DNA Cleavage Potency, Cytotoxicity, and Mechanism of Action of a Novel Class of Enediyne Prodrugs

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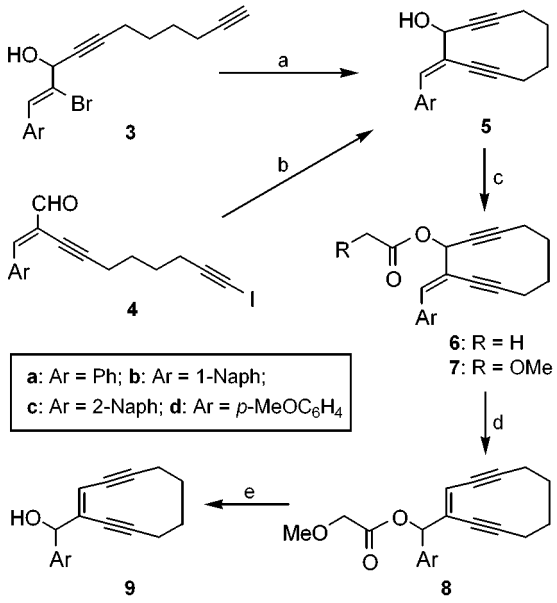
Received November 19, 2001

Abstract: We have discovered a novel class of (*E*)-3-acyloxy-4-(arylmethylidene)cyclodeca-1,5-diynes which exhibit promising enediyne-like DNA cleavage and cytotoxic activities. LC-MS analysis of the incubation mixture (pH 8.5, 37 °C) confirmed formation of 10-membered ring enediyne presumably via an allylic cation and suggested that the 1,4-benzenoid diradical might be one of the active species for DNA damage and cytotoxicity.

Introduction. The enediyne antitumor antibiotics represent a family of structurally unusual natural products that consist of a (*Z*)-hex-1,5-diyn-3-ene moiety embedded in either a 9- or a 10-membered ring.[1] An apoprotein is normally associated with the labile nine-membered ring enediyne through noncovalent interaction. It prevents the enediyne moiety from decomposition and facilitates its delivery to the target DNA in the nucleus for cleavage chemistry to occur.[2] On the other hand, the naturally occurring 10-membered ring enediynes are stabilized by various "locking devices" strategically engineered in their molecular architectures. Bioactivation is generally required for the action of these enediyne antitumor antibiotics,[1a,e,f] and they are referred to as prodrugs.[3]

It has been well demonstrated that acyclic and cyclic derivatives of (*Z*)-hex-1,5-diyn-3-ene undergo cycloaromatization to generate 1,4-benzenoid diradicals.[1a−d,4] The latter species are capable of hydrogen atom abstraction from donors such as deoxyribose[5] to form benzene derivatives. The temperature for inducing cycloaromatization depends on the enediyne structure. As the consequence of increased strain energy in a 10-membered ring, the parent cyclodeca-1,5-diyn-3-ene cyclizes at 37 °C with a half-life of 18 h and causes DNA cleavage and cell death.[6] However, the relatively short lifetime of monocyclic 10-membered ring enediynes renders their handling, storage, and application difficult. A strategy to overcome the instability issue targets at in situ formation of bioactive enediynes from suitable precursors. As a matter of fact, the Maier[7] and Myers[8] groups have cleverly utilized the enzymatic redox systems to generate the 10-membered ring enediynes from the cyclic 1,5-diynes. A variety of chemical methods have been successfully developed for laboratory syntheses, but the reaction conditions are not compatible with biological system.[9]

Scheme 1. Synthesis of Enediyne Derivatives **6**−**10**[a]



[a] Reagents and conditions: (a) 10 mol % $Pd(PPh_3)_4$, 20 mol % CuI, Et_2NH-CH_3CN (1:5), 80−90 °C, 1.5 h (ref 12a); (b) 3 equiv of $CrCl_2$, 1 equiv of $NiCl_2$, THF, 20 °C, 8 h (ref 12b); (c) for **5** → **6**: 5 equiv of Ac_2O, 10 equiv of DMAP, CH_2Cl_2, 20 °C, 1.5−2.5 h (**6a**, 81%; **6b**, 67%; **6c**, 68%; **6d**, 44%); for **5** → **7**: 1.6 equiv of $MeOCH_2CO_2H$, 2 equiv of DCC, 10 equiv of DMAP, CH_2Cl_2, 20 °C, 1 h (**7a**, 79%; **7b**, 65%; **7c**, 65%; **7d**, decomposed to **9d** during chromatographic purification over silica gel, 43%); (d) for **7** → **8**: 10 mol % $Eu(fod)_3$, $CHCl_3$, 20 °C, 24−48 h (ref 12b); (e) for **8a** → **9a**: 2 equiv of K_2CO_3, aq MeOH, 0 °C, 30 min (84%).

In a recent study, we found that the cyclic 1,5-diyne **1** cleaved DNA to an extent comparable with a structurally related 10-membered ring enediyne.[10] We assumed that an allylic rearrangement occurred during incubation to convert **1**, via an allylic cation **2** (R = anthraquinone-2-carbonyloxy) followed by attack of H_2O at the γ position, into a cyclic enediyne. The latter underwent the diradical-forming reaction cascade, leading to DNA damage. We now report on DNA cleavage potency and cytotoxicity of (*E*)-3-acyloxy-4-(arylmethylidene)cyclodeca-1,5-diynes **6** and **7**, the evidence of allylic rearrangement through allylic cation **2** (R = H), and the significant substituent effects of the aryl group (Ar) on biological activities.[11]

Synthetic Chemistry. As shown in Scheme 1, (*E*)-3-hydroxy-4-(arylmethylidene)cyclodeca-1,5-diynes **5** can be synthesized either from the bromides **3** via an intramolecular Sonogashira cross-coupling catalyzed by Pd(0)−Cu(I)[12a] or from the aldehydes **4** via an intramolecular Nozaki−Hiyama−Kishi reaction using $CrCl_2$−$NiCl_2$.[12b] Acetylation of **5** using Ac_2O and DMAP in CH_2Cl_2 at 20 °C gave the acetates **6a**−**d** in 44−81% yields. Because the esters are quite sensitive to weak acidic conditions, use of excess DMAP was necessary for minimizing product decomposition. Low yield of the *p*-methoxy substituted **6d** is due to its ready conversion into the allylic cation **2** (R = H) over silica gel. We also experienced difficulty in isolating the methoxyacetate

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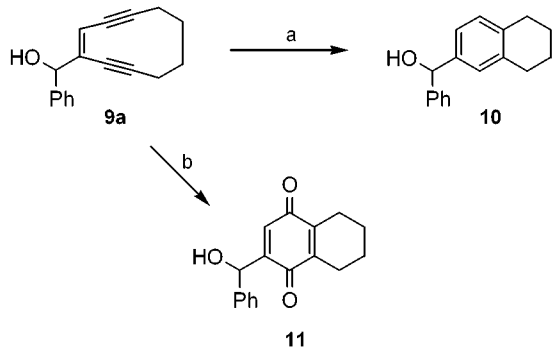
10.1021/jm015588e CCC: $22.00
Published on Web 01/17/2002

Scheme 2. Reactions of Enediyne **9a**[a]



[a] Reagents and conditions: (a) degassed toluene:1,4-cyclohexadiene (5:1), 90 °C, 3 h (85%); (b) 2,2,6,6-tetramethylpiperidinooxy [TEMPO], PhH, reflux, 6 h (42%).

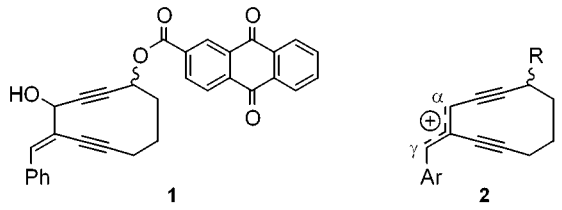


Figure 1. Structures of the DNA-cleaving cyclic 1,5-diyne **1** and the proposed allylic cation intermediate **2**.

7d which completely decomposed over silica gel during column chromatography to give the rearranged **9d** in 43% yield. The methoxyacetates **7a**−**c** were prepared from **5** and methoxyacetic acid using DCC-DMAP in 65−79% yields. Conversion of **7a**−**c** into the enediynes **8a**−**c** was carried out in the presence of 10 mol % $Eu(fod)_3$ in $CHCl_3$ at 20 °C in good yields.[12b,13] Alkaline hydrolysis of **8a** furnished the enediyne alcohol **9a** in 84% yield.

Cycloaromatization of **9a** was carried out in a degassed mixture of toluene and 1,4-cyclohexadiene (5:1) at 90 °C for 3 h to give the product **10** in 85% yield (Scheme 2). Kinetic studies on the cycloaromatization of **8a** were also performed at 60, 70, 80, and 90 °C, respectively, in toluene-d_8 and monitored by ^{1}H NMR spectroscopic analyses on a 400 MHz instrument. These kinetic data provided an estimated half-life of ca. 62.5 h and a free energy of activation of ca. 26.0 kcal/mol for **8a** at 37 °C. In contrast, heating a toluene-d_8 solution of **6a** at 90 °C for 8 h did not cause any decomposition as monitored by ^{1}H NMR spectroscopy.

Very recently, formation of a quinone from an enediyne in aqueous buffers and air was reported as a possible alternative therapeutic mechanism.[14] We synthesized the quinone **11** from **9a** using TEMPO in refluxing benzene in 42% isolated yield (Scheme 2).[15]

Results and Discussion. DNA cleavage of **8a** and related compounds was assayed using ΦX174 RFI supercoiled DNA (form I). We found a time-dependent DNA scission, and the potency increased with incubation time over a period of 48 h. Therefore, in the following experiments the samples were allowed to react with DNA for 72 h at 37 °C. The reaction mixtures were analyzed by electrophoresis over 1% agarose gel. The separated DNA fragments were then visualized by ethidium bromide stain and quantified by scanning densitometry. Figure 2 illustrates the concentration-dependent breakage of the DNA substrate by **7a** and

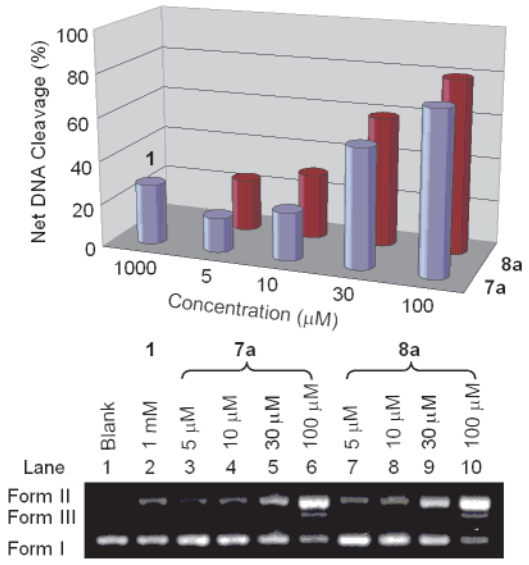
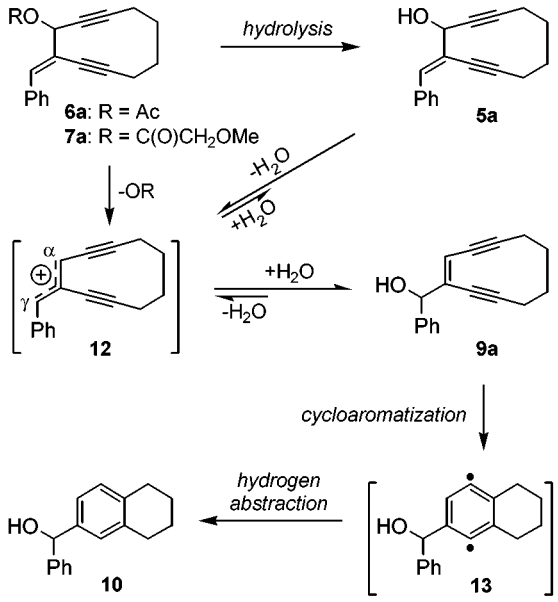


Figure 2. Comparison of DNA cleavage potency of the alcohol **1** with the ester **7a** and the enediyne **8a**. ΦX174 RFI DNA (54.3 μM/bp) was incubated with the samples at the indicated concentrations in TEA buffer solution (pH 8.5) containing 20% DMSO at 37 °C for 72 h and then analyzed by gel electrophoresis over 1% agarose gel and visualized by ethidium bromide stain. The gel picture is shown in the bottom, and the scanning densitometry results of the gel picture are plotted on the top. The percentage of net DNA cleavage was calculated by the following equation: $\{[(\text{form II})_s + 2 \times (\text{form III})_s]/[(\text{form I})_s + (\text{form II})_s + 2 \times (\text{form III})_s] \times 100\} - \{(\text{form II})_c/[(\text{form I})_c + (\text{form II})_c] \times 100\}$. The subscripts "s" and "c" refer to the samples and controls, respectively.

Scheme 3. Proposed Mechanism of Action of **6a** and **7a**



8a in comparison with **1**. We found that the ester **7a** was ca. 100-fold much more potent than the alcohol **1**. Moreover, over the concentration range of 5−100 μM, **7a** exhibited *almost* the same level of activity as the enediyne **8a**. These results suggested that **7a** might dissociate in the aqueous media into the allylic cation **12**. The latter was trapped by H_2O preferentially at the γ position[9,10] to furnish **9a** (Scheme 3). According to the known enediyne chemistry,[1,4] at the incubation temperature, **9a** should cyclize to the diradical **13** which

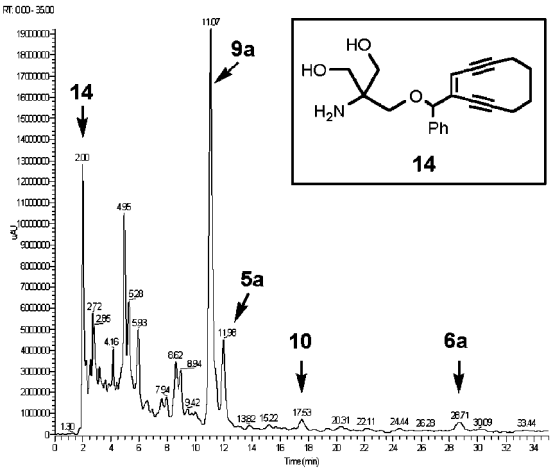


Figure 3. HPLC chromatogram of the organic extract of the reaction mixture of **6a** after incubating at 37 °C for 48 h under N_2 atmosphere in TEA buffer solution (pH 8.5) containing 10% DMSO. The molecular structures of the marked peaks are confirmed by LC-MS in comparison with those authentic samples synthesized in Schemes 1 and 2. The structure **14** given in the inset is deduced from its LC-MS data. See the Supporting Information for details of the LC-MS analysis.

abstracted hydrogen atoms from the sugar moiety of DNA[5] to afford the aromatic product **10**.

To confirm formation of **9a** from the precursor, we analyzed the reaction products of **6a** in TEA buffer solution (pH 8.5) containing 10% DMSO by LC-MS. Thus, a solution of **6a** was incubated in the absence of the DNA substrate at 37 °C for 48 h under N_2 atmosphere, and the organic extract of the reaction mixture was subjected to LC-MS analysis. Figure 3 shows the HPLC chromatogram where the peaks corresponding to **5a**, **6a**, **9a**, and **10** are identified by the retention times and mass spectra in comparison with the authentic samples synthesized in Schemes 1 and 2 (see the Supporting Information for details). We found that hydrolysis of **6a** to the alcohol **5a** competed against formation of the enediyne **9a**. The relationship among **6a**, **5a**, and **9a** is proposed in Scheme 3, indicating that both **5a** and **6a** can be converted into **9a** through the allylic cation **12**.[10] However, the route from **5a** to **12** should be much slower because the acetate moiety in **6a** is a better leaving group. Indeed, **6a** is a much more powerful DNA cleaver than the alcohol **5a** which causes only 22% net DNA cut at 5.0 mM. Once the enediyne **9a** is formed, it enters the radical-forming reaction cascade leading to the benzene derivative **10**. The peak intensity of **10** in Figure 3 is rather weak probably due to the absence of an efficient hydrogen atom donor in the buffer solution for trapping **13**. We also confirmed formation of a very polar component **14**, having a retention time of 2.0 min, by the LC-MS analysis. Compound **14** is the trapping product of the allylic cation **12** by tris(hydroxymethyl)aminomethane used in the buffer. We were not able to identify the quinone **11**, possessing a retention time of 10.2 min, from the reaction mixture of **6a**. There are many unidentified polar components having retention times less than 10 min. Mass spectra indicate that they seem not related to the structures derived from **6a**. Nevertheless, the LC-MS data given in Figure 3 and the Supporting Information clearly demonstrate transformation of (*E*)-3-acyloxy-4-(arylmethylidene)-cyclodeca-1,5-diynes such as **6a** into the bioactive enediynes under the DNA assay conditions.

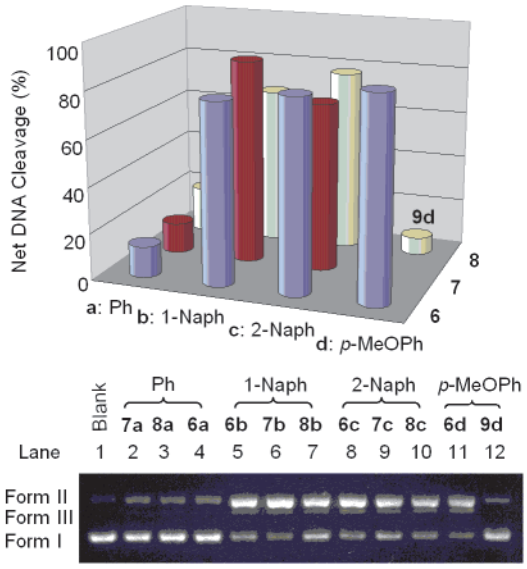


Figure 4. DNA cleavage results of compounds **6a**−**d**, **7a**−**c**, **8a**−**c**, and **9d** at 20 μM, respectively. The incubation conditions are identical to those given in Figure 2. The gel picture is shown in the bottom, and the scanning densitometry results of the gel picture are plotted on the top. Definition of the percentage net DNA cleavage is the same as that given in Figure 2.

Table 1. IC_{50} Data (μM) against P388 Cancer Cell Line[a]

Ar	alcohols	acetates	esters	enediynes
Ph	**5a**: 25	**6a**: 48	**7a**: 23	**8a**: 7.7
1-naph	**5b**: 41	**6b**: 2.4	**7b**: 12	**8b**: 3.8
2-naph	**5c**: 3.6	**6c**: 2.8	**7c**: 28	**8c**: 9.5
p-$MeOC_6H_4$	**5d**: 5.8	**6d**: 15		**9d**: 13

[a] MTT assay was used. See ref 16.

Figure 4 shows the DNA cleavage results of **6**−**8** at 20 μM. In general, the phenyl derivatives **6a**, **7a**, and **8a** are much weaker cleavers compared to the naphthyl and *p*-methoxyphenyl analogues **6b**−**d**, **7b**,**c**, and **8b**,**c**. The esters **6b**−**d** and **7b**,**c** are 5.6−6.8-fold more potent than **6a** and **7a**. Also, it was revealed that the two naphthyl-derived enediynes **8b** and **8c** are 3.5- and 4.0-fold more potent than the phenyl analogue **8a**. To examine the possibility of intercalation with DNA, we measured the DNA binding constants K' of **5**−**8** as given in the Supporting Information. Because the K' values for the phenyl and naphthyl analogues are on the same order of magnitude, a clear conclusion on the enhanced DNA cleavage potency for **6b**,**c**, **7b**,**c**, and **8b**,**c** cannot be reached at this stage. Nevertheless, the remarkably increased activity of the naphthyl analogues is very promising for further development. In contrast, the enediyne **9d** gave an unusual result compared to **6d**. We suspected that **9d** could dimerize via an O-methylated quinone methide intermediate. This side reaction might be suppressed when it was generated in situ from **6d**. We observed such dimerization reaction of a similar compound when the solvent of the sample solution was evaporated to dryness.

Cytotoxicity was assayed using the P388 mouse T cell leukemia cell line, and the results are listed in Table 1. In general, the phenyl derivatives **5a**, **6a**, and **7a** exhibit

less potent in vitro cytotoxicity in parallel to their low DNA cleaving activity. The two naphthyl substituted acetates **6b** and **6c** are the most promising compounds, with IC_{50} values in the low micromolar range. We found that the quinone **11** neither causes DNA cleavage at 100 μM nor exhibits cytotoxicity against the P388 cell line. The latter result is different from the reported observation on a similar quinone compound.[14]

Conclusion. We have discovered a novel series of (*E*)-3-acyloxy-4-(arylmethylidene)cyclodeca-1,5-diynes **6** and **7** as the promising DNA cleaving and cytotoxic agents. Experimental evidences have confirmed formation of 10-membered ring enediynes from cyclic 1,5-diynes presumably through an allylic cation intermediate **2** (R = H). It suggests that the enediyne pathway should be one of the possible mechanisms of action for this novel class of cyclic 1,5-diyne compounds. Manipulation on the aryl substituent could finely tune the biological potency although the exact factor for the enhanced DNA damage and cytotoxicity of the naphthyl compounds is unclear. We did not observe formation of the quinone **11** in the reaction mixture of **6a** by LC-MS analysis. Moreover, **11** failed to exhibit both DNA cleavage and cytotoxicity. It is unlikely that our results described above involve action of the quinone intermediates.

Acknowledgment. Financial supports to A. Wu through a postdoctoral fellowship from the Department of Chemistry, HKUST, to W. Hamaguchi through an RGC Direct Allocation Grant (DAG97/98.SC12), and to M. Y. H. Lee through a HKUST Postdoctoral Fellowship Matching Fund (PDF99/00) are acknowledged.

Supporting Information Available: DNA binding constants *K′* of selected compounds and spectral, analytical, and LC-MS data. This information is available free of charge via the Internet at http://pubs.acs.org.

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JM015588E