has been synthesized and was found to cleave the supercoiled DNA under photoirradiation in the presence of NADH. Although the mechanism of action is not very clear, superoxide radical generated on photoirradiation seems to be the reactive oxygen species (ROS) behind the DNA cleavage. This work opens up interesting prospects in the field of DNA cleavage by this novel class of fullerene-based amino acids.

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Supplementary data

Supplementary data (general methods, synthetic procedure and characterization data of intermediates **1** and **2**. A copy of FAB-MS and FTIR spectra of **3**, dynamic light scattering data of compound **3**. General method for DNA cleavage, effect of photoirradiation time, concentration of **3** and concentration of NADH on DNA cleavage) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.09.027.

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15. *Synthesis of compound* **3**. The N-protected fulleropyrrolidine **2** (30 mg, 0.03 mmol) was dissolved in a 1:1 mixture of toluene/trifluoroacetic acid and stirred for 12 h. The reaction was monitored by TLC (SiO_2; toluene/propanol, 9:1). After completion of the deprotection, the solvents were evaporated, and some MeOH was added and evaporated again. The residue was taken up in CH_2Cl_2, and the solution was added dropwise to excess hexane. The precipitated solid was separated by centrifugation, washed with a small amount of Et_2O and then dried under high vacuum to obtain **3** as brownish solid product. Yield 25 mg (83.3%) mp 251 °C; Anal. Calcd for $C_{78}O_4H_{24}N_3F_3$: C, 83.35; H, 2.15; N, 3.74. Found: C, 83.26; H, 2.14; N, 3.76; IR(KBr; cm^{-1}) 528 (C_{60}), 1600 (CH=N), 1659 (C=O stretching), 3257 (NH stretching), 3497 (NH stretching); ^{1}H NMR (400 MHz, DMSO-d_6, Me_4Si, 298 K) δ 1.50–1.97 (m, 8H, $-CH_2-$), 2.82 (3H, s, N–CH_3), 4.1(s, 1H, –CH–), 4.45 (d, ^2J = 9.3 Hz, 1H, H*HC*–*N*–), 4.90 (s, 1H, HC–N–), 5.25 (d, ^2J = 9.3 Hz, 1H, *HHC*–*N*–), 7.16 (d, ^3J = 8.0 Hz, 2H, ArH), 7.57(d, ^3J = 8.0 Hz, 2H, ArH), 7.80 (s, 3H, NH_3^+), 8.23 (s, 1H, CH=N), 12.1(s, 1H, –COOH) ppm; ^{13}C NMR(125 MHz, DMSO-d_6, Me_4Si, 298 K) δ 177.1(1C, –COO), 164.3(1C, CH=N), 154.2(2C, C_{60}), 153.4(2C, C_{60}), 151.6(2C, C_{60}), 150.7(2C, C_{60}), 150.5(2C, C_{60}), 149.6(2C, C_{60}), 149.2(2C, C_{60}), 148.9(2C, C_{60}), 148.3(2C, C_{60}), 147.7(2C, C_{60}), 147.4(2C, C_{60}), 146.9(2C, C_{60}) 146.2(2C, C_{60}), 145.9(4C, C_{60}), 145.8(2C, C_{60}), 145.6(2C, C_{60}), 145.4(2C, C_{60}), 144.6(2C, C_{60}), 143.2(1C, ArCq), 142.8(2C, C_{60}), 142.2(2C, C_{60}), 141.8(2C, C_{60}), 141.7(2C, C_{60}), 141.5(2C, C_{60}), 140.7(2C, C_{60}), 139.8(1C, ArCq), 139.5(2C, C_{60}), 138.9(2C, C_{60}), 136.4(2C, C_{60}), 135.9(2C, C_{60}), 135.9(2C, C_{60}), 130.1(2C, ArCH), 129.8(2C, ArCH), 83.5(1C, NCH of the pyrrolidine ring), 75.2(1C, sp^3 C– of C_{60}), 69.1(1C, NCH_2 of pyrrolidine ring), 68.4(1C, sp^3 C– of C_{60}), 67.9 (1C, –CH–COOH), 66.5 (1C, $-CH_2-NH_3^+$), 44.8(1C, $-CH_2$), 42.3(1C, CH_3 linked to N of the pyrrolidine ring),

31.5(1C, $-CH_2$), 30.7(1C, $-CH_2$), 22.5(1C, $-CH_2$) ppm; FAB-MS *m/z* (%) 1011 (100) $[M-CF_3COO]^+$.

16. *DNA cleavage procedure*: Thirty microlitres of aqueous solution of DNA pBR322 (0.50 $\mu g\ \mu L^{-1}$) were diluted by adding 270 μL of water. Typically, 10 μL of aqueous solution of **3** (1.0×10^{-5} M), 10 μL of aqueous solution of DNA pBR322, NADH (5 μL, 0.126 M) and 8 μL of tris-EDTA buffer (10 μL, 150 × TE, pH 8.0) were mixed in a micro test tube under dark conditions. The samples were incubated under irradiation with visible light for 3 h at 298 K, mixed with 10 μL of loading buffer (0.1% bromophenol blue and 30% glycerol in TBE buffer) and loaded onto a 1% agarose gel containing ethidium bromide (1 $\mu g\ mL^{-1}$). The gels were run at a constant voltage of 70 V for 2 h in TBE buffer, washed with distilled water, visualized under a UV transilluminator and photographed using an instant camera.