Bioorganic Chemistry 48 (2013) 8–15

Contents lists available at SciVerse ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

PEG-mediated one-pot multicomponent reactions for the efficient synthesis of functionalized dihydropyridines and their functional group dependent DNA cleavage activity

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ARTICLE INFO

Article history:
Received 11 July 2012
Available online 9 April 2013

Keywords:
Dihydropyridine derivatives
PEG
MCRs
pUC 19
DNA cutting activity

ABSTRACT

Polyethylene glycol (PEG) has been found to be an inexpensive, non-toxic and useful medium for the one pot synthesis of highly functionalized dihydropyridines using multicomponent reactions (MCRs) at room temperature under catalyst free conditions. The notable features of this protocol are: mild reaction condition, applicability to wide range of substrates, reusability of the PEG and good yields. The interaction of the synthesized compounds with pUC19 plasmid DNA was also analyzed. Some of the synthesized compounds showed interesting functional group dependent nuclease activity for plasmid DNA cleavage under physiological conditions.

1. Introduction

Dihydropyridine (DHP) derivatives are abundant in various natural products as well as in synthetic pharmaceuticals. Substituted DHPs exhibit diverse biological activities and have been used for the treatment of Alzheimer's disease, angina pectoris and cardiovascular diseases including hypertension [1]. They also demonstrate anticancer, antitumor, antioxidant, antimutagenic and antidiabetic activities [2]. In addition to these, substituted DHPs exhibit other therapeutic properties including Platelet antiaggregatory activities, neuroprotection, radioprotection, noncompetitive inhibition of topoisomerase I and HIV protease inhibition [3]. DHPs can also be used as hydrogen transfer reagents similar to coenzymes NADH and NADPH [4]. In view of the prominent pharmaceutical significance of DHPs, considerable attention have been paid in recent times by organic as well as medicinal chemists for the design and development of newer and greener methodologies for the efficient synthesis of highly functionalized DHPs [5].

In continuation to our endeavor to develop multicomponent reactions (MCRs) for the access to new functionalized small molecules [6], we were interested to develop a greener protocol for the synthesis of highly functionalized 6-amino DHP derivatives using one-pot MCRs.

Polyethylene glycol (PEG) has gained significant interest as a powerful solvent system as well as a phase transfer catalyst for various organic transformations under mild reaction conditions [7]. PEG is considered as a non-ionic hydrophilic polymer which can be used in drug delivery and in bioconjugates as tools for diagnostics [8]. Most importantly, it is an inexpensive, non-toxic, thermally stable, easy to handle and recyclable medium. Considering its green chemical aspects, we were inspired to test the usefulness of PEG in multicomponent reactions for the synthesis of highly functionalized 6- amino DHPs.

Metal-free small molecules that can interact with DNA through recognition, binding, modifying, cleaving or crosslinking have been shown to have widespread applications [9]. Though DHPs are widely used in the pharmaceutical and pharmacological fields, report of interaction of these interesting small molecules with DNA is scarce [10]. This prompted us to investigate the interaction between our synthesized DHPs with DNA. A number of simple in vitro experiments [11], including evaluation of plasmid DNA-compound interaction [12] can be performed to determine whether the compounds physically interact with DNA. Many chemical compounds show nuclease activity on supercoiled plasmid (Form I) by introducing nicks (relaxed circular plasmid, Form II) or double strand breaks (linear plasmid, Form III) [13]. The compounds recognize specific DNA sequences, intercalate into DNA double strands or catalyze the hydrolysis of phosphodiester bonds [14]. Hydrolytic cleavage of DNA by small molecules is less common than by transition metal ions, lanthanide ions, actinide ions or their complexes [15]. Herein we report, a catalyst free synthesis of 6-amino dihydropyridine derivatives and their functional group dependent DNA cleaving activities [16].

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http://dx.doi.org/10.1016/j.bioorg.2013.03.003

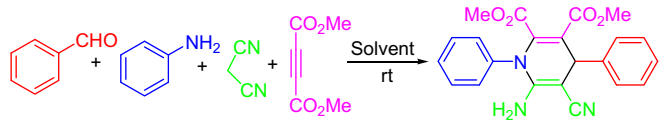
2. Results and discussion

2.1. Synthesis

Initially, benzaldehyde, malononitrile, aniline and dimethylacetylene dicarboxylate (DMAD) were chosen as model substrates and reacted in presence of various solvents at room temperature (Table 1). Under catalyst free condition, in presence of different organic solvents (Table 1, entries 1–5), as well as in water, the experiments did not provide the desired product even after stirring at room temperature for 24 h. We also failed to achieve the desired DHP **(1)** under solvent free condition (Table 1, entry 7). Interestingly, the same set of substrates provided 72% yield of the desired DHP (**1**) in presence of PEG-200 as a reaction medium within 10 h. This result prompted us to study this reaction with other higher molecular weight PEGs. Both PEG 400 and 600 were tested under the similar reaction condition and better yields were achieved (Table 1, entries 9 and 10). Among all the screened solvents, PEG-400 provided the best result.

The structure of the compound **1** was established using usual spectroscopic techniques (IR, ^{1}H and ^{13}C NMR) and CHN analysis.

Table 1
Optimization of reaction condition



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Entry	Solvent	Reaction time (h)	% Yields
1	Ethanol	24	0
2	Methanol	24	0
3	Dichloromethane	24	0
4	Acetonitrile	24	0
5	DMF	24	0
6	Water	24	0
7	Solvent free	24	0
8	PEG-200	10	72
9	PEG-400	10	81
10	PEG-600	10	79

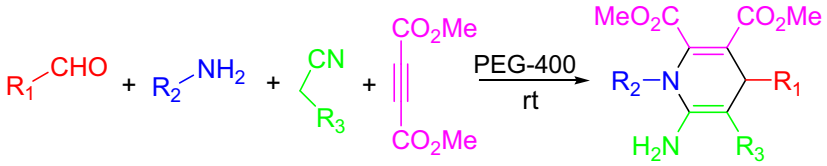
Next, we checked the scope and general applicability of this PEG-mediated MCR, through substrate variability of aldehyde, amine and malononitrile derivatives under similar reaction conditions. This methodology was found to be applicable to aromatic aldehydes tethered with both electron withdrawing as well as electron releasing groups. Similarly, a wide range of substituted anilines have been found to be pertinent for this methodology. Interestingly, bulky amine such as β-naphthyl amine also reacted under similar reaction condition to provide the corresponding DHP **2a** in 82% yield (Table 2, entry 2). As evident from Table 2, benzyl amine offered moderate yield of the corresponding DHP (**2e**). Malononitrile derivatives such as 2-cyanoacetamide (Table 2, entry 3), ethyl 2-cyanoacetate (Table 2, entries 9 and 10), and benzoyl acetonitrile (Table 2, entries 4 and 11) were also found suitable for this multicomponent reaction.

The exact role of PEG in this multicomponent reaction is not yet clear, however we believe that PEG acts as a promoter by its two active sites (ethereal oxygen linkages and free hydroxyl group). The free hydroxyl group can form hydrogen bonding with the aldehydes and make it more electrophilic, as a result it helps in the formation of the alkene (**A**). At the same time, the ethereal linkages of oxygen enhance the nucleophilicity of the amines for the formation of **B**. In addition to this, we believe that these ethereal linkages of oxygen also help in the cyclization process of **D** followed by tautomerization, leading to the formation of the final product **F** as shown in Scheme 1.

2.2. Evaluation of nuclease activity

Concentration dependent nuclease activity was observed for **2a–2d**, as well as for the bromo derivatives, **3a** and **3b** as shown in Fig. 1 (data not shown for 3b). Effect of concentration on nuclease activity was moderate for **2a**, **2b** and the unsubstituted DHP derivative **1**, but considerable increase in nuclease activity was observed for **2c**, **2d**, **3a** and **3b** with increase in compound concentration. However, **2e** with a Benzyl group as R_2 showed similar nuclease activity as the unsubstituted analogue **1** (data not shown). Replacement of –CN group by $–CONH_2$ group in the DHP ring (R_3) and $–C_6H_5$ group by 4-OMe–C_6H_4 group (R_1), as in **2b**, decreases the nuclease activity considerably as compared to the unsubstituted DHP **1**. Comparison between **2c** and **2d** reveals that –CN group has more contribution in the nuclease activity of the

Table 2
PEG-mediated synthesis of substituted dihydropyridines with different aromatic aldehydes, anilines, malononitrile derivatives and dimethyl acetylenedicarboxylate



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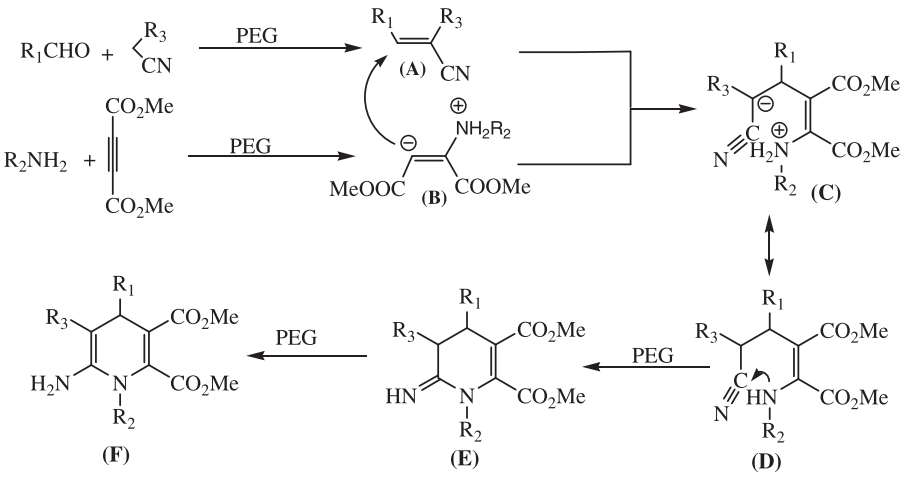
Entry	R_1	R_2	R_3	Product	Reaction time (h)	Yield (%)
1	C_6H_5	C_6H_5	CN	1	10	81
2	4-Cl–C_6H_4	β-Naphthyl	CN	2a	8	82
3	4-Cl–C_6H_4	4-OMe–C_6H_4	$CONH_2$	2b	16	52
4	4-Cl–C_6H_4	4-Me–C_6H_4	COPh	2c	10	55
5	4-Cl–C_6H_4	4-Me–C_6H_4	CN	2d	10	80
6	4-Cl–C_6H_4	$C_6H_5CH_2$	CN	2e	16	50
7	4-Br–C_6H_4	4-Me–C_6H_4	CN	3a	10	78
8	4-Br–C_6H_4	C_6H_5	CN	3b	10	80
9	3-NO_2–C_6H_4	C_6H_5	COOEt	4a	8	83
10	3-NO_2–C_6H_4	4-Cl–C_6H_4	COOEt	4b	10	77
11	3-NO_2–C_6H_4	C_6H_5	COPh	4c	12	60
12	3-NO_2–C_6H_4	C_6H_5	CN	4d	7	85
13	3-NO_2–C_6H_4	4-Me–C_6H_4	CN	4e	8	83
14	4-OMe–C_6H_4	4-OMe–C_6H_4	CN	5	8	81



Scheme 1. Plausible reaction mechanism of PEG mediated MCRs for the synthesis of dihydropyridine derivatives.

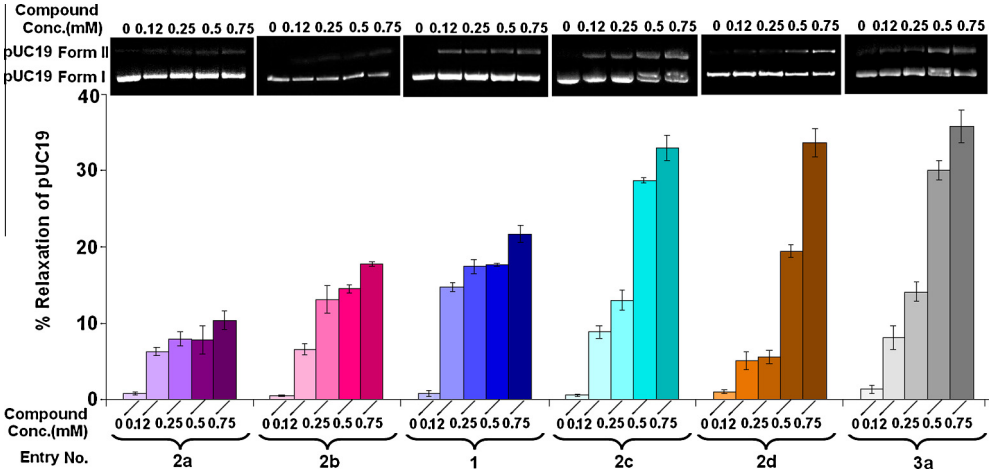


Fig. 1. Effect of concentration of compounds **1**, **2a–2d** and **3a** on the cleavage reaction of pUC19. Amount of plasmid relaxation are shown in agarose gels, and the corresponding quantitative analysis in the bar chart below; numbers above the gels and below the bars indicate concentration of the compounds used during plasmid incubation and the corresponding lane used in the gel electrophoresis.

compounds than –COPh group at the R_3 position. Though both **2a** and **2d** have –CN and –NH_2 group in the DHP ring, in all probability, the presence of Naphthyl moiety as R_2, render **2a** less reactive. The bulky naphthalene group in **2a** might interrupt proper interaction of the compound with the DNA [17]. The bromo derivative **3a** with a 4-Me–C_6H_4 group as R_2 shows slightly greater overall nuclease activity (ca. 38%) as compared to the non-methylated analogue **3b** (ca. 36%, see supporting information). Comparison of nuclease activity between **1** and **3b** clearly reveals the effect of the presence of a halogen atom in R_1 in augmenting nuclease activity, while slight differences in nuclease activity between **2d** and **3a** shows that bromo has a slim edge over the chloro analogue. In summary, nuclease activity of the DHP derivatives **2a–2e** (R_1 = 4-Cl–C_6H_4) and **3a–3b** (R_1 = 4-Br–C_6H_4) were found to be greatly dependant on the functional groups present in the 1st position (R_2), 3rd-position (R_3) and as well as 4th position (R_1) of the DHP ring.

For the compounds of the nitro series, nuclease activity for all the compounds was found to be greater than the unsubstituted DHP derivative **1** (Fig. 2). Comparison between **4a** and **4c** clearly indicates that the presence of –COPh group at R_3 in the DHP ring enhances nuclease activity of the compound as compared to –COOEt group. Substitution of the –C_6H_5 group at R_2 with 4-Cl–C_6H_4 does not change the nuclease activity as evident in **4a** and **4b**. Nuclease activity increases by ~7% when –C_6H_5 group as R_2 was replaced by 4-Me–C_6H_4 (ca. 47% for **4e** vs. 40% for **4d**). This shows that one methyl group in the R_2 can have substantial influence in increasing the nuclease activity in this class of compounds. As observed earlier also, nuclease activity was on the higher side for those compounds among all other entries from **4a–4e** when the DHP ring contains –CN group (R_3) along with the usual –NH_2 functional group. However, no compound was found to have nuclease activity beyond 47% or form linear DNA (form III) from the supercoiled plasmid. Nevertheless, it is reasonable to infer that presence of cyano (–CN) functional group was found to enhance the nuclease activity of different dihydropyridine derivatives. In addition, presence of –NO_2 group in the phenyl ring of R_1 supplemented by a methyl group in the phenyl ring in R_2 can have considerable contribution in augmentation of nuclease activity of the compounds. As observed, **4e** showed highest nuclease activity among all the entries (ca. 47%). The most important generalization that might follow regarding the enhancement of nuclease activity in the presence of –NO_2 group is that the nitronium ions probably neutralize the excess negative charge on phosphates of the DNA backbone, which in turn enhances the cleaving activity of the analogues [18]. Alternatively, nitro group can initiate the nuclease activity of the compounds by producing DNA-Sugar damaged

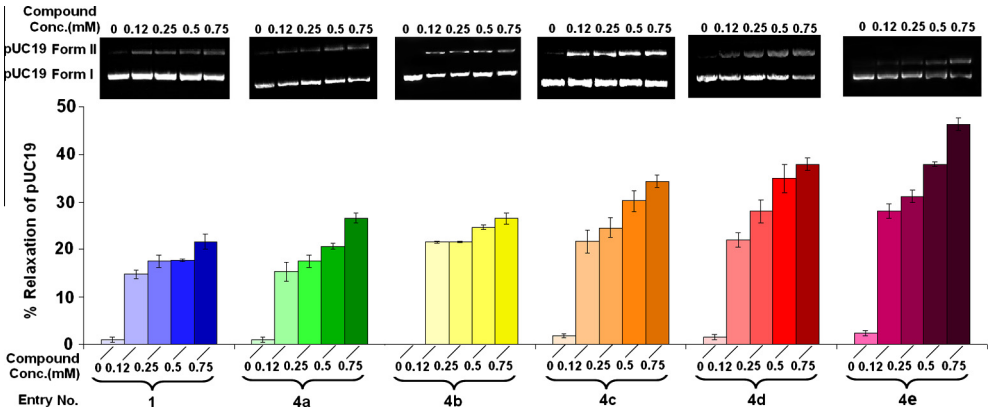


Fig. 2. Effect of concentration of compounds **4a**–**4e** on the cleavage reaction of pUC19. Convention and denotations followed in this figure is similar to Fig. 1.

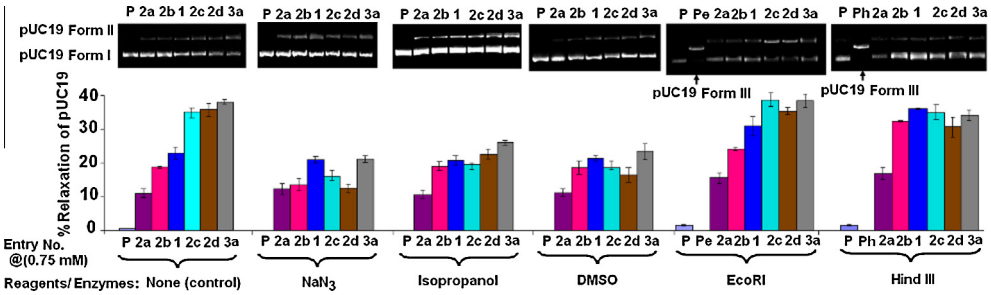


Fig. 3. Effect of radical quenchers NaN_3, Isopropanol and DMSO on the nuclease activity of the compounds (0.75 mM) and inhibition of endonucleases activity of EcoRI and Hind III by the compounds **1**, **2a–2d** and **3a**. In the gel lanes and the bar chart, P: pUC19 only (no compound, reagent or enzyme present), Pe: pUC19 + Eco RI (no compound present), Ph: pUC19 + Hind III (no compound present).

products also [19]. Presence of electron donating groups like –OMe on phenyl ring in the R_1 and R_2 position as in **5**, shows nuclease activity comparable to the unsubstituted DHP **1**, while –Me groups on phenyl ring at R_2 was found to have important contribution in increasing the potency of a compound's nuclease activity.

The unsubstituted DHP, **1** along with **2a** and **2b** did not exhibit reduction in nuclease activity either in presence of singlet oxygen scavenger NaN_3, or hydroxyl radical quenchers like Isopropanol and DMSO. However, the relaxation of the plasmid during incubation with **2c**, **2d** and **3a** (R_2 = 4-Me–C_6H_4) was found to be noticeably reduced in the presence of NaN_3, Isopropanol and DMSO (Fig. 3). This suggest that hydroxyl radical or singlet oxygen mediated oxidative cleavage might occur in those reactions, leading to DNA damage by oxidative pathway [20], in addition to nucleophilic attack leading to hydrogen abstraction [21] and hydrolysis of phosphodiester bonds in the plasmid by those compounds [22].

Nuclease activity of the compounds of the Nitro series **4a–4d** was not appreciably reduced in presence of radical quenchers NaN_3, Isopropanol and DMSO (Fig. 4). Similar to the case of **2c**, **2d** and **3a**, we also found that there is ~8–10% decrease in nuclease activity for **4e** in presence of radical scavengers like NaN_3, Isopropanol and DMSO, which may be attributed to the presence of –Me group in R_2. In general, in the presence of the nitro group, the dominant mechanism responsible for creation of nicks in the plasmid seems to follow a hydrogen abstraction route [23], where electron are being shuttled from the functional groups present in the dihydropyridine ring to initiate the process.

In order to assess whether the dihydropyridine derivatives shows affinity towards any particular region (for example GC or AT rich) in a plasmid, we carried out restriction endonuclease analysis [24,25]. Compound-pUC19 preincubated solutions were treated with restriction endonucleases EcoRI and Hind III, each enzyme having single restriction site in pUC19. One interesting observation was that while native pUC19 was totally converted to the linear form (Form III), presence of any one of the compounds from **2a–2e**, **3a–3b** including **1**, completely inhibited the incision

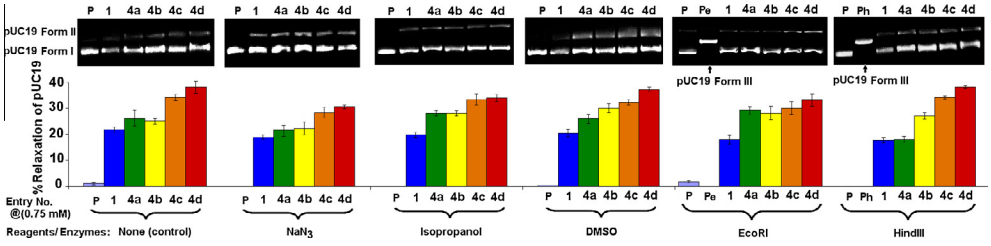


Fig. 4. Effect of radical quenchers NaN_3, Isopropanol and DMSO on the nuclease activity of the compounds (0.75 mM) and inhibition of endonucleases activity of EcoRI and Hind III by the compounds **4a–4d**. In the gel lanes and the bar chart, P: pUC19 only (no compound, reagent or enzyme present), Pe: pUC19 + Eco RI (no compound present), Ph: pUC19 + Hind III (no compound present).

activity on the plasmid by both EcoRI (cutting site G↓A, recognition sequence 5′-AATT) and Hind III (cutting site A↓A, recognition sequence 5′-AGCT) (Fig. 3). Inhibitions of endonuclease activity of EcoRI and Hind III in the compound-pUC19 preincubated solutions suggest that the compounds can interact with recognition sites of both the restriction endonucleases in the plasmid. Thus, site specificity or selectivity for any nucleoside or sequence in compound-DNA interaction was not observed. With Eco RI and Hind III restriction enzymes, similar results were observed for **4a–4e** and **5** (Fig. 4, 4e and 5 not shown), where none of them showed presence of the linear form (Form III) of the plasmid upon incubation with the restriction endonucleases.

To see whether Dihydropyridine compounds interact directly with restriction endonuclease and inhibit their enzymatic activity, plasmid DNA was incubated with restriction enzyme that were pretreated with one of the compound. 100 units of Hind III enzyme was incubated with 0.75 mM of **4d** (one of the nitro derivatives having high nuclease activity) at 37 °C overnight. The enzyme compound mixture was subjected to dialysis for 24 h in a dialysis cassette (MWCO 7 K, Slide-A-Lyzer™, Thermofisher Scientific). After completion of the dialysis, the enzyme was recovered from the dialysis membrane. Plasmid pUC19 was incubated following the usual procedure with calculated amount of the recovered enzyme. Agarose gel electrophoresis with the DNA incubated with recovered Hind III showed complete restriction digestion of the plasmid (see Supplementary section). This proves that compound has no effect on the activities of the restriction enzymes, and inhibition of endonuclease activity of the restriction enzyme is due to interaction of the compounds with the recognition sites of the enzyme and not due to compound–enzyme interaction.

To summarize the interaction of the compounds **1**, **2a–2e**, **3a–3b**, **4a–4e** and **5**, from the experiments performed herein, it is reasonable to say that the dominant mechanism responsible for plasmid relaxation by the compounds is electron transfer from DHP ring substituents followed by hydrolysis of phosphodiester bonds in DNA. However, single electron oxidative pathway has been found to be operative also in few entries particularly with R_2 as 4-Me–C_6H_4. Efficiency of electron donation and hence nuclease activity is dependent on the functional group present in the DHP ring where –CN was found to be the most effective among all others tested. In addition, R_1 groups can have important role in dictating nuclease activity, whereby it was found that 3-NO_2–C_6H_4, where –NO_2 can exist as a nitronium ion are more efficient to promote nuclease activity than halides. Presence of methyl group in R_2 tends to enhance nuclease activity. Also, bulky substituents at R_2 probably tend to decrease nuclease activity by increasing the distance from the nucleophilic centre in the compound and the electrophilic centres in the DNA. Overall, few DHP derivatives showed appreciable nuclease activity in absence of any transition or other heavy metal ions, and the nuclease activity can be tuned by appropriate substitution at R_1, R_2 and R_3 positions.

3. Conclusions

We have described a highly atom economic multicomponent reaction for the efficient synthesis of functionalized amino dihydropyridine derivatives under mild and neutral catalyst free conditions using PEG-400 as promoter. The notable advantages of this protocol are: (a) applicability to a wide range of aromatic aldehydes, amines as well as malononitrile derivatives, (b) reusability of PEG and its low cost and (c) simple reaction conditions with good yields. Some of the DHPs demonstrate appreciable metal-free nuclease activity, whereby –CN group on the heterocyclic ring has been found to be very efficient in initiating the cleavage of pUC19 DNA. Additionally, functional groups attached to the 1 and 4 position of the DHP ring can also play significant role in deciding the efficiency of the compound as a catalyst in nuclease activity leading to plasmid DNA damage. In particular, **3a**, **3b**, **4d** and **4e** were found to be very effective nucleases, yielding DNA cleavage product (nicked, open circular form of plasmid) from supercoiled plasmid.

4. Experimental

4.1. General

All the reagents were procured from commercial sources and used as received. All the reactions were monitored by TLC on Merck GF 254 plates and spots were visualized by UV light and/or using iodine vapor chamber. IR spectra were recorded on a Shimadzu Affinity 1, FTIR spectrophotometer. ^{1}H and ^{13}C NMR was either recorded on a Jeol 500 or Bruker 500/400 MHz spectrometer in $CDCl_3$ using TMS as internal reference. CHN analysis was carried out either on an Elementar Vario EL III or Perkin–Elmer 2400 II elemental analyzers. Melting points were obtained manually by capillary methods and uncorrected.

4.2. Preparation of substituted DHPs

A mixture of aromatic aldehyde (1.0 mmol), malononitrile or its derivative (1.0 mmol) in 2.0 mL PEG-400 was stirred at room temperature for half an hour. A solution of arylamine (1.0 mmol) and dimethylacetylenedicarboxylate (1.0 mmol) in 2.0 mL PEG was then added to the mixture. The whole solution was stirred at room temperature for appropriate time. After completion of the reaction, as indicated by TLC, the reaction mixture was extracted with diethyl ether and PEG was separated from the products. The combined organic layers were evaporated under reduced pressure, and the crude product was either purified by column chromatography (silica gel, 60–120 mesh and hexane: ethyl acetate (8:2) as eluent) or by recrystalisation technique to obtain the desired DHPs. The recovered PEG was dried under vacuum and reused for 3 cycles without significant loss of activity.

4.2.1. Dimethyl 6-amino-5-cyano-1,4-diphenyl-1,4-dihydropyridine-2,3-dicarboxylate (*1*)

Yield 81%; Light yellowish solid; mp 161–163 °C; IR (KBr): 3465, 3376, 3336, 2950, 2175, 1746, 1707, 1650, 1568, 1492, 1413, 1353, 1247, 1212, 1109, 1076 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.48–7.49 (m, 3H, Ar–H), 7.34–7.38 (m, 6H, Ar–H), 7.25–7.27 (m, 1H, Ar–H), 4.65 (s, 1H, CH), 4.11 (bs, 2H, NH_2), 3.58 (s, 3H, OMe), 3.42 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 165.9, 163.6, 149.7, 144.8, 141.9, 135.2, 130.6, 130.3, 130.0, 128.9, 127.3, 127.1, 120.6, 105.3, 62.9, 52.7, 52.1, 38.5; Anal. calcd for $C_{22}H_{19}N_3O_4$ (389.40): C, 67.86; H, 4.92; N, 10.79; Found: C, 67.94; H, 4.95; N, 10.88.

4.2.2. Dimethyl 6-amino-4-(4-chlorophenyl)-5-cyano-1-(naphthalen-2-yl)-1,4-dihydro pyridine-2,3-dicarboxylate (*2a*)

Yield 82%; Light yellowish solid; mp 171–173 °C; IR (KBr): 3455, 3327, 3220, 2945, 2178, 1753, 1712, 1651, 1571, 1422, 1351, 1223, 1120, 1009 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.95 (d, J = 8.4 Hz, 2H, Ar–H), 7.89 (t, J = 6.8 Hz, 2H, Ar–H), 7.83 (s, 1H, Ar–H), 7.58–7.64 (m, 2H, Ar–H), 7.31–7.36 (m, 4H, Ar–H), 4.68 (s, 1H, CH), 4.12 (s, 2H, NH_2), 3.58 (s, 3H, OMe), 3.33 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 165.7, 163.5, 149.9, 143.4, 142.2, 133.5, 133.0, 132.3, 130.3, 129.8, 129.1, 128.5, 128.4, 128.0, 127.7, 126.5, 120.4, 104.9, 62.6, 52.8, 52.2, 38.2; Anal. calcd for $C_{26}H_{20}ClN_3O_4$ (473.90): C, 65.89; H, 4.25; N, 8.87; Found: C, 65.93; H, 4.29; N, 8.96.

*4.2.3. Dimethyl 6-amino-5-carbamoyl-4-(4-chlorophenyl)-1-(4-methoxyphenyl)-1,4-dihydropyridine-2,3-dicarboxylate (**2b**)*

Yield 52%; Light yellowish solid; mp 221–223 °C; IR (KBr): 3451, 3327, 3182, 2950, 2843, 1748, 1685, 1656, 1571, 1480, 1436, 1404, 1335, 1298, 1255, 1218, 1185, 1109, 1062 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.38 (d, J = 8.4 Hz, 2H, Ar–H), 7.26–7.32 (m, 4H, Ar–H), 6.97 (d, J = 8.8 Hz, 2H, Ar–H), 6.74 (bs, 2H, NH_2), 4.95 (s, 2H, NH_2), 4.74 (s, 1H, CH), 3.85 (s, 3H, OMe), 3.68 (s, 3H, OMe), 3.42 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 125 MHz): 171.9, 165.9, 163.7, 160.6, 151.4, 144.2, 141.5, 132.8, 131.7, 129.1, 128.6, 127.2, 114.9, 105.9, 79.2, 55.6, 52.4, 51.9, 38.1. Anal. calcd for $C_{23}H_{22}ClN_3O_6$ (471.89): C, 58.54; H, 4.70; N, 8.90. Found: C, 58.43; H, 4.76; N, 8.78.

*4.2.4. Dimethyl 6-amino-5-benzoyl-4-(4-chlorophenyl)-1-p-tolyl-1,4-dihydropyridine-2,3-dicarboxylate (**2c**)*

Yield 55%; Yellow solid; mp 184–186 °C; IR (KBr): 3420, 3049, 2951, 1736, 1703, 1639, 1596, 1450, 1355, 1224, 1111, 1048 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.35–7.39 (m, 3H, Ar–H), 7.32 (d, J = 8.3 Hz, 2H, Ar–H),7.22–7.25 (m, 4H, Ar–H), 7.14 (d, J = 8.6 Hz, 2H, Ar–H), 6.84 (d, J = 8.6 Hz, 2H, Ar–H), 4.99 (s, 1H, CH), 3.62 (s, 3H, OMe), 3.45 (s, 3H, OMe), 2.43 (s, 3H, Me); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 194.5, 165.7, 163.7, 154.4, 144.7, 141.5, 141.2, 132.2, 131.8, 131.0, 130.2, 129.9, 129.5, 129.2, 128.6, 128.0, 126.8, 108.7, 89.5, 52.7, 52.2, 38.0, 21.4; Anal. calcd for $C_{29}H_{25}ClN_2O_5$ (516.97): C, 67.38; H, 4.87; N, 5.42; Found: C, 67.43; H, 4.82; N, 5.51.

*4.2.5. Dimethyl 6-amino-4-(4-chlorophenyl)-5-cyano-1-p-tolyl-1,4-dihydropyridine-2,3-di carboxylate (**2d**)*

Yield 80%; Light yellowish solid; mp 186–188 °C; IR (KBr): 3472, 3360, 3221, 2950, 2175, 1742, 1699, 1646, 1574, 1508, 1413, 1355, 1301, 1248, 1216, 1176, 1016 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.33 (d, J = 8.5 Hz, 2H, Ar–H), 7.29 (d, J = 7.9 Hz, 2H, Ar–H), 7.27 (d, J = 7.6 Hz, 2H, Ar–H), 7.20 (d, J = 7.9 Hz, 2H, Ar–H), 4.65 (s, 1H, CH), 4.09 (bs, 2H, NH_2), 3.58 (s, 3H, OMe), 3.46 (s, 3H, OMe), 2.40 (s, 3H, Me); ^{13}C NMR ($CDCl_3$, 125 MHz): 165.6, 163.4, 149.9, 143.5, 142.2, 141.1, 132.9, 132.2, 130.6, 129.9, 128.8, 128.5, 120.4, 104.6, 62.1, 52.6, 52.1, 38.1, 21.3; Anal. calcd for $C_{23}H_{20}ClN_3O_4$ (437.87): C, 63.09; H, 4.60; N, 9.60. Found: C, 63.18; H, 4.67; N, 9.72.

*4.2.6. Dimethyl 6-amino-1-benzyl-4-(4-chlorophenyl)-5-cyano-1,4-dihydropyridine-2,3-dicarboxylate (**2e**)*

Yield 50%; Yellow solid; mp: 146–148 °C; IR (KBr): 3466, 3346, 3038, 2955, 2188, 1743, 1705, 1645, 1569, 1493, 1434, 1343, 1290, 1215, 1116, 1018 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.34–7.41(m, 3H, Ar–H), 7.22–7.29 (m, 4H, Ar–H), 7.10 (d, J = 8.4 Hz, 2H, Ar–H), 4.78 (d, J = 17.2 Hz, 1H, Ar–CH_2), 4.66 (d, J = 17.2 Hz, 1H, Ar–CH_2), 4.57 (s, 1H, CH), 4.15 (s, 2H, NH_2), 3.77 (s, 3H, OMe), 3.63 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 165.6, 164.6, 151.3, 142.6, 142.2, 135.0, 132.9, 131.9, 130.1, 129.3, 128.8, 128.4, 126.7, 120.3, 106.9, 65.9, 53.3, 52.2, 50.8, 37.9; Anal. calc. for $C_{23}H_{20}ClN_3O_4$ (437.87): C, 63.09; H, 4.60; N, 9.60. Found: C, 63.01; H, 4.67; N, 9.72.

*4.2.7. Dimethyl 6-amino-4-(4-bromophenyl)-5-cyano-1-p-tolyl-1,4-dihydropyridine-2,3-dicarboxylate (**3a**)*

Yield 78%; Light yellowish solid; mp 185–187 °C; IR (KBr): 3475, 3364, 3033, 2952, 2174, 1741, 1701, 1646, 1575, 1509, 1414, 1356, 1326, 1250, 1113, 1071 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.48 (d, J = 8.6 Hz, 2H, Ar–H), 7.27 (d, J = 8.0 Hz, 2H, Ar–H), 7.23 (d, J = 8.0 Hz, 2H, Ar–H), 7.19 (d, J = 8.0 Hz, 2H, Ar–H), 4.62 (s, 1H, CH), 4.12 (bs, 2H, NH_2), 3.58 (s, 3H, OMe), 3.45 (s, 3H, OMe), 2.39 (s, 3H, Me); ^{13}C NMR ($CDCl_3$, 125 MHz): 165.6, 163.4, 149.9, 143.9, 142.2, 141.1, 133.1, 132.2, 132.0, 131.8, 129.9, 129.6, 121.1, 120.4, 104.5, 62.1, 52.6, 52.1, 38.1, 21.3; Anal. calcd for $C_{23}H_{20}BrN_3O_4$ (482.33): C, 57.27; H, 4.18; N, 8.71. Found: C, 57.37; H, 4.24; N, 8.83.

*4.2.8. Dimethyl 6-amino-4-(4-bromophenyl)-5-cyano-1-phenyl-1,4-dihydropyridine-2,3-dicarboxylate (**3b**)*

Yield 80%; Light yellowish solid; mp 194–196 °C; IR (KBr): 3486, 3386, 3065, 2954, 2185, 1746, 1710, 1652, 1575, 1491, 1421, 1355, 1326, 1251, 1112, 1010 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.48–7.51 (m, 5H, Ar–H), 7.32–7.34 (m, 2H, Ar–H), 7.23–7.25 (m, 2H, Ar–H), 4.64 (s, 1H, CH), 4.18 (s, 2H, NH_2), 3.59 (s, 3H, OMe), 3.43 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 100 MHz): 165.5, 163.4, 149.7, 143.8, 141.9, 134.9, 131.9, 130.7, 130.2, 130.0, 128.8, 121.1, 120.3, 104.6, 62.1, 52.6, 52.1, 38.1; Anal. calcd for $C_{22}H_{18}BrN_3O_4$ (468.30): C, 56.42; H, 3.87; N, 8.97. Found: C, 56.35; H, 3.83; N, 8.89.

*4.2.9. 5-Ethyl 2,3-dimethyl 6-amino-4-(3-nitrophenyl)-1-phenyl-1,4-dihydropyridine-2,3,5-tricarboxylate (**4a**)*

Yield 83%; Light yellowish solid; mp 172–173 °C; IR (KBr): 3472, 3264, 2979, 2951, 1749, 1709, 1667, 1639, 1600, 1530, 1506, 1436, 1354, 1213, 1123, 1095, 1017 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 8.54 (s, 1H, Ar–H), 8.25 (d, J = 8.0 Hz, 1H, Ar–H), 7.94 (d, J = 7.5 Hz, 1H, Ar–H), 7.70–7.73 (m, 3H, Ar–H), 7.64–7.67 (m, 3H, Ar–H), 6.50 (bs, 2H, NH_2), 5.31 (s, 1H, CH), 4.24–4.30 (m, 2H, OCH_2), 3.61 (s, 3H, OMe), 3.82 (s, 3H, OMe), 1.41–1.44 (m, 3H, Me); ^{13}C NMR ($CDCl_3$, 125 MHz): 169.1, 165.7, 163.6, 151.4, 149.3, 148.3, 142.2, 134.9, 134.0, 130.7, 130.5, 130.0, 128.8, 123.0, 121.5, 106.6, 79.4, 61.9, 59.6, 52.5, 52.0, 51.9, 37.3, 14.4; Anal. calcd for $C_{24}H_{23}N_3O_8$ (481.45): C, 59.87; H, 4.82; N, 8.73. Found: C, 59.96; H, 4.90; N, 8.84.

*4.2.10. 5-Ethyl 2,3-dimethyl 6-amino-1-(4-chlorophenyl)-4-(3-nitrophenyl)-1,4-dihydropyridine-2,3,5-tricarboxylate (**4b**)*

Yield 77%; Light yellowish solid; mp 186–188 °C; IR (KBr): 3442, 3221, 3107, 2985, 2954, 1751, 1710, 1663, 1602, 1523, 1440, 1401, 1345, 1236, 1095, 1042 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ = 8.31 (s, 1H, Ar–H), 8.06 (d, J = 7.6 Hz, 1H, Ar–H), 7.71 (d, J = 7.6 Hz, 1H, Ar–H), 7.51 (d, J = 8.4 Hz, 2H, Ar–H), 7.45 (t, J = 7.6 Hz, 1H, Ar–H), 7.39 (d, J = 8.4 Hz, 2H, Ar–H), 6.25 (bs, 2H, NH_2), 5.10 (s, 1H, CH), 4.05–4.13 (m, 2H, OCH_2), 3.67 (s, 3H, OMe), 3.49 (s, 3H, OMe), 1.23 (t, J = 6.8 Hz, 3H, Me); ^{13}C NMR ($CDCl_3$, 125 MHz): 169.0, 165.6, 163.6, 151.1, 149.0, 148.4, 141.8, 136.9, 133.9, 133.4, 132.1, 130.3, 128.9, 122.9, 121.5, 107.1, 79.8, 62.2, 59.7, 52.7, 52.1, 37.2, 14.4; Anal. calcd for $C_{24}H_{22}ClN_3O_8$ (515.90): C, 55.87; H, 4.30; N, 8.15. Found: C, 55.94; H, 4.35; N, 8.29.

*4.2.11. Dimethyl 6-amino-5-benzoyl-4-(3-nitrophenyl)-1-phenyl-1,4-dihydropyridine-2,3-dicarboxylate (**4c**)*

Yield 60%; Yellow solid; mp 156–157 °C; IR (KBr): 3424, 3068, 2951, 2928, 2853, 1745, 1706, 1643, 1608, 1530, 1452, 1354, 1220, 1123, 1076, 1053, 1025 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.90–7.91 (m, 1H, Ar–H), 7.80–7.81 (m, 1H, Ar–H), 7.46–7.51 (m, 3H, Ar–H), 7.36–7.39 (m, 2H, Ar–H), 7.33–7.35 (m, 1H, Ar–H), 7.28–7.31 (m, 2H, Ar–H), 7.22 (t, J = 8.0 Hz, 1H, Ar–H), 7.13 (d, J = 8.5 Hz, 2H, Ar–H), 7.00 (d, J = 7.5 Hz, 1H, Ar–H), 5.02 (s, 1H, CH), 3.40 (s, 3H, OMe), 3.37 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 194.2, 165.3, 163.4, 154.2, 148.4, 148.3, 141.4, 141.2, 134.3, 133.3, 130.9, 130.4, 130.3, 129.4, 129.1, 128.5, 128.4, 126.5, 122.3, 121.7, 108.2, 89.2, 52.7, 52.2, 38.6; Anal. calcd for $C_{28}H_{23}N_3O_7$ (513.50): C, 65.49; H, 4.51; N, 8.18. Found: C, 65.57; H, 4.57; N, 8.30.

*4.2.12. Dimethyl 6-amino-5-cyano-4-(3-nitrophenyl)-1-phenyl-1,4-dihydropyridine-2,3-dicarboxylate (**4d**)*

Yield 85%; Yellow solid; mp 217–219 °C; IR (KBr): 3446, 3336, 3229, 2951, 2178, 1755, 1704, 1651, 1577, 1521, 1421, 1349, 1250, 1213, 1112, 926 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.84 (s, 1H, Ar–H), 7.73 (d, J = 8.2 Hz, 1H, Ar–H), 7.34 (d, J = 7.9 Hz, 1H, Ar–H), 7.20 (t, J = 7.9 Hz, 1H, Ar–H), 7.12–7.14 (m, 3H, Ar–H), 6.96–6.98 (m, 2H, Ar–H), 4.75 (bs, 2H, NH_2), 4.36 (s, 1H, CH), 3.20 (s, 3H, OMe), 3.01 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 165.0, 162.9, 150.6, 148.3, 147.3, 142.4, 134.7, 133.2, 130.4, 130.1, 129.7, 129.7, 121.9, 121.7, 120.4, 103.8, 59.7, 52.4, 51.9, 38.6; Anal. calcd for $C_{22}H_{18}N_4O_6$ (434.40): C, 60.83; H, 4.18; N, 12.90; Found: C, 60.89; H, 4.24; N, 13.03.

*4.2.13. Dimethyl 6-amino-5-cyano-4-(3-nitrophenyl)-1-p-tolyl-1,4-dihydropyridine-2,3-dicarboxylate (**4e**)*

Yield 83%; Yellow solid; mp 212–214 °C; IR (KBr): 3424, 3374, 3180, 2960, 2185, 1752, 1703, 1653, 1574, 1525, 1423, 1351, 1250, 1218, 1109, 1052 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ = 8.26 (s, 1H, Ar–H), 8.14 (dd, J = 8.2 and 1.4 Hz, 1H, Ar–H), 7.71 (d, J = 7.6 Hz, 1H, Ar–H), 7.54 (t, J = 7.9 Hz, 1H, Ar–H), 7.31 (d, J = 8.1 Hz, 2H, Ar–H), 7.26 (d, J = 8.3 Hz, 2H, Ar–H), 4.78 (s, 1H, CH), 4.29 (bs, 2H, NH_2), 3.61 (s, 3H, OMe), 3.46 (s, 3H, OMe), 2.42 (s, 3H, Me); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 165.2, 163.1, 150.4, 148.7, 147.1, 142.5, 141.3, 133.4, 131.8, 130.8, 129.8, 129.7, 122.4, 122.2, 120.1, 104.2, 61.3, 52.7, 52.2, 38.6, 21.3; Anal. calcd for $C_{23}H_{20}N_4O_6$ (448.43): C, 61.60; H, 4.50; N, 12.49; Found: C, 61.66; H, 4.53; N, 12.56.

*4.2.14. Dimethyl 6-amino-5-cyano-1,4-bis(4-methoxyphenyl)-1,4-dihydropyridine-2,3-dicarboxylate (**5**)*

Yield 81%;Yellow solid; mp: 159–161 °C; IR (KBr): 3449, 3315, 3215, 2179, 1745, 1709, 1647, 1568, 1510, 1416, 1318, 1256, 1221, 1119, 1028 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.29 (d, J = 6.8 Hz, 2H, Ar–H), 7.26 (d, J = 7.2 Hz, 2H, Ar–H), 6.96 (d, J = 8.8 Hz, 2H, Ar–H), 6.90 (d, J = 8.4 Hz, 2H, Ar–H), 4.61 (s, 1H, CH), 4.05 (s, 2H, NH_2), 3.88 (s, 3H, OMe), 3.81 (s, 3H, OMe), 3.59 (s, 3H, OMe), 3.48 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 166.0, 163.8, 149.8, 142.0, 137.5, 137.4, 131.7, 131.5, 128.2, 127.4, 127.3, 120.8, 115.0, 105.3, 62.0, 55.4, 52.7, 52.1, 52.0, 37.7; Anal. calc. for $C_{24}H_{23}N_3O_6$ (449.45): C, 64.13; H, 5.16; N, 9.35. Found: C, 64.23; H, 5.22; N, 9.48.

4.3. Nuclease activity

Agarose, NaN_3, Isopropanol and DMSO were purchased from Sigma Aldrich and were used as received. pUC19 plasmid DNA, EcoRI and HindIII restriction enzymes were purchased from New England Biolabs. Solutions of crystalline DHP derivatives were made by dissolving calculated amount of the compounds in water either directly or as their hydrochloride salt and pH adjusted to 7.0. 250 ng of pUC19 was incubated with appropriate concentration of the compounds in a total reaction volume of 20 μl in 10 mM Na-Phosphate buffer (Ph 7.0) at 37 °C for 24 h. The interaction of DHP derivatives with pUC19 was monitored on 1.5% low melting Agarose gels run at 70 V for 2–2.5 h in 1X TAE buffer and stained with Ethidium bromide. Gel images were captured by UVP Gel-Doc-It 310 imaging system and the quantitative damages were estimated using Vision Work Ls Image Acquisition and Analysis software from UVP (UK). All experiments were performed in triplicate and error bars were generated by calculation of standard deviation from average values. As the time of incubation was increased from 24 h to 48 or 72 h, the amount of relaxed form of plasmid increased. Incubation studies in presence of radical scavengers was performed with 250 ng of pUC 19 in 10 mM Na-Phosphate buffer in presence of 10 mM of NaN_3, DMSO or Isopropanol and 0.75 mM of the compounds at 37 °C for 24 h. For restriction endonucleases studies, plasmid DNA was preincubated with 0.75 mM of the compounds for 24 h and then the reaction mixture was subjected to 8 units of either EcoRI or Hind III restriction enzymes along with the appropriate enzyme buffer for 1 h at 37 °C.

Nuclease activity was calculated as follows (1.42 is the correction factor for supercoiled DNA):

$$\%\ \text{Activity} = \frac{\text{Amount of nicked plasmid} \times 100}{(\text{Amount of supercoiled plasmid} \times 1.42) + \text{amount of nicked Plasmid}}$$

Acknowledgments

L.H.C is thankful to Department of Science and Technology, India for financial support with sanction No. SR/FT/CS-042/2009 for carrying out this study. S.P. and V.S. thank UGC and CSIR New Delhi respectively for their research fellowships. The authors are also grateful to IIT Patna for providing basic research facilities as well as SAIF, CDRI Lucknow and SAIF, IIT Madras for providing analytical facilities.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.03.003.

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