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Benzofused N-substituted cyclic enediynes: Activation and DNA-cleavage potential

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Abstract—The effect of electron withdrawal on the reactivity of N-substituted cyclic enediynes has been studied. These were synthesized via an intramolecular Mitsunobu reaction. The electron withdrawing effect of the nitro groups or the positive charge on the free ammonium salts was found to lower the cyclization temperature for Bergman cyclization. The ammonium salts cleave ds-DNA at nanomolar concentrations.

1. Introduction

Various structural changes are believed to lower the activation barrier for the Bergman cycloaromatization[1] process either by bringing the terminal acetylenic carbon atoms (*c*, *d* distance)[2] closer or by easing the overall conformational restrictions.[3] It is difficult to judge the effect of one factor independent of the other on the overall kinetics of the reaction and quite often, both *c*, *d* distance and strain factors act synergistically to drive the process of diradical generation at physiologically relevant temperature. In reality, both the factors need to be considered while designing new enediynes as possible therapeutic agents. Cyclic aliphatic enediynes with a ring size of 10 have low half-life[4] and as such are not easy to handle. Thus, elaboration of these molecules into a drug candidate will pose problems because of limited shelf-life. Aromatic enediynes, on the other hand, are stable at room temperature. For example,[5] the aliphatic N-substituted enediyne **1** cyclizes spontaneously at room temperature of 30 °C with a half-life of 36 h. The corresponding aromatic counterparts **2a**–**2c** are stable under ambient conditions and cyclize only when heated at 65–70 °C[6] (Scheme 1). Our long standing objective was to activate aromatic fused cyclic enediynes towards BC to bring down the cyclization temperature. In principle, this can be achieved in three ways: (i) by incorporating electron withdrawing groups in the sulfonamide aryl ring (Type 1), (ii) by attaching electron withdrawing groups in the aromatic ring,[7] which is a part of the enediyne system (Type 2) and (iii) by keeping the N atom in the protonated amine form (Type 3). All these approaches are expected to lower the activation barrier to BC. The electron withdrawing effect (mainly I) from the substituents in the aryl ring or from the positively charged N atom reduces the repulsion between the in-plane alkyne π-orbitals (Koga–Morokuma hypothesis)[8] thus helping the process of cycloaromatization leading to a planar aromatic ring. Moreover, the electron withdrawal lowers the singlet–triplet gap[9] and thus favors the triplet state. The triplet, being a better H-abstractor, then produces cleavage of DNA. The first strategy was attempted earlier[10] and the results indicated that incorporation of electron withdrawing groups in the aryl ring of the sulfonamide had some influence upon the BC kinetics. However, the effect is not sufficient enough to have the BC occurring under ambient conditions. Thus, we became interested to study the activity of molecules belonging to Type 2 and **3** categories. In this communication, we report our results.

The synthesis of Type 2 molecules starting from the nitro substituted dibromobenzene[11] is shown in Scheme 2. The salient features of the synthesis are mentioned below: (a) nitration of dibromo benzene[11] using a mixture of concd HNO_3 and H_2SO_4 (1:3 by volume) at 0 °C for 15 min yielded the 4-nitro-1,2-dibromo benzene **9a** as the major product, isolated by chromatography. This on further nitration and fractional crystallization from hexane–CH_2Cl_2 furnished the dinitro derivative **9b**; (b)

Keywords: Enediynes; Mitsunobu reaction; Bergman cyclization; DNA cleavage; pH.
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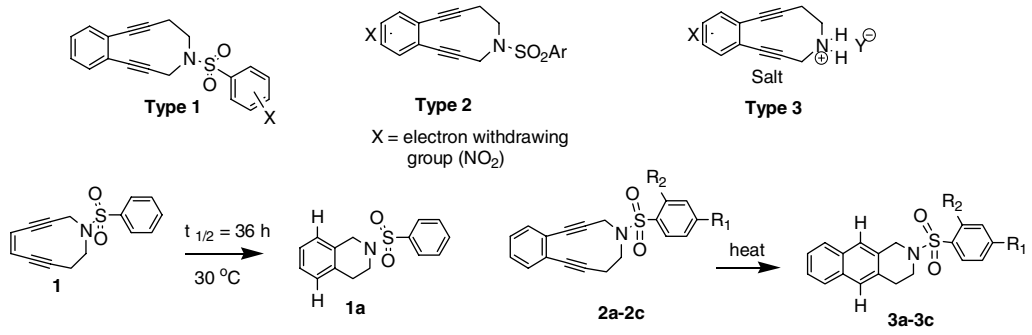
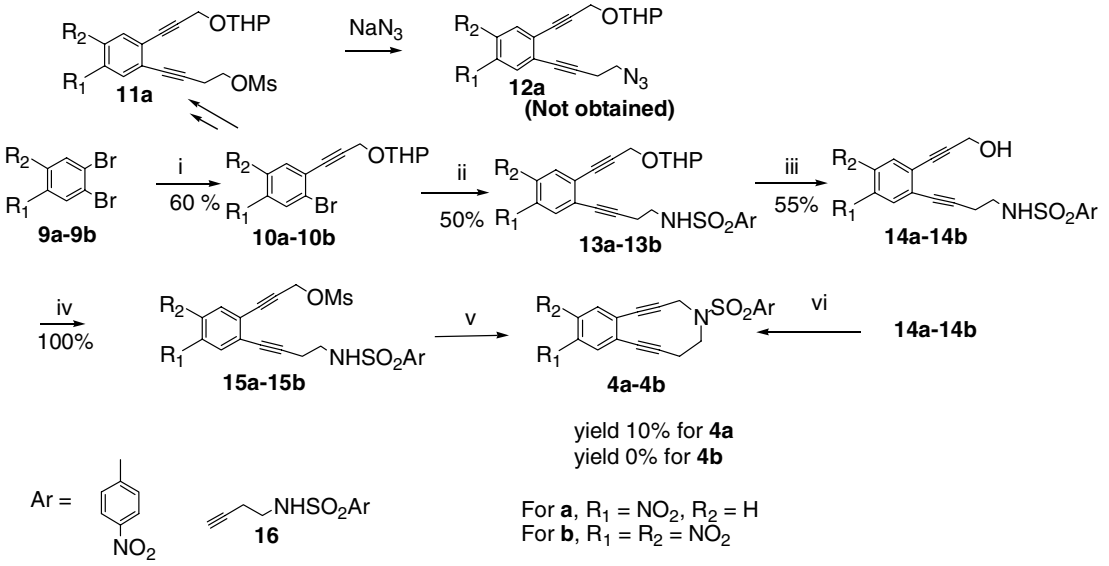


Scheme 1. Reactivity of N-substituted enediynes. Reagents and conditions: (a) R_1 = Me, R_2 = H, $t_{1/2}$ = 72 h, 65 °C; (b) R_1 = R_2 = NO_2, $t_{1/2}$ = 28 h, 70 °C (c) R_1 = NO_2, R_2 = H, $t_{1/2}$ = 49 h, 70 °C.

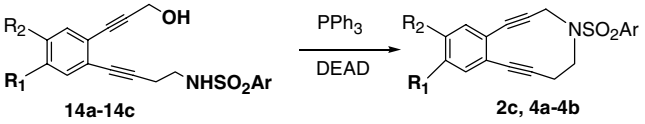


Scheme 2. Synthesis of enediynes via intramolecular N-alkylation from mesylate. Reagents and conditions: (i) Propargyl OTHP ether, Pd (0)/CuI/Et_3N/5 h (for **10a**), 1 h (for **10b**)/rt; (ii) 16, Pd (0)/CuI/Et_3N/THF (3:1)/reflux, 1 h (for **13a**)/rt, 5 h (for **13b**); (iii) PPTS/Dry EtOH/40 °C/12 h; (iv) MsCl/CH_2Cl_2/0 °C/20 min; (v) K_2CO_3/Dry DMF/2 h; (vi) PPh_3/DEAD/THF/30 min/rt.

Compound **9a** was subjected to sequential Sonogashira coupling[12] with THP-protected propargyl alcohol and 3-butyn-1-ol. Treatment with mesyl chloride in the presence of Et_3N gave mesylate **11a**; (c) attempted displacement of the mesylate with NaN_3 in DMF met with failure. The azide probably underwent cycloaddition[13] with the acetylenic arm in conjugation to the nitro group; so the N-4-nitrophenyl sulfonamido homopropargyl amine **16** was used as the second coupling[14] partner; (d) the basic condition usually employed to effect cyclization via intramolecular N-alkylation led to formation of side products. This is probably due to the base-induced isomerization of propargylic hydrogen (which is more acidic because of the nitro group) that allowed its decomposition via Myers–Saito pathway.[15] In case of the mononitro derivative, only a 10% yield of the cyclized product was obtained while for the dinitro system, we could not isolate any cyclized product, the reaction producing insoluble polymeric material (Scheme 2). Thus, the final cyclization needed avoidance of strongly basic conditions. Previously, Reitz et al.[16] have reported the synthesis of benzo fused N-substituted cyclic enediynes via an intramolecular Mitsunobu reaction. Based on that report,[16] as well as by others,[17] sulfonamide was treated with PPh_3 and diethylazido dicarboxylate (DEAD) (2.5 equiv) in THF, for 30 min. Gratifyingly, only intramolecular cyclization took place and the cyclic sulfonamide was isolated in up to 60% yield after column chromatography over Si-gel. The reaction also worked with other systems and the yields range from 60% to 70% (Table 1).

The chemical reactivity of sulfonamides **4a**–**4b** was then checked to find out the effect of the electron withdrawing nitro group on the onset temperature and kinetics

Table 1. Intramolecular Mitsunobu reaction



Compound	R_1	R_2	Ar	Product	Yield
14c	H	H	4-Tolyl	**2c**	60
14a	NO_2	H	4-Nitrophenyl	**4a**	70
14b	NO_2	NO_2	4-Nitrophenyl	**4b**	67

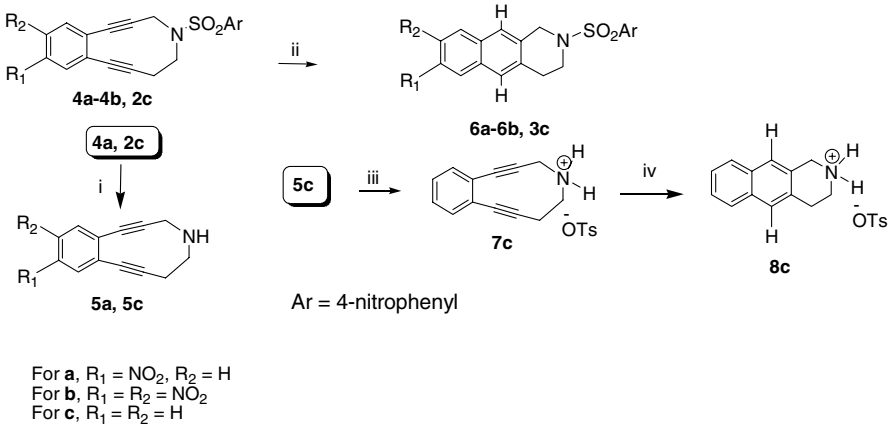
of BC. First, the DSC (differential scanning calorimetry) theromogram[18] was recorded for **4a** which showed a strong exothermic peak starting at around 166 °C which can be regarded as the onset temperature for BC. For the corresponding nonnitrobenzene fused enediyne **2c**, the onset temperature as recorded in DSC was found to be 240 °C thus showing significant lowering of onset temperature for BC in case of the nitroenediyne. Kinetic studies in solution were carried out by heating a DMSO-d_6 solution of enediyne at 70 °C and checking the ^{1}H NMR at different time points. The singlet at δ 4.44 was slowly replaced by a broad singlet at δ 4.58 typical of a methylene adjacent to the isoquinoline derivative, thus pointing to the formation of cycloaromatized product **6a** which was also proved by the appearance of peaks at *m*/*z* 415, 414, 413 (major) in the ESI mass spectrum corresponding to the abstraction of two deuterium, one deuterium one hydrogen, and two hydrogen atoms, respectively. The cyclization followed first order kinetics like the benzene fused analogue. The half-life for **4a** at 70 °C was found to be 12 h as compared to 48 h for **2c** at the same temperature. The half-life of **4a** was also recorded at 60 °C, which came out to be 138 h. For the dinitro enediyne **4b**, the onset temperature was further lowered to 128 °C and the half-life at 70 °C was found to be 9.5 h.

Thus, by incorporating electron withdrawing nitro group(s) we could significantly enhance the reactivity towards Bergman cyclization as revealed in DSC as well as in the rate of solution phase cyclization. However, the molecules still remain stable at room temperature and hence, did not show any DNA cleavage under biological conditions. It may be noted that the reactivity trend obtained from DSC measurements was in agreement with that obtained from solution phase kinetic studies.

Having failed to bring down the temperature for the BC to the level of biological conditions for nitroaromatic systems, we turned our attention to the cyclic enediynes in the protonated form (Type 3). Thus, both the cyclic enediynes **4a** and **2c** were deprotected[19] using thiophenol and K_2CO_3 in DMF and the free amines **5a** (yield 20%) and **5c** (yield 70%) were isolated pure by Si-gel chromatography (Scheme 3). Use of basic conditions produced poor yield of **5a**. Deprotection of the dinitroenediyne system **4b** was not successful, we could not isolate any free amine and that precluded any reactivity study using **5b**. DSC measurement on the tosylate **7c** indicated significant lowering of the cyclization temperature (Table 2). Solution phase kinetics for **7c** in DMSO-d_6 showed the half-life of 130 h at 50 °C, close to the biological temperature. Indeed, at 30 °C it showed a measurable rate of BC (half-life of 770 h). The tosylate salt of amine **5a** could not be isolated as the electron withdrawal by both the positively charged nitrogen and the nitro group made it reactive under ambient conditions. Even the free amine slowly decomposed in $CDCl_3$ at room temperature leading to unidentifiable products. However, generation of signals in the aromatic region is suggestive of occurrence of BC. It may be noted that sulfonamides **4a**–**4b** did not produce any cyclization product at 50 °C even when kept for 7 days. Inspired by these results, we then checked the DNA-cleavage activity of amines **5a** and **5c** at pH 8.0 in which the amine should be mostly present in the conjugate acid form. Both these compounds now showed cleavage of plasmid DNA (pBR 322) at micromolar concentrations (Fig. 1). Most interestingly, amine **5c** even showed the generation of Form III with pBR 322 which was not observed with *p*-BlueScript SK+ plasmid.[20] If the ammonium salt is really responsible for the cleavage, then the cleavage efficiency should depend upon the extent of conjugate acid formation which in turn should depend upon the pH. Thus, the DNA-cleavage study was carried out with the tosylate salt **7c** at three different pHs (7.5, 8.0, and 8.5). The gel pattern (Fig. 2) clearly showed increase of cleavage efficiency as the pH is lowered thus supporting the involvement of the ammonium salt as the key

Table 2. Results of DSC studies

Compound	Onset temperature (T_1) for BC (°C)	Difference in onset temperature (ΔT)
4a	166	74 (T_1**2c** − T_1**4a**)
4b	128	112 (T_1**2c** − T_1**4b**)
2c	240	—
7c	110	130 (T_1**2c** − T_1**7c**)



Scheme 3. BC of various enediynes. Reagents and conditions: (i) PhSH, K_2CO_3, DMF, rt, 30 min (20% for **5a**, 70% for **5c**); (ii) DMSO-d_6, 70 °C; (iii) 4-toluene sulphonic acid, ether, MeOH, 90%; (iv) DMSO-d_6, 50 °C.

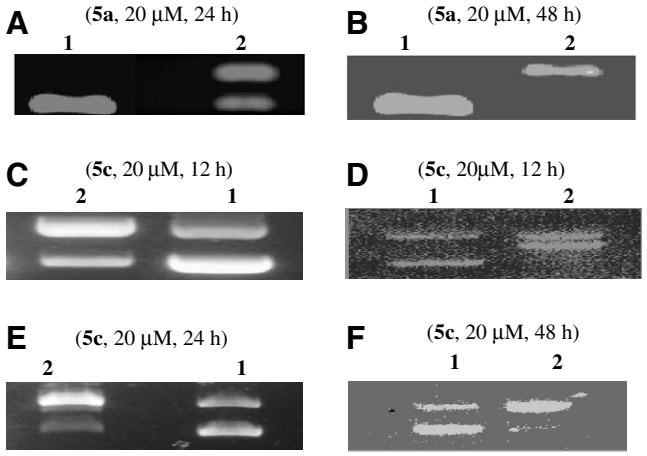
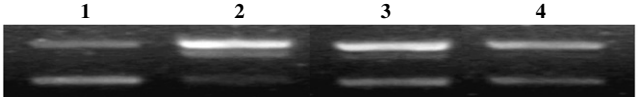


Figure 1. DNA cleavage with compounds **5a** and **5c** at 37 °C; for A, B, C, E pBR 322 and for D, F pBluscript+ were, respectively, used: lane 1, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + CH_3CN (5 μl); for A: lane 2, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + **5a** (20 μM) in CH_3CN (5 μl); for B: lane 2, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + **5a** (20 μM) in CH_3CN (5 μl); for C: lane 2, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + **5c** (20 μM) in CH_3CN (5 μl); for D: lane 2, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + **5c** (20 μM) in CH_3CN (5 μl); for E: lane 2, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + **5c** (20 μM) in CH_3CN (5 μl); for F: lane 2, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + **5c** (20 μM) in CH_3CN (5 μl).



G (**7c**, 20 μM, 24 h)

Figure 2. pBR 322 DNA cleavage with **7c** at 37 °C after 24 h; lane 1, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + CH_3CN (5 μl); for lane 2, DNA in TAE buffer (pH 7.5, 0.4 μm bp) (7 μl) + **7c** (0.4 μM) in CH_3CN (5 μl); lane 3, DNA in TAE buffer (pH 8.0, 0.4 μm bp) (7 μl) + **7c** (0.4 μM) in CH_3CN (5 μl); lane 4, DNA in TAE buffer (pH 8.5, 0.4 μm bp) (7 μl) + **7c** (0.4 μM) in CH_3CN (5 μl).

cleaving agent.[21] The stabilizing ionic interaction between the negatively charged DNA and the positively charged ammonium salt may be aiding to the efficiency of cleavage.

In conclusion, we have demonstrated that by simply using N-substituted enediyne as protonated amine, one can bring down the activation barrier for BC to such an extent that the molecules can inflict damage to ds-DNA under ambient conditions. This observation is important as we can now think of better selectivity of these molecules in showing cytotoxic activity against cancer cells, which has much lower pH[22] as compared to the normal cell.

2. Experimental

2.1. General procedure for intramolecular Mitsunobu reaction

Diethyl azodicarboxylate (0.15 mmol) was added to a solution of **14a**–**14c** (0.06 mmol) and triphenylphosphine (0.15 mmol) in 10 mL of dry tetrahydrofuran, and the solution was stirred under a dry nitrogen atmosphere for 30 min at room temperature. The solvent was concentrated and worked up with EtOAc and water. The title compounds were isolated by column chromatography (hexane/Ethyl acetate 4:1) followed by crystallization from $CHCl_3$–hexane.

2.1.1. 1-[4-Nitrophenylsulphonyl]-[5,6]-[4-nitrobenz]-1-azacyclodec-3,7-diyne (4a). Yield 70%; white solid, mp 125 °C; ν_{max} (KBr, cm^{-1}) 3430, 1526, 1348, 1159, 1095, 852, 744, 607; δ_H (DMSO-d_6) 8.38 (d, J = 8.8 Hz, 2H), 8.17 (d, J = 8.4 Hz, 4H), 7.67 (d, J = 8.4 Hz, 1H), 4.44 (s, 2H), 3.55 (t, J = 4.6 Hz, 2H), 2.85 (t, J = 4.8 Hz, 2H); δ_C (DMSO-d_6) 150.0, 146.7, 143.1, 133.7, 131.7, 129.8, 128.8, 128.8, 124.7, 123.1, 122.6, 101.6, 99.9, 85.4, 81.2, 54.9, 50.7, 41.9; Mass (ES^+) 412.06 (MH^+); HRMS calculated for $C_{19}H_{13}N_3O_6S + H^+$: 412.06042; found: 412.06040.

2.1.2. 1-[4-Nitrophenylsulphonyl]-[5,6]-[3,4-dinitrobenz]-1-aza cyclodec-3,7-diyne (4b). Yield 67%; white solid, mp 120 °C, ν_{max} (KBr, cm^{-1}) 3676, 3295, 2370, 1605, 1529, 1421, 1350, 1305, 1159, 1085, 854, 799, 734, 683, 619, 464; δ_H (acetone-d_6) 8.44 (d, J = 8.8 Hz, 2H), 8.25 (d, J = 8.8 Hz, 2H), 8.16 (s, 1H), 8.11 (s, 1H), 4.56 (s, 2H), 3.74 (t, J = 5.2 Hz, 2H), 3.00 (t, J = 5.2 Hz, 2H); δ_C (acetone-d_6) 143.9, 142.5, 134.7, 133.3, 132.3, 130.4, 128.8, 124.5, 124.5, 124.3, 106.1, 100.8, 83.9, 80.6, 50.8, 42.1, 22.1; Mass (ES^+) 456 (M^+), 380 ($M^+-NO-NO_2$), 326 (M^+-NO); HRMS calcd for $C_{19}H_{12}N_4O_8S + H^+$: 457.04549; found: 457.04530.

2.2. General procedure for deprotection of sulfonamides: synthesis of free amines 5a and 5c

To a solution of compound (0.1 mmol) (**2c**, **4a**) in DMF (15 mL), thiophenol (1.2 equiv) and K_2CO_3 (3 equiv) were added and the mixture was stirred for 40 min at room temperature. After partitioning between water and EtOAc, the organic layer was evaporated using liquid N_2 in the vacuum pump and the free amine (**5a**/**5c**) was isolated by column chromatography (Si-gel, 20% CH_3OH in CH_2Cl_2).

2.2.1. [5,6]-Benz-1-azacyclodec-3,7-diyne (5c). Yield 70%; brown oil; δ_H 7.24–7.20 (m, 2H), 7.15–7.12 (m, 2H), 3.78 (NH), 3.58 (s, 2H), 3.18 (t, J = 5.4 Hz, 2H), 2.48 (t, J = 5.4 Hz, 2H); Mass (ES) m/z 182 (MH^+).

2.2.2. [5,6]-(4-Nitrobenz)-1-azacyclodec-3,7-diyne (5a). Yield 20%; brown oil; δ_H 8.09 (s, 1H), 8.01 (d, J = 8.4 Hz, 2H), 7.26 (d,J = 8.4 Hz, 1H), 3.45 (s, 2H), 3.11 (bs, 2H), 2.46 (t, J = 5.2 Hz, 2H); Mass (ES) m/z 227 (MH^+).

2.3. Synthesis of [5,6]-benz-azacyclodec-3,7-diyne 4-toluene sulfonate (7c)

The diethyl ether solution of the amine compound (**5a**, **5c**) was added drop wise to the solution of p-toluene sulfonic acid in diethyl ether with few drops of methanol mixture. The reaction mixture was stirred for few minutes until the

white precipitate appeared. The solid material was isolated by washing with diethyl ether several times; Yield: 100%, white solid, mp 235 °C (decomposed); ν_{max} (neat): 3464, 2979, 2924, 2786, 2629, 2524, 1461, 1213, 1154, 1030 and 1006; δ_H (DMSO-d_6) 9.39 (br s, 2H), 7.50–7.39 (m, 6H), 7.09 (d, $J = 8$ Hz, 2H), 4.20 (s, 2H), 3.46 (t, $J = 5.1$ Hz, 2H), 2.82 (t, $J = 5.1$ Hz, 2H), 2.27 (s, 3H); δ_C(DMSO-d_6) 145.5, 137.8, 129.2, 128.5, 128.1, 128.0, 127.8, 126.9,125.5, 98.2, 90.3, 88.1, 83.8, 48.7, 39.5 (obscured by DMSO), 20.8, 18.7; Mass (ES^+) *m/z* 182.11 (MH^+), 153.08 ($M^+ - CH_2{=}NH$); HRMS calcd for $C_{13}H_{12}N$: 182.0971; found: 182.0977.

2.4. Spectral data of cycloaromatized products

For **6a**: δ_H (DMSO-d_6) 8.5–8.2 (9H, m), 4.58 (2H, m), 3.56 (2H, m), 3.10 (2H, m); Mass 413 (M^+).

For **6b**: δ_H (DMSO-d_6) 8.6–8.2 (8H, m), 4.60 (2H, m), 3.58 (2H, m), 3.14 (2H, m); Mass 458 (M^+).

For **8c**: δ_H (DMSO-d_6) 8.95 (1H, br s), 7.35 (8H, m), 7.05 (2H, d, $J = 8.0$ Hz), 4.40 (2H, m), 3.40 (2H, obscured), 3.12 (2H, m), 2.14 (3H, s); Mass 184 (MH^+).

2.5. Differential calorimetric studies

DSC measurements were recorded in Perkin-Elmer Jade instrument (Jade DSC). The rate of heating was fixed at 10 °C/min. Heating was carried out in an atmosphere of nitrogen (99.49% purity) in aluminum crucibles. Calibration was done with indium.

2.6. DNA-cleavage experiment

Sample solution (5 μl) in acetonitrile was mixed with the DNA solution (7 μl) in TAE (Tris–Acetate–EDTA) buffer (pH 8.0) and was incubated at 37 °C for 12–48 h. The solution was then mixed with aqueous sucrose (40%, 20 μl)) and bromophenol blue (0.25%, 5 μl). From the above mixture, 15 μl was loaded on 0.7% agarose gel and was subjected to electrophoresis in a horizontal slab gel apparatus in TAE buffer for 1 h under 75 V. After electrophoresis, the bands were visualized under UV-transilluminator and photographed using GELDOC. The cleavage efficiency was measured by densitometry using image processing software (Kodak 1D version V.3.6.3).

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20. Although **5a** is more reactive than **5c** in the protonated form, the lower basicity of the former may be responsible in generating less number of cleavage events. This might explain the DNA-cleavage result observed with the amines.
21. Considering the pK_a of the cyclic amine **5c** to be ~8.6 (assumed to be close to *N*-ethyl *N*-propargyl amine), it has

been estimated that the percentage of protonated form of the amine **5c** at pH 7.5, 8.0 and 8.5 to be approximately 92, 80, and 55, respectively. Thus, there is a definite correlation between extent of cleavage and percentage of protonated form. The pK_a of *N*-ethyl *N*-propargyl amine has been obtained from SciFinder search which used ACD software for calculating the pK_a.

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