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Synthesis of highly efficient pH-sensitive DNA cleaving aminomethyl N-substituted cyclic enediyne and its L-lysine conjugate

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ABSTRACT

Two 10-membered benzo-fused N-substituted cyclic enediynes, one an amino methyl and the other, a C-lysine conjugated derivative **2** and **3**, respectively were synthesized (as a 1.2:1 mixture of regioisomers) and their DNA-cleavage efficiency studied. Both the compounds showed much better DNA-cleavage profile than that of the parent unsubstituted enediyne **1**. The lysine conjugate **3** showed an efficient pH dependent cleavage to the extent of ~50% of linear DNA formation under ambient conditions.

Designing anticancer agents with a high degree of selectivity has been a great challenge.[1] Distinguishing cancer cells from normal ones requires use of molecules[2] which target protein receptors or enzymes that are over-expressed in cancer cells. An alternate strategy is to capitalize the difference in the microenvironment like pH in the two types of cells.[3–6] Previously, we have reported[7] the DNA cleavage efficiency of a benzofused *N*-substituted cyclic enediyne **1** (Fig. 1) in the ammonium salt form that underwent a Bergman Cyclization (BC)[8] under ambient conditions. The DNA-cleavage potential was shown to be dependent upon pH; however, being a primary amine, the pH window had to be restricted to the range of 7.5–8.5. Although the compound exhibited formation of linear strands of DNA, the extent of cleavage was very low. It may be mentioned that for designing a pH-sensitive anticancer agent, two important parameters should be kept in mind: high efficiency in terms of cleavage to produce Form III (Linear) which should increase with lowering of pH to values attainable in a cancer cell[9] as opposed to pH of 7.2 in normal healthy cells. Recently, Alabugin et al.[10] have reported a clever design of C-lysine conjugates of various acetylenes and acyclic enediynes which showed double-stranded DNA-cleavage under photoirradiation. The fully protonated lysine at pH <7 assured stronger DNA binding and less photo quenching thereby showing better efficiency of cleavage. Encouraged by this report and also based on our intention to increase the DNA-cleavage efficiency under lower pH range, specifically the pH range of cancer cell, two cyclic enediynes, one an aminomethyl N-substituted enediyne **2** (obtained as a 1.2:1 mixture of **2a** and **2b**) and the other a corresponding L-lysine conjugated enediyne **3** (again as a 1.2:1 mixture of **3a** and **3b**) were synthesized. Their DNA-cleavage efficiencies were studied and compared with the parent enediyne **1**. To our delight the lysine conjugate **3** showed high cleavage efficiency (to an extent of 50% of linear form) at a pH range of 5.5–6.5. This is quite remarkable for a small molecule, a property which has high relevance to anticancer drug development.

Compound **1** was synthesized as reported earlier.[7] It was isolated as the TFA salt. The synthesis of both the enediynes **2** and **3** required 4-aminomethyl 1,2-diiodo benzene **10** which was prepared from 4-amino benzoic acid **4** via a sequence of steps as shown in Scheme 1. The free amine **10** was Boc-protected and then subjected to sequential Sonogashira coupling,[11] first with 1-butyne-3-ol followed by THP-protected propargyl alcohol. The resulting acyclic enediyne **12** (isolated as an inseparable mixture of two regioisomers in the ratio of 1.2:1) was successfully converted into the cyclic system **18** via a series of reactions already reported.[12] The fully protected enediyne **18** was then deprotected, first with thiol[13] (for Nosyl removal) and then with TFA (for Boc removal)

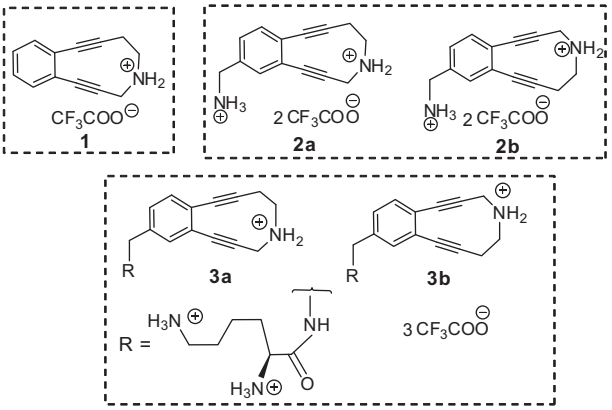

Figure 1. The target enediynes as ammonium salts.

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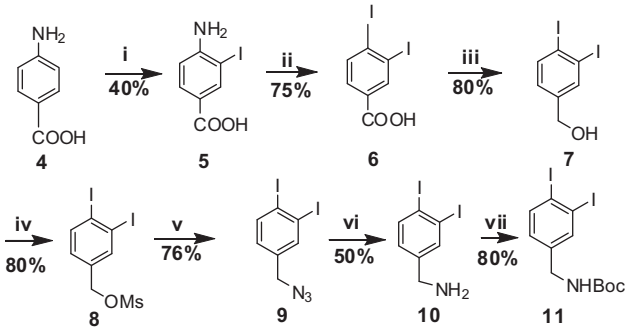


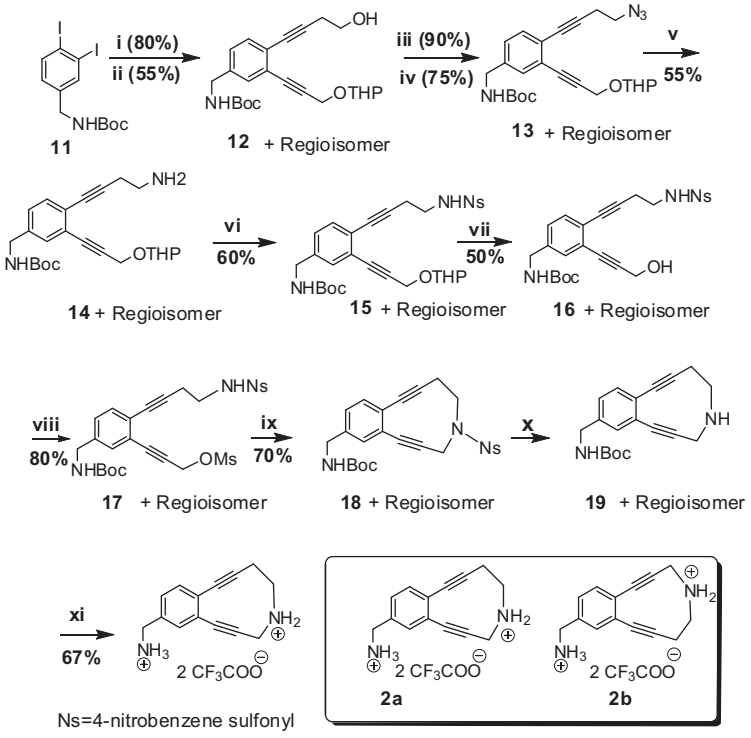
Reagents & Conditions: $NaIO_4$, KI, MeOH, r.t.; ii) $NaNO_2$, KI, 0 °C, 2 h; iii) BH_3SMe_2, THF, r.t., 6 h; iv) MsCl, CH_2Cl_2, 0 °C, 30 min; v) NaN_3, DMF, r.t.; 12 h; vi) PPh_3, THF, H_2O, r.t.; 12 h; vii) $CHCl_3$, Boc-anhydride, r.t., 12 h

Scheme 1. Synthesis of 4-aminomethyl 1,2-diiodo benzene.

and the target enediyne **2** was finally isolated as the bis-TFA salt (as 1.2:1 mixture of regioisomers **2a** and **2b)** (Scheme 2).

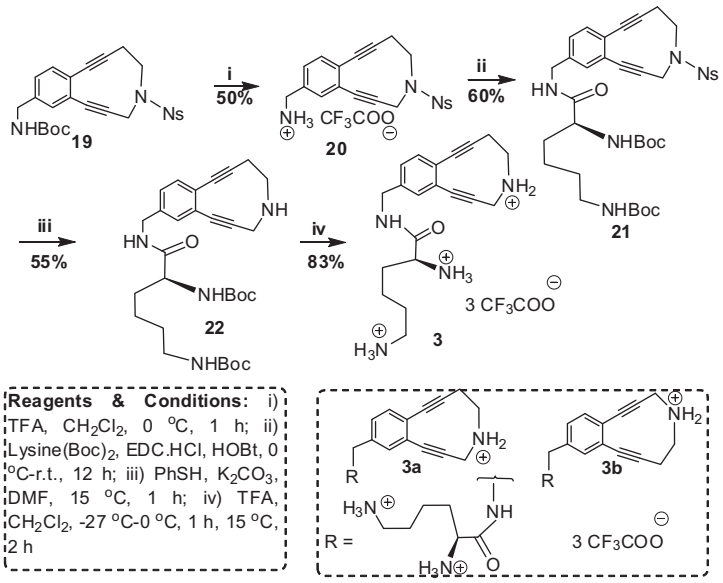
The synthesis of the L-lysine conjugate **3** which was also isolated as a mixture of regioisomers **3a** and **3b** in a ratio of ~1.2:1 is shown in Scheme 3. Thus the enediyne **19** was first deprotected with TFA and the free amine **20** was coupled with bis-Boc L-lysine (from L-lysine monohydrochloride of 99% purity) in the presence of EDCI.[14] Thiol mediated deprotection of the resulting lysine conjugate **21** produced the monoamine **22**. Further deprotection with TFA led to the final compound **3**, isolated pure as the tris-TFA salt. All the new compounds were fully characterized by high field ^1H and ^{13}C NMR analysis.[15]

Before checking the DNA-cleavage activity of the synthesized molecules **2** and **3**, their chemical reactivity towards BC was evaluated. These compounds showed higher reactivity towards BC as revealed by the onset temperature observed in DSC (~65 °C for both) as compared to ~110 °C reported for **1**. The ability of the enediynes **1**–**3** to cleave DNA was then investigated[16] using the conversion of supercoiled plasmid DNA into the respective relaxed circular and linear forms (Forms II and III, respectively). A densitometric analysis[17] of the gel electrophoresis bands provided the relative amounts of the three DNA forms. Enhancement of DNA-cleaving ability has been observed for both the amino methyl enediyne **2** as well as the lysine conjugate **3** as compared to the parent enediyne **1** (Fig. 2). The most remarkable observation is the increase, often quite dramatic, in the efficiency of cleavage leading to Form III of DNA by both compounds **2** and **3** when pH changes from slightly alkaline to slightly acidic. Control experiments clearly indicated that no additional cleavage was caused by DNA alone under the incubation conditions upon lowering of pH. For compound **2**, at pH 7.5, only 60% of intact DNA (Form I) remained after 18 h of incubation in contrast to 45% and 20% of unreacted DNA at pH 6.5 and 5.5, respectively. Even more remarkable is the fact that while there was no Form III at pH 7.5 at the concentrations of 20 μM, there was formation of Form III at a lower pH of 6.5, as is reflected in the *nicked/linear* ratio[18] of 5.7:1. This ratio improves further to 2.26:1 at pH 5.5. Compound **3** showed even better cleavage efficiency at lower pH (*nicked/linear* ratio varied from 1.35 at pH 6.5 to 0.80 at pH 5.5) (Fig. 3). This favourable *nicked/linear* ratio is quite important for a small molecule-based



Reagents & Conditions: i) 3-Butyn-1-ol, $Pd(PPh_3)_4$, Et_3N, CuI, 0 °C-r.t., 3 h ii) THP-propargyl alcohol, CuI, $Pd(PPh_3)_4$, Et_3N, r.t., 12 h; iii) MsCl, Et_3N, CH_2Cl_2, 0 °C, 30 min; iv) NaN_3, DMF, r.t., 15 h; v) PPh_3, THF, H_2O, r.t., 12 h; vi) NsCl, Et_3N, DCM, 0 °C, 1 h; vii) PPTS, EtOH, H_2O, 40 °C; viii) MsCl, CH_2Cl_2, Et_3N, 0 °C, 30 min; ix) K_2CO_3, DMF, r.t., 12 h; x) PhSH, K_2CO_3, DMF, 15 °C, 1 h; xi) TFA, CH_2Cl_2, -27 °C-0 °C, 1 h, 15 °C, 2 h

Scheme 2. Synthesis of aminomethyl enediyne **2** as bis-triflate salt.



Scheme 3. Synthesis of L-lysine conjugated enediyne **3** as tris-triflate salt.

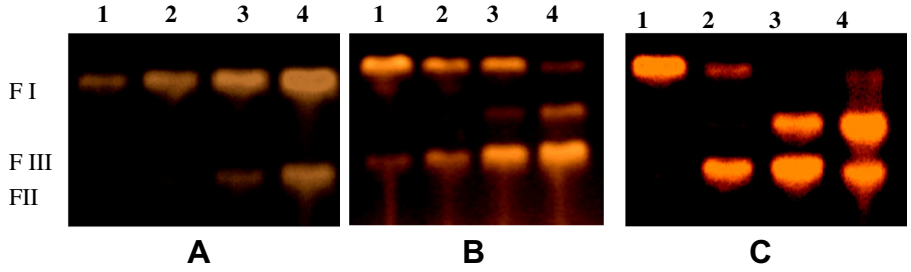


Figure 2. Qualitative Plasmid Relaxation Assays carried out with compounds **1**, **2**, **3** (5 μL each from a stock of 20 μM in DMSO) and pBR 322 Plasmid DNA (7 μL from a stock of 0.03 μg/μl at pH 8.0). These were separately mixed with 20 mM phosphate buffer of pH 7.5, 6.5 and 5.5 and incubated for 18 h at 37 °C; **A** for Compound **1**, **B** for Compound **2** and **C** for compound **3**; Lanes **1**: DNA alone, **2**: at pH 7.5, **3**: at pH 6.5 and **4**: at pH 5.5.

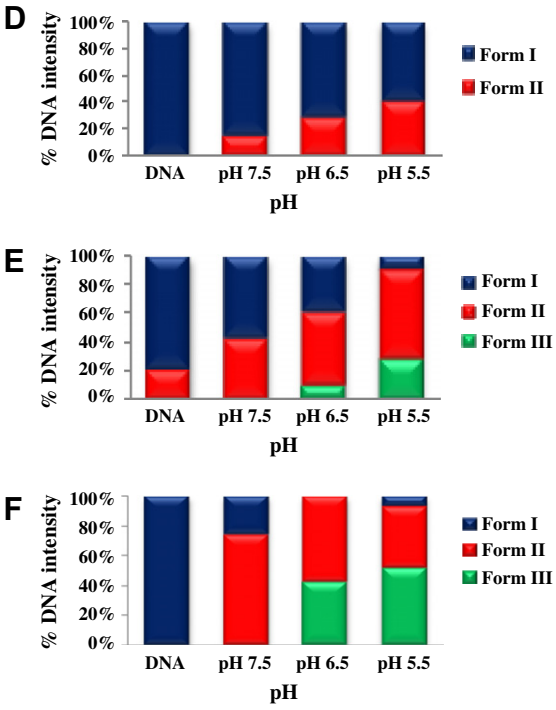


Figure 3. Quantified cleavage data are presented: **D** for Gel **A**, **E** for Gel **B** and **F** for Gel **C**.

DNA cleaver which works at biological temperatures. However, in the absence of any study based on statistical analysis,[19,10] to determine the nature of cuts (number of *ss* and *ds* cuts), the cleavage efficiency of **2** and **3** could not be compared with that of the natural enediynes. However, the observed switch from negligible to high efficiency of the most therapeutically useful form of DNA cleavage (formation of linear DNA) occurring upon a relatively small change in the pH opens up their potential use to differentiate healthy cells from hypoxic cancer tissues.

In silico molecular docking of the biradical intermediates from enediyne **1** and from the regioisomers **2a** and **3a** with CT-DNA segment 5′-CGCGAATTCGCG-3′ revealed that all the three compounds bind to the minor groove. The free energy of docking (Fig. 4) for the compounds was calculated (Table 1) which showed most efficient binding of biradical intermediate from compound **3a** because of the greater number of H-bond interactions resulting in strongest binding.[20] These results supported the original assumption that the side chain lysine moiety of the enediyne plays an important role in the DNA binding process. UV titration assay for compound **3** also showed better binding at lower pH as compared to pH of 7.5.

In conclusion, the previously synthesized *N*-substituted cyclic enediyne **1** has been made much more effective DNA-cleaving agent, especially at acidic pH by incorporating more ammonium moieties in the form of amino methyl and lysine side chains. The present study is aimed towards further screening of the compounds.

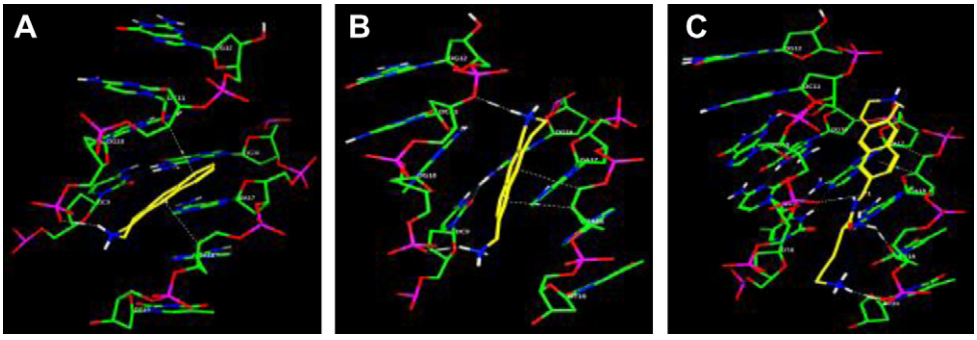


Figure 4. Docking of biradical intermediates (A) from **1**, (B) from **2** and (C) from **3**

Table 1
Calculated free energy of binding

Compd	$\Delta G_{binding}$ (Kcal/mol)
1-Biradical	−9.19
2a-Biradical	−11.7
3a-Biradical	−16.86

Acknowledgement

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.11.102.

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15. Spectroscopic data (^{1}H NMR and ^{13}C NMR recorded at 400 MHz and 100 MHz, respectively)
*For **1***: δ_H (d_4-CD_3OD): 7.42–7.32 (4H, m), 4.19 (2H, s), 3.64, br s), 2.88 (2H, t, J = 5.0 Hz); δ_C (d_4-CD_3OD): 159.5, 128.6, 128.1, 127.8, 127.8, 126.9, 95.3, 88.9, 87.4, 83.4, 49.3, 39.3, 18.4.
*For **2***: δ_H (d_4-CD_3OD): 7.54–7.43 (3H, m), 4.21 (2H, s), 4.12 (2H, s), 3.64 (2H, t, J = 5.0 Hz), 2.9 (2H, t, J = 5.2 Hz); δ_C (d_4-CD_3OD): *For isomer I (**2a** or **2b**)*: 160.5, 134.1, 129.2, 128.9, 128.4, 128.3, 127.6, 96.6, 88.7, 88.3, 88.2, 49.2, 42.2, 39.2, 18.4; *For isomer II (**2b or 2a**)*: 160.5, 133.3, 129.2, 128.8, 128.4, 128.3, 127.7, 96.8, 88.5, 88.1, 83.3, 49.2, 42.2, 39.2, 18.4; HRMS (done on the mixture) Calcd for $C_{14}H_{14}N_2 + H^+$ 211.1230. Found 211.1230.
*For **3***: δ_H (d_6-DMSO): 9.73 (1H, br s), 9.08 (1H, s), 8.25 (3H, s), 7.92–7.87 (3H, m), 7.49–7.27 (3H, m), 4.43–4.23 (2H, m), 4.18 (2H, s), 3.47 (2H, s), 3.14 (1H, s), 2.86–2.82 (2H, m), 2.72–2.70 (3H, m), 1.71 (2H, br s), 1.51 (2H, s), 1.29 (2H, s); δ_C (d_6-DMSO): *For isomer I (**3a or 3b**)*: 168.9, 158.9, 140.2, 128.4, 128.2, 127.5, 127.0, 125.8, 98.6, 90.2, 88.3, 83.9, 52.3, 48.9, 42.1, 36.3, 31.2, 30.7, 26.7, 21.6, 18.9. *For isomer II (**3b or 3a**)*: 163.0, 158.5, 139.5, 130.6, 128.3 127.2, 127.1, 126.8, 98.3, 90.4, 82.3, 83.9, 52.2, 48.9, 42.0, 36.3, 31.2, 30.6, 26.7, 21.6, 18.9; HRMS (done on the mixture) Calcd for $C_{20}H_{26}N_4O + H^+$ 339.2180.1230. Found 339.2178.
16. *DNA cleavage experiment* Solutions of sample (5 μL, from a stock of 20 μM in DMSO) and DNA (7 μL, from the stock solution of 0.03 μg/μL) were taken in 20 mM phosphate buffer (pH 7.5, 6.5, 5.5) and incubated at 37 °C for 18 h. Aqueous solution of sucrose (40%, 20 μL) and bromophenol blue (0.25%, 5 μL) were added and mixed thoroughly.[21] 30 μL of the above mixture was loaded on 1.0% agarose gel and subjected to electrophoresis in a horizontal slab gel apparatus in TAE buffer (pH 8) for 1 h under 75 V. After electrophoresis, the bands were visualized under an UV-transilluminator and photographed using GELDOC. The cleavage efficiency was measured by densitometry using Image J software.
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18. The ratio of nicked circular DNA to linear DNA as observed in the gel was determined by densitometric analysis after taking care of the reduced intercalation of ethydium bromide into Form I.
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20. The results from docking with the biradical intermediates from the other set of regioisomers **2b** and **3b** showed similar trends (see SI).
21. The protocol for cleavage experiment was designed in such a way that the ratio of drug: DNA concentration/bp is maintained below 1.