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An Acyclic Enediyne Anticancer Compound Attributed to a Bergman Cyclization at Physiological Temperature

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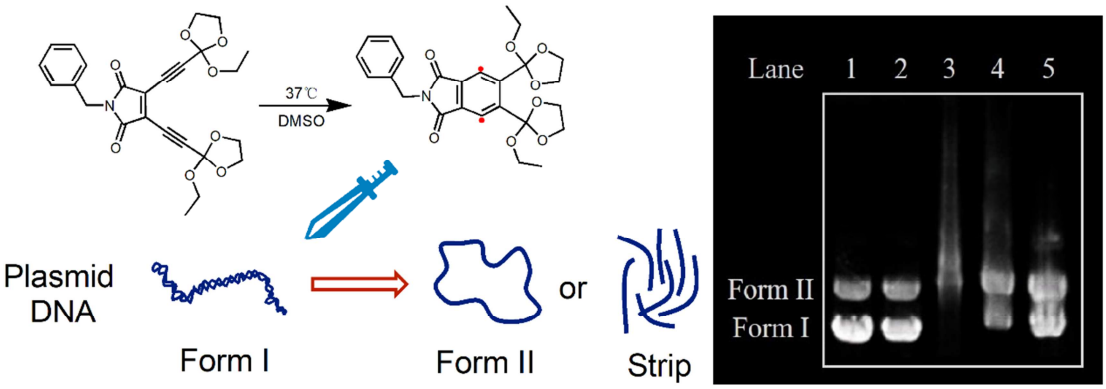

An acyclic enediyne functions possibly through spontaneous Bergman cyclization to induce DNA cleavage, inhibit cancer cell growth, and lead cancer cells to apoptosis pathway.

An Acyclic Enediyne Anticancer Compound Attributed to a Bergman Cyclization at Physiological Temperature

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Abstract Enediyne cytotoxic drugs have attracted much attention because of their unique structure and potent anticancer activity. However, acyclic enediynes are long considered as inactive at physiological temperature due to their long C1-C6 distance. By adjusting the steric bulkiness of the functional groups at the alkynyl termini and the electron-withdrawing effect at the ene moiety, herein, a simple acyclic enediyne was designed and synthesized to achieve the onset of thermal Bergman cyclization at physiological temperature in polar solvents. The spontaneous generation of diradical intermediates was confirmed through EPR analysis and further supported by spin trapping experiment, radical indicator experiment, and high resolution MS analysis. The reactive diradicals generated in aqueous media induced single and double stranded cleavage of DNA, and showed high cytotoxicity to Hela cells. The IC_{50} value of the enediyne compound is comparable to many clinical used anti-tumor agents.

Keywords: Anticancer Agents • Bergman cyclization • Cytotoxicity • DNA cleavage • Enediyne

Introduction

Cancer is one of the major diseases seriously threatening the lives of human beings. Searching for medicines employed in cancer treatment is therefore of great importance in medicinal chemistry.[1-3] A number of anticancer agents have been used for clinical practice,[4-8] and there have been many ways to load these drugs.[9-11] Unfortunately, many conventional anticancer agents face failures in their development due to the insurgence of multidrug resistance.[12-14] Enediyne antitumor agents, on the other hand, are promising to solve this problem thanks to their unique radiomimetic cytotoxic mechanism.[15-18] Indeed, enediyne compounds are acknowledged as the strongest antitumoral agents, with their half maximal inhibitory concentration (IC_{50}) ranging from submicromolar to picomolar towards a wide spreading of cancer cell lines.

Neocarzinostatin (NCS) is the first member of natural enediyne family secreted from *Streptomyces macromomyceticus* in 1965.[19] Afterwards, more than 20 kinds of natural enediyne compounds have been discovered, such as Calicheamicins,[20] Dynemicins,[21] and Esperamicins.[22] These compounds are named for an enediyne structure, which induce DNA cleavage in cancer cells through diradicals generated from a Bergman or Bergman-like cyclization. At present, NCS and Calicheamicins have been employed in clinical treatment; studies on the modification of natural enediynes also emerge.[23,24] However, due to the complicated molecular structure, the difficulty in chemical synthesis of most natural enediyne compounds isolated from microorganisms brings a great obstacle to the development of new types of enediynes antitumoral drugs. For example, Nicolaou et al. calls for 35 steps of organic reactions to the total synthesis of Calicheamicins.[25] Besides, the yield of natural enediynes or analogues is also problematic due to the tedium synthetic procedure,[24] hindering the industrialization of enediyne anticancer agents. To this end,

developing new enediyne anticancer compounds with simple structure and high tumor cell suppression efficiency is of great significance.

In most cases, the chemically synthesized enediynes with simple molecular structure are inert towards thermal Bergman cyclization at physiological temperature. Nicolaou[26] has pointed out that the C1-C6 distance of an enediyne compound should be shorter than 3.35 Å (which was later on expanded by Schreiner et al. to be 3.4 Å[27]) for a spontaneous Bergman cyclization to take place at ambient temperature. Many strategies have been adopted to lower down the onset temperature of enediyne compounds, such as introducing strong electron-withdrawing groups,[28-31] coordinating with metal ions[32-36] or embedding the enediyne moiety into a highly strained 9- or 10-member ring.[37,38] Recently, our work revealed that by incorporating the ene moiety of enediyne into electron-deficient maleimide framework, the onset temperature of thermal Bergman cyclization is drastically lowered down.[39] The maleimide-based enediyne compounds with triethoxy group at alkyne termini are active at around 100 ºC.[40,41] When the orthoester groups were hydrolyzed in an acidic environment, the enediyne was activated towards Bergman cyclization at low temperature, leading to the formation of reactive diradicals for DNA-cleavage and tumor cell suppression.

Herein, by sophisticated adjusting the steric bulkiness of the substituents at the alkyne termini, the onset temperature of an acyclic enediyne was modulated to close to physiological temperature. Spontaneous Bergman cyclization which was attributed has arisen in polar solvents, leading to diradical formation. The acyclic enediyne showed remarkable cytotoxicity towards Hela cell line, and induced apoptosis in the cancer cells.

Experimental

Materials

Toluene and tetrahydrofuran (THF) were dried over calcium hydride and distilled before use. Other chemicals were of commercial grade and used as received. 3,4-Diiodo-N-benzylmaleimide (**1**),[42] 3,3,3-triethoxy-1-propyne,[43] and catalyst NHC-$PdCl_2$-3-chloropyridine[44] were synthesized according to literature procedures with minor modification. The detailed synthesis of these precursors is presented in the ESI.

Synthesis of enediyne compounds

2-Ethoxy-2-ethynyl-[1,3]dioxolane (compound 2). 3,3,3-triethoxy-1-propyne (4.776 g, 27.76 mmol), succinic acid (0.147 g, 1.24 mmol), and ethylene glycol (3.923 g, 63.20 mmol) are successively weighed in a 50 ml sealed bottle. The solution system was stirred for 2 h at 120 ºC and purified by silica chromatography with hexane/ethyl acetate = 10/1 as eluent to give a colorless liquid (2.01 g 51.2%). ^{1}H NMR ($CDCl_3$, ppm): 4.03-4.07 (m, 4 H, -O-CH_2-CH_2-), 3.63-3.69 (q, 2 H, J = 7.2 Hz, -CH_2), 2.55 (s, 1 H, C≡CH), 1.15-1.19 (t, 3 H, J = 7.2 Hz, CH_3) ^{13}C NMR ($CDCl_3$, ppm): 113.1, 77.5, 71.1, 64.8, 59.6, 14.8. HR-MS (ESI): m/z calcd. for $C_7H_{10}O_3Na$ $(M + Na)^+$: 165.0528; found: 165.0525.

1-Benzyl-3,4-bis-(2-ethoxy-[1,3]dioxolan-2-ylethynyl)-pyrrole-2,5-dione (enediyne 3). Compound **1** (263.4 mg, 0.6 mmol), CuI (45.7 mg, 40%), NHC-$PdCl_2$-3-chloropyridine (102.0 mg, 25%), DIPEA (244.4 mg, 1.8 mmol) were successively added to a solvent mixture of dry THF (3 mL) and toluene (6 mL) under nitrogen. Then, compound **2** (0.2558 g, 1.8 mmol) was added dropwisely. The mixture was stirred at 20 ºC for 15 h. After the completion of the reaction as detected by TLC, the mixture was directly purified by column chromatography over magnesium silicate (hexane/ethyl acetate = 4 : 1) to yield a red viscous liquid (0.12 g, 42.8%). The product was stored at low temperature. ^{1}H NMR ($CDCl_3$, ppm): 7.29-7.32 (m,

5 H, Ph), 4.69 (s, 2 H, CH_2), 4.16-4.14 (m, 8 H, CH_2), 3.76-3.82 (q, 4 H, J = 7.1 Hz, CH_2), 1.24-1.27 (t, 6 H, J = 7.1 Hz, CH_3). ^{13}C NMR ($CDCl_3$, ppm): 165.6, 135.5, 129.3, 128.9, 128.7, 128.6, 128.3, 128.0, 114.2, 103.3, 72.0, 65.4, 60.5, 42.8, 15.2. HR-MS (EI): m/z calcd. for $C_{25}H_{25}NO_8$ (M + 2H): 469.1738; found: 469.1739.

Characterizations

^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded in chloroform ($CDCl_3$) on an Ultra Shield 500 spectrometer (BRUKER BIOSPIN AG, Magnet System 500 MHz/54 mm) and referenced to Me_4Si. Mass spectra were recorded on a Micromass LCTTM mass spectrometer using the ESI method or EI method. Differential scanning calorimetry (DSC) was carried out with a Pyris Diamond thermal analysis workstation equipped with a model 822e DSC module under a constant nitrogen flow. The electron paramagnetic resonance (EPR) study was carried out with the enediynes dissolved in DMSO or chloroform at a concentration of 8 mM. Measurements were performed with an X-band EMX-8/2.7C EPR spectrometer (Bruker, Germany). The settings of the spectrometer were as follows: sweep width, 200G; time constant, 163.84 ms; conversion time, 40.96 ms; resolution, 1024 points; modulation frequency, 100.00 kHz; modulation amplitude, 1.00 G; and microwave power, 6.370 mW.

DNA cleavage experiments

The freshly prepared enediyne **3** was respectively dissolved in DMSO (2 μL) at concentrations of 200 mM, 100 mM and 50 mM, and added to a solution of supercoiled plasmid FX174 RF1 DNA (0.5 mg/mL) in TE buffer (pH 7.6, 4 μL). Control samples were separately incubated with pure DMSO and DNA. All the systems were incubated at 37 ºC for 48 h. After incubation, each system (6 μL) was mixed with a 6×loading buffer (1 μL) and subjected to a 0.8% agarose gel electrophoresis at 128 V (101 mA) for 30 min, stained by ethidium bromide and then the gel was photographed on the UV transilluminator (FR-200A) and analyzed by scanning densitometry.

Cell viability

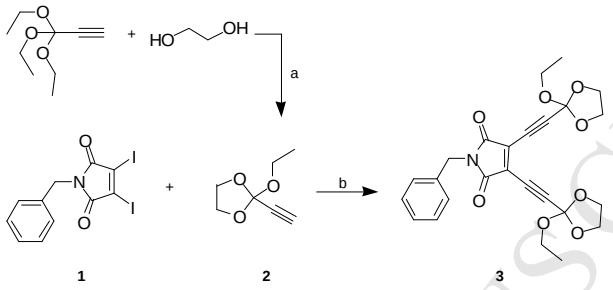
The cell viability tests were investigated by an MTT assay.[45] Human uterine cervix carcinoma HeLa cells were respectively cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 ºC under a humidified atmosphere containing 5% carbon dioxide. The cells were trypsinized until it reaching 70% confluence in the tissue culture flasks with buffered saline solution containing 0.25% trypsin and 0.03% EDTA.

Cells were seeded into a 96-well plate at a density of 5000 cells per well in 200 μL of the cell culture medium. After 24 h of incubation, the culture medium was removed and the cells were then exposed to 0.75, 1.5, 3.13, 6.25, 12.5, 25 and 50 μM concentrations of enediyne **3**. The enediyne **3** solution was sterilized by a 0.22 μm membrane filter (Sartorius Stedim Biotech, France). The concentration of DMSO was 0.1 %. The culture medium (0.05% DMSO) was used as a blank control. After incubation for another determined period of 48 h, 20 μL of sterile filtered MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliumbromide) stock solution (5 mg/mL) in PBS (pH 7.4) was added to each well and the cells were further incubated for 4 h at 37 ºC to allow the yellow dye to be transformed into blue crystals. Then, the unreacted dye was removed by aspiration, and 200 μL of DMSO was added to each well to dissolve the dark blue crystals. Finally, the optical density was measured by a microtitre reader at a wavelength of 492 nm. The spectrophotometer was normalized using culture medium without cells. Cell viability (%) related to control wells containing cell culture medium without enediyne was calculated by $[A]_{test}/[A]_{control}$.

Flow cytometric analysis

Cell death was analyzed by fluorescence-activated cell sorting (FACS) using Annexin V and propidium iodide (PI) staining assay. HeLa cells were seeded in a 6-well plate at a density of 1×10^5 cells per well and incubated in DMEM/FBS incubation medium and allowed to settle for 24 h. The medium was replaced with the fresh one containing enediyne **3** (30μM). After incubation for 48 h, the cells were washed twice with cold PBS, trypsinized and centrifuged. Then the cells were collected for Annexin V and propidium iodide (PI) staining and analyzed by flow cytometry.

Results and discussion



Scheme 1. Synthesis of enediyne **3**. a) succinic acid, 120 ºC, 2h; b) CuI, NHC-$PdCl_2$-3-chloropyridine, DIPEA, THF/ toluene, 20 ºC, 15 h.

The terminal alkyne with dioxolane-type orthoester substituent (**2**) was firstly synthesized from orthoester exchange reaction between 3,3,3-triethoxy-1-propyne and ethylene glycol with succinic acid as the catalyst. The Sonogashira reaction between **1** and **2** to produce **3** was achieved at 20 ºC using an air-stable and highly reactive Pd catalyst, NHC-$PdCl_2$-3-chloropyridine, developed by Michael et al.[44] It should be noted that this reaction should be run at low temperature to reduce the consumption of the enediyne **3** through the reactions like thermal Bergman cyclization and the formation of side products (see below) in the reaction mixture. The obtained enediyne **3** was characterized by NMR spectroscopy and high resolution mass spectroscopy (ESI† Figure S1 and S2), and stored in a refrigerator.

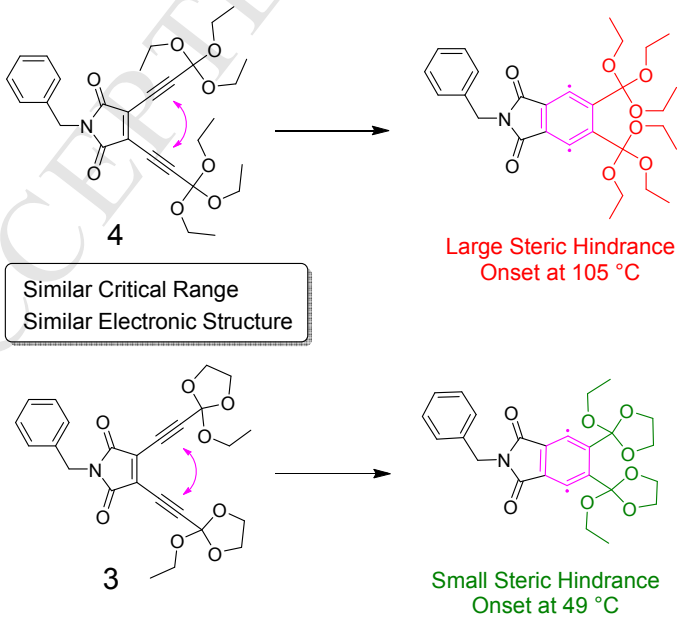


Figure 1. Comparison of enediyne **3** and another acyclic enediyne (**4**) with a similar molecular structure.

The attributed thermal Bergman cyclization of enediyne **3** is characterized with differential scanning calorimetry (DSC) experiment (ESI† Fig. S3). A strong exothermal peak was found starting at 49 ºC, which, to the best of our knowledge, is among the lowest onset temperature for thermal Bergman cyclization of an acyclic enediyne, and is very close to physiological temperature, implying a spontaneous Bergman cyclization would occur in physiological environment. Comparing the molecular structure of enediyne **3** with a previously reported enediyne **4** (Fig. 1),[40] both with an electron-deficient maleimide moiety and two orthoester substituents at the alkynyl termini, it is striking to find out that by simply releasing the steric hindrance raised by the relatively bulky triethoxy groups, the thermal cyclization is drastically activated, which is indispensable for further applications in DNA-cleavage and tumor cell suppression.

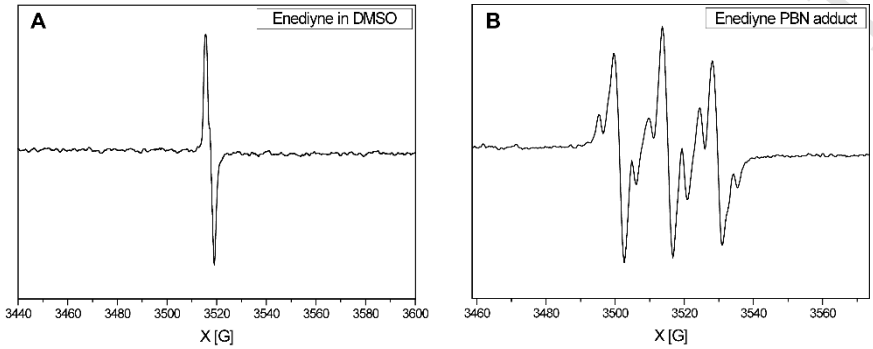
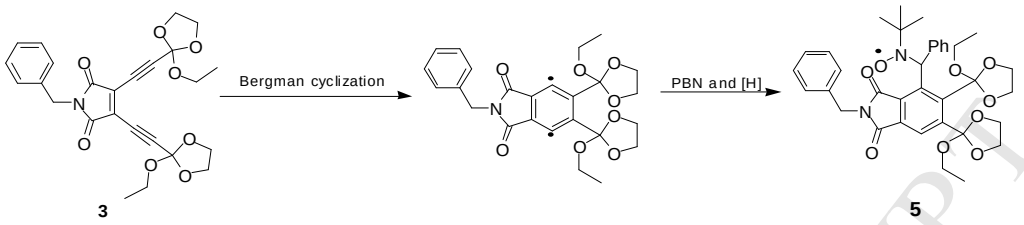


Figure 2. EPR spectra of enediyne **3** (A) and PBN adduct **5** (B) in DMSO at ambient temperature.

Electron paramagnetic resonance (EPR) spectroscopy is typically used to verify the radical nature of Bergman cyclization. Freshly prepared enediyne **3** was dissolved in dimethylsulfoxide (DMSO) at ambient temperature and immediately subjected to EPR analysis. As shown in figure 2a, an intense single peak at 3520 G is clearly observed, indicating the formation of carbon-centered free radicals.[39] Even after the sample was stored for one week, the EPR peak corresponding to free radicals had not decayed significantly (ESI† Fig. S4). The spectrum became slightly complicated, probably due to the generation of polymeric species[46] through the coupling of the free diradicals. Alabugin et al. showed that if there was an alkoxyl group at the ortho-position to the free radical, intra-molecular H-transfer would occur, helping to stabilize the free radical.[47] At the same time, this process renders the cyclization step essentially irreversible and produce other kinds of free radicals.[48] Interestingly, when enediyne **3** was dissolved in chloroform, no free radical signal was observed in EPR spectrum (ESI† Fig. S5), implying that the polarity of the solvent also shows strong influences on the thermal cyclization of this kind of enediyne compounds. This unexpected solvent effect is still under investigation, we currently believe that it is related to the polarization of enediyne system by the solvent molecules.[49] Nevertheless, this phenomena suggests a simple yet efficient drug loading approach, in other words, the enediyne is safely preserved in nonpolar environments (for example, in the nonpolar core of a micellar drug delivery system) and then activated when they are released in cancer cell with highly polar cytosol microenvironments.

The reactive free diradicals generated from thermal cyclization can be converted into stable free radical in the presence of spin traps.[50] To this end, freshly prepared enediyne **3** and excess of N-tert-butyl-α-phenylnitrone (PBN) were dissolved in DMSO and kept at 37 ºC for 1 h (Scheme 2). The diradical intermediates might interact with 1 or 2 equivalent of PBN to produce two different types of nitroxide radicals. ESI-MS analysis showed an intense peak at m/z of 645.2811 (ESI† Figure S6), corresponding to the radical molecular ion ($M^{\cdot+}$) of the single PBN-adduct. Meanwhile, EI-MS analysis showed a peak at m/z of 821.3881 (ESI† Figure S7), corresponding to the double PBN-adduct. The single PBN-adduct of enediyne (**5**) was separated out and subjected to EPR analysis. As shown in figure 2b,

three sets of peaks with an intensity ratio of 1 : 1 : 1 and hyperfine splitting constant (A_N) of 14.05 G, similar to that of the calicheamicin-derived phenyl radical PBN monoadduct (A_N = 14.6),[51] confirming the conversion of carbon-free radicals to nitrogen oxygen radicals. The hyperfine splitting of each set of peaks is probably originated from the neighboring methine group.



Scheme 2. Bergman cyclization of enediyne **3** and related PBN addition (**5**).

It is difficult to monitor the generation of free radicals in aqueous solution with EPR experiment due to the absorption of microwave irradiation by water molecules. To this end, a free-radical-indicator recently developed by Li. et al.[52] was adopted. A mixture of enediyne **3** (1 mg) and the indicator (2 mg) in DMSO (1 ml) was suspended in water (5 ml) and maintained at 25 ºC. The color of the mixture slowly turned into pink in 10 min (ESI† Fig. S8), indicating the formation of free radicals through the reaction that was attributed to Bergman cyclization. This color change originated from the attack of the spirocyclic rhodamine amide (the indicator) by the diradical species, resulting in the restoring of the π-conjugation system.[52] In comparison, in the absence of the indicator, the color of the enediyne **3** in aqueous media stayed unchanged under the same condition.

The high reactivity of enediyne **3** was indirectly evidenced by ESI-MS analysis of an unexpected reaction mixture during the synthesis of enediyne **3**. In this Sonogashira reaction, the temperature was not restrictively controlled below room temperature (roughly 25 ºC), and a complicated mixture of various radical addition and abstraction products was obtained. As indicated in the high resolution mass spectrometry spectrum (ESI† Figure S9), hydrogen donors in the reaction system and the raw material alkynes were attacked by the highly reactive free diradicals generated from spontaneous Bergman cyclization of enediyne **3**, generating at least three different kinds of addition products (ESI† Scheme S4).

The radical-forming processes of enediyne **3** under mild conditions is fascinating and puzzling as most of the other enediyne compounds with similar structural features, like acceptor substitution the ene part, small cycle annealed at the ene part, and bulky terminal groups, were considered to be inactive at low temperature. We acknowledge that our findings go against the current understanding of electronic effects in Bergman cyclization, and we have also investigated other possible mechanisms of generating radical species from a close shell enediyne compound, like C1-C5 cyclization,[53] enediyne dimerization.[54] Since the calculated barrier for C1-C5 cyclization is even higher and the dimerization of enediyne is not supported by mass spectroscopy analysis in this work, we tentatively consider the generation of radical species is most likely from the Bergman cyclization of enediyne compounds. The discrepancy between theoretical calculation and experimental results are common. In an early report by P. Schreiner et al.,[55] the authors found that while the carbonyl-substituted enediynes showed high tendency to polymerize under room temperature, the calculated energy barrier (28.8 kcal/mol) of these compounds for Bergman cyclization were comparable or even higher than those of many stable enediyne compounds. Therefore, at the current stage, we prefer to base more on the experimental results. The high activity of enediyne **3** is probably due to the contribution of the electron-withdrawing effect action by maleimide group and OR substituents which facilitate Bergman cyclization by helping the enediyne system to distort and also by decreasing electron-electron repulsion between the in-plane alkyne π-bonds.[56] Herein, we tentatively

attribute the formation of radicals to the Bergman cyclization but we cannot exclude other radical-forming processes. Deeper investigations into the reaction mechanism of this kind of enediyne compounds are still underway in our group. Nevertheless, the high reactivity of enediyne **3** under low temperature to produce free radicals is essential for its DNA-cleavage ability and tumor cell cytotoxicity.

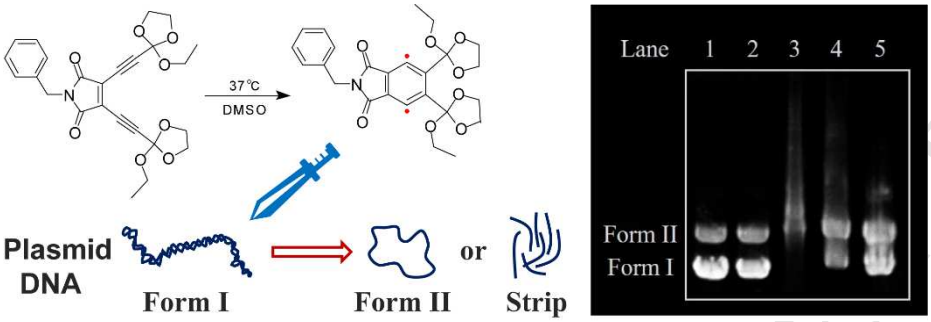


Figure 3. Schematic illustration of the cleavage of DNA by enediyne **3** (left) and agarose gel electrophoresis of supercoiled plasmid DNA (right). Lane 1, free DNA; Lane 2 DNA + DMSO; Lane 3, DNA + enediyne **3** (66.7 mM); Lane 4, DNA + enediyne **3** (33.3 mM); Lane 5, DNA + enediyne **3** (16.7 mM). The solvent system is [Water : DMSO = 2 : 1].

The DNA-cleavage ability of enediyne **3** was estimated by measuring the conversion of supercoiled plasmid DNA from native supercoiled form (Form I) to circular relaxed form (Form II, single-strand cleavage).[57] The DNA samples (ΦX174 supercoiled plasmid RF1 DNA 0.5 μg/μL in TE buffer) with different concentration of enediyne **3** were incubated for 48 h at 37 °C, which were subjected to agarose gel electrophoresis to analyze the conversion of DNA by photographed scanning. As shown in figure 3, the supercoiled DNA is significantly converted to Form II in the presence of 16.7 mM of enediyne **3** (Lane 5). In comparison, the control samples were not affected (Lane 1, free DNA; Lane 2 DNA+DMSO). When the concentration of enediyne increased, the DNA was completely fractured into strips (33.3 mM, Lane 4; 66.7 mM, Lane 3), demonstrating the strong DNA-cleavage performance of enediyne in physiological environments.

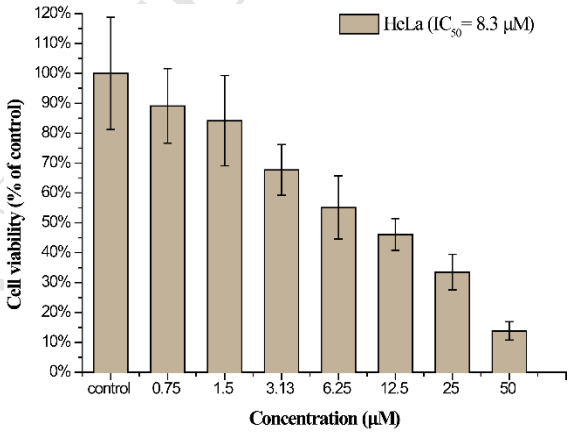


Figure 4. MTT assay of the cell viability of Hela cell in the presence of enediyne **3**.

The cytotoxicity of enediyne **3** was investigated by using the MTT method[45] in a tumor model line cell. Firstly, freshly prepared enediyne **3** was dissolved in DMSO, diluted with cell culture medium to a series of concentrations and then respectively cultured with Hela cells for 48 h. A control sample was involved with the addition of the same amount of DMSO to the cell culture media. As shown in figure 4, the cell viabilities of Hela cell are lower than 90% when the concentrations of enediyne **3** are beyond 0.75 μM. In addition, the cell viability clearly shows concentration dependence of enediyne **3**. In contrast, the previously reported maleimide based enediynes with high onset temperature (150 ºC) exhibited no

cytotoxicity, suggesting that the cytotoxicity of enediyne **3** is derived from the free radicals generated probably through Bergman cyclization. Specifically, the IC_{50} value of enediyne **3** fit from the cell variability curve is 8.3 μM, comparable to many clinical anticancer drugs, like cisplatin,[58] Irinotecan,[59] and doxorubicin.[60] It is speculated that enediyne **3** with a low molecular weight shows great potential of being a bullet in a drug carrier to increase cytotoxicity like nature enediyne antibiotics with multi-functional groups.

Flow cytometry analysis is a common method of detecting drug cytotoxic pathways. Hela cells were used as model cancer cells and co-cultured with enediyne **3** (30 μM) for 48 h, followed by staining with annexin V-FITC and propidium iodide (PI) to evaluate the percentage of live (annexin V-/PI-), early apoptotic (annexin V+/PI-), late apoptotic (annexin V+/PI+) and necrotic (annexin V-/PI+) cells. The cellular density plots that treated with enediyne **3** are shown in figure 5. Compared with the untreated control group, enediyne **3** exhibits the ability to induce apoptosis. Similar to commonly used anti-tumor agents such as cisplatin and its biotin-tagged complexes[61]. This result unambiguously confirms that enediyne **3** acts on DNA-damage in cells to cause cancer cell death through apoptosis pathway.

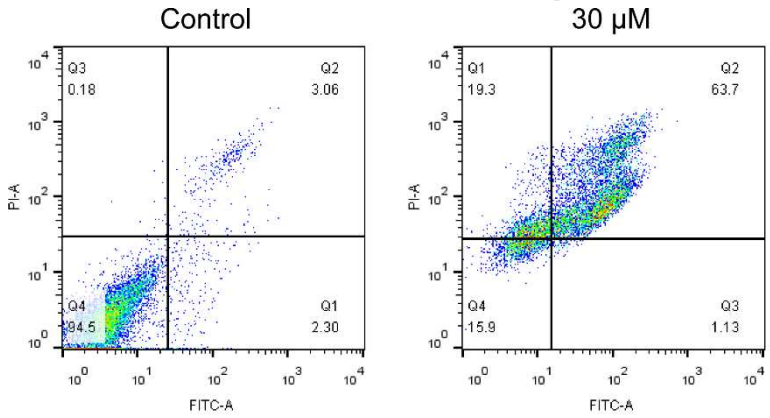


Figure 5. Flow cytometry analysis of enediyne **3**.

Conclusions

A novel acyclic enediyne functions at physiological temperature was designed and synthesized with subtle balancing of the steric and electronic effects in molecular design. Spontaneous Bergman cyclization which was attributed of the enediyne has arisen to produce diradical in a polar solvent DMSO at ambient temperature. The free radicals are highly reactive towards spin trapping agents and hydrogen donors, showing effective DNA-cleavage abilities and high cytotoxicity towards tumor cells. Flow cytometry analysis showed that the inhibition of the cancer cell variability by the enediyne is mainly through apoptosis pathway. In conclusion, thanks to its spontaneous thermal cyclization at physiological temperature, this highly reactive enediyne is promising to act as a radiomimetic agent with strong activity on DNA-cleavage and high cytotoxicity to cancer cells.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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