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Thiol-independent DNA Cleavage by a Leinamycin Degradation Product

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Abstract—To understand the mechanism of action of a novel antitumor antibiotic leinamycin (**1**) which induces single-strand scission of DNA in the presence of thiol, the reaction of **1** with thiol in aqueous conditions was investigated. Two major degradation products were obtained from **1** in the presence of thiol. **2** was an inactive product, while **3** caused DNA cleavage in the absence of thiol. The DNA-cleaving activity of their synthetic derivatives indicates that the DNA alkylation and subsequent strand scission by leinamycin require the conversion of leinamycin to an electrophilic episulfonium species. © 1997 Elsevier Science Ltd.

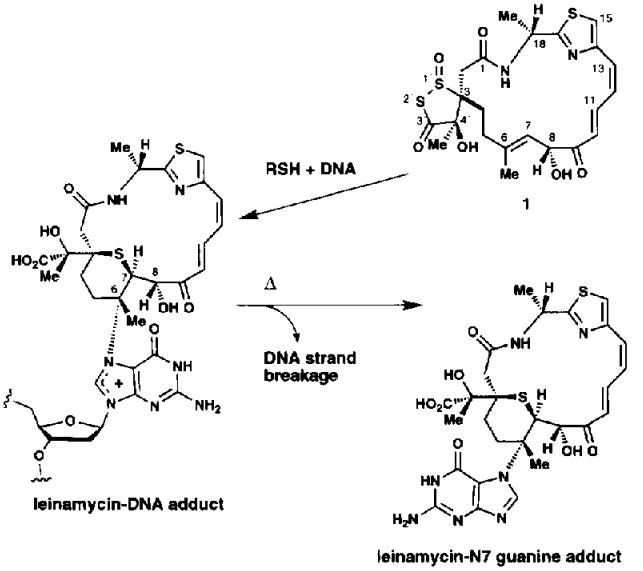
Introduction

Leinamycin (**1**) was isolated from the culture broth of *Streptomyces* sp in 1989.[1,3] Its structure was elucidated by spectroscopic analysis,[1] X-ray crystallography,[4] and chemical synthesis.[5] This antibiotic contains an unusual 1,3-dioxo-1,2-dithiolane moiety, which is connected to the 18-membered lactam through a spiro linkage, and appeared to be a new class of natural products. Leinamycin exhibited significant antitumor activity in some murine tumor models.[2] The mode of action is thought to be the thiol-mediated single-strand scission of DNA.[3] Further, our recent efforts have revealed that the thiol-dependent N7-guanine DNA alkylation is responsible for the DNA-cleaving activity of leinamycin (Scheme 1).[6] Some natural products have been reported to induce the single-strand scission of DNA by the covalent adduct formation with nucleobase followed by the generation of the unstable apurinic sites on DNA.[7–10] However, although these classes of DNA-alkylating agents contain the electrophilic functional groups exemplified by epoxide or active cyclopropane, leinamycin does not appear to fall into any of these classes of agent. On the other hand, Gates et al. proposed that the mechanism of DNA cleavage by dithiolane oxide subunit apparent in leinamycin involves thiol-mediated conversion of molecular oxygen to DNA-cleaving oxygen radicals from the results of their experimental system employing the simple dithiolane oxide compound.[11] Herein we report the isolation and DNA-damaging activity of the products generated from leinamycin treated with thiol along with their derivatives. Our results demonstrate that an electrophilic subunit, presumably an episulfonium subunit, could be generated from leinamycin by thiol treatment, which readily reacts with the nucleophile such as hydroxyl anion or nucleobase of DNA.

Results

HPLC analysis of the degradation process

To investigate how leinamycin (**1**) would be activated by thiol, we examined the stability of **1** under aqueous conditions in the absence or presence of thiol by reverse-phase HPLC analysis. Figure 1A shows the time course of the loss of drug residue in the absence of thiol. **1** was slightly stable in 10% MeOH/10 mM phosphate buffer (pH 7) at 37 °C ($t_{1/2}$ = 8 h) and gradually disappeared to give some unidentified degradation products. The stability of **1** was pH dependent. At lower pH, **1** was stable ($t_{1/2}$ > 20 h at pH 6), while the



Scheme 1. DNA alkylation and subsequent strand cleavage by leinamycin.

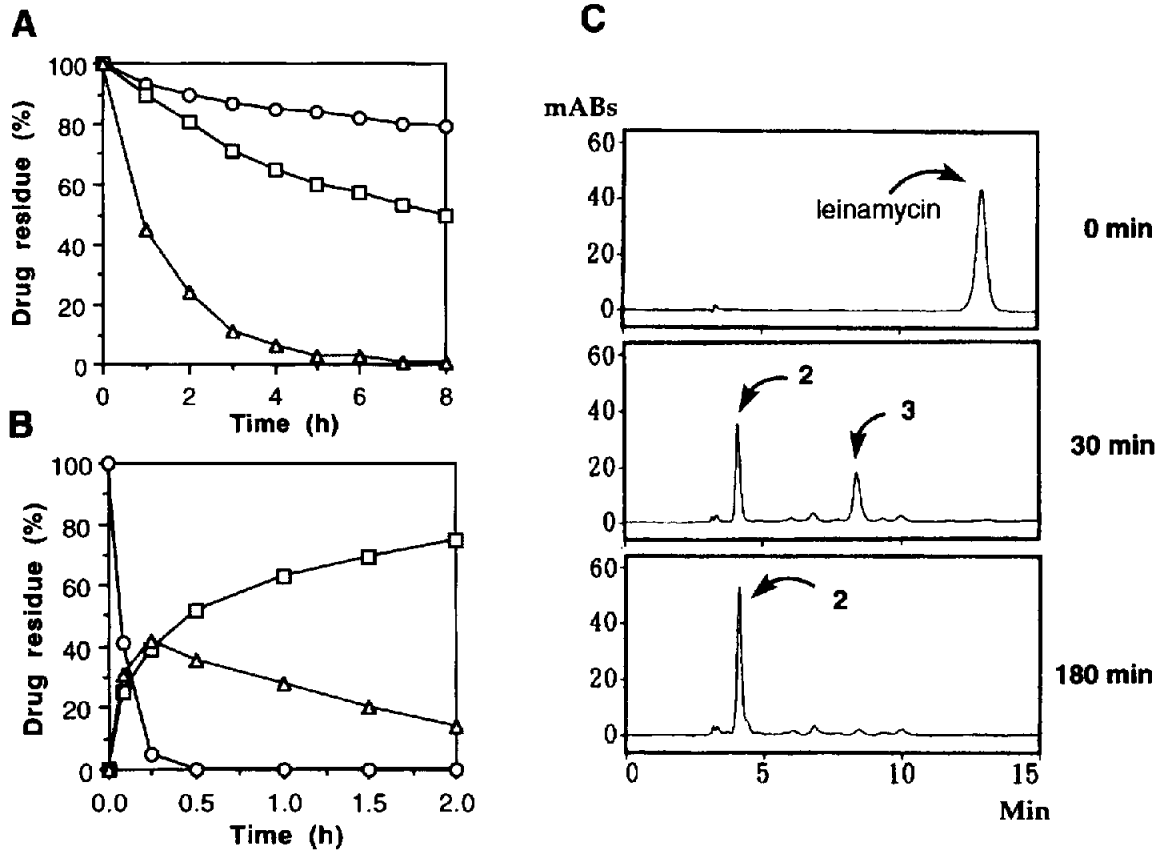


Figure 1. HPLC analysis of the degradation of leinamycin in the absence or presence of thiol. Assay conditions are described in Experimental. (A) Effect of pH on the stability of leinamycin. Leinamycin was incubated at pH 6 (circle), pH 7 (square) or pH 8 (triangle). (B) Disappearance of leinamycin (circle) and generation of **2** (square) and **3** (triangle) in the presence of 2-ME. (C) Chromatograms of the degradation process of leinamycin in the presence of 2-ME.

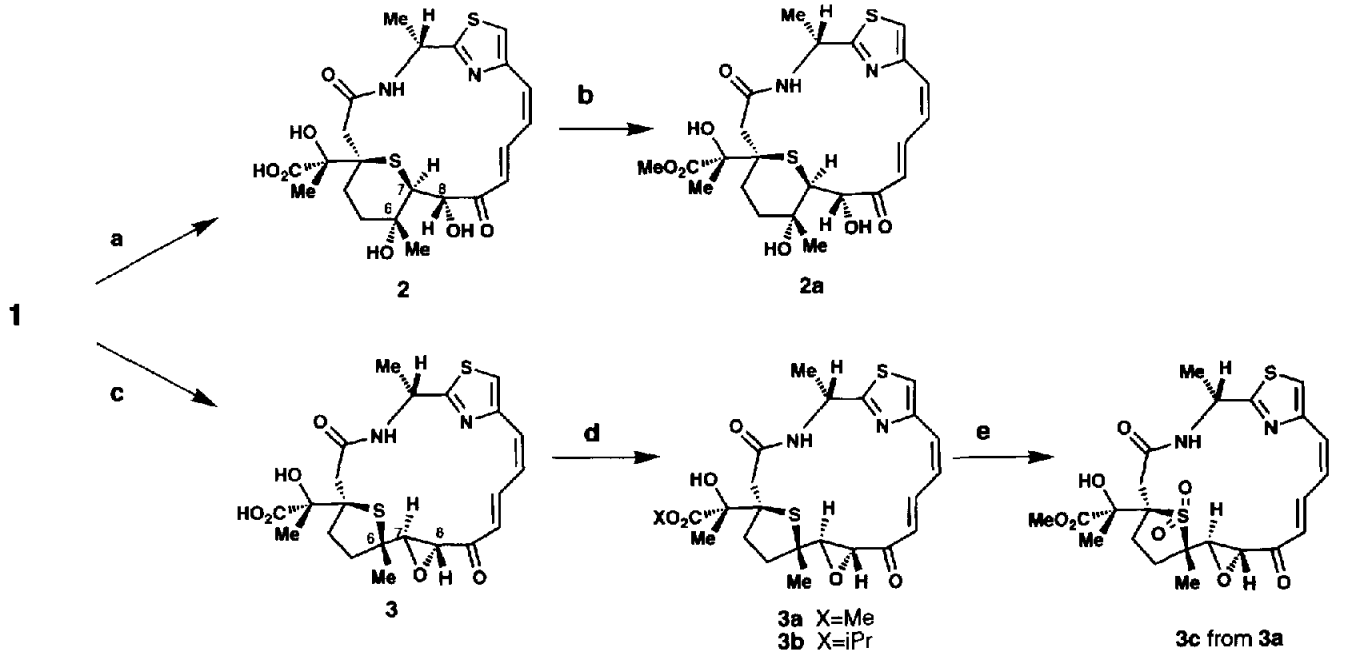
higher pH resulted in the rapid degradation ($t_{1/2}$ < 1 h at pH 8). On the other hand, with the addition of 1.5 equiv of 2-mercaptoethanol (2-ME) to the buffered solution of pH 7, the rate of disappearance of **1** was accelerated with the generation of the degradation products **2** and **3** as shown in Figure 1B and C. **3** was initially observed and gradually disappeared with the increase in the amount of **2**, while **2** was so stable in this condition that the accumulation of **2** was observed without further degradation. Since the degradation process appeared to be finished after sufficient accumulation of **2**, **2** is thought to be a main final product. Some other products were detected as minors in this condition (Fig. 1C). Two of them have been identified with the methanol- and 2-hydroxyethyldisulfide-adduct[6] respectively from the retention time and UV spectrum. Other thiols including ethanethiol, dithiothreitol, cysteine and glutathione afforded approximately equal amounts of **2**, suggesting the thiol functional group alone in these reagents is sufficient to generate **2**.

Isolation and analogue synthesis

We conducted the large preparation and isolation of these degradation products. **2** was readily obtained from **1** treated with 2-ME. The structure of **2**, including relative stereochemistry was elucidated by 2-D NMR studies as previously reported.[6] The treatment of **2** with K_2CO_3 and methyl iodide resulted in the conversion to the methyl ester derivatives **2a**. Isolation of **3** from the aqueous conditions resulted in the low yields (<10%) because of its inability to generate **2** readily. However, different reaction conditions and careful isolation allowed us to terminate the conversion to **2** and to obtain product **3** sufficiently. Treatment of **1** with 2-ME and excess molar of triethylamine in alcohol solvents resulted in the generation of more amounts of **3** without conversion to **2** or the alcohol adduct. Purification was done by reverse phase HPLC at neutral pH, and a collected fraction containing **3** was extracted with ethyl acetate at acidic conditions. Full characterization of isolated **3** by 2-D NMR revealed the structure as previously described.[6] The treatment of **3** with K_2CO_3 and alkylhalide resulted in the formation of the ester derivatives **3a** and **3b**. **3a** was further oxidized with m-CPBA to afford sulfone derivative **3c** (Scheme 2).

DNA-alkylation property

Our previous efforts revealed that **1** alkylates N7 guanine of DNA in the presence of DNA. The DNA alkylation activity was easily observed by HPLC. **1** disappeared immediately when 2-ME was added to the solution in the absence or presence of calf thymus



Scheme 2. (a) 2-ME, 10% MeOH 10 mM phosphate buffer (pH 7), 70%; (b) MeI, K_2CO_3, DMF, 68%; (c) 2-ME, NEt_3, 2-propenol, 68%; (d) (1) MeI, K_2CO_3, DMF, 67%; (2) iPrI, K_2CO_3, DMF, 64%; (e) m-CPBA, CH_2Cl_2, 60%.

DNA. However, in the presence of DNA, **1** gave a smaller amount of degradation product **2** than when in the absence of DNA without any other degradation products (Fig. 2A). Further incubation at 37 °C induced the generation of the leinamycin–N7 guanine adduct. From our previous report[6] along with the investigation of the duocarmycin analogues,[12] the decrease in the generation of inactive product **2** in the presence of DNA indicates the covalent binding of **1** to DNA.

We examined the stability of the degradation products in the absence or presence of DNA by 3-D HPLC analysis for investigating the DNA-alkylating activity of these compounds. **2** was so stable that no degradation product was observed in the phosphate buffer (pH 7) in the absence or presence of DNA at 37 °C after incubation for 8 h. Another product **3** readily generated **2** quantitatively in the absence of DNA (Fig. 2B). Acidic conditions (pH 5) resulted in the acceleration of the

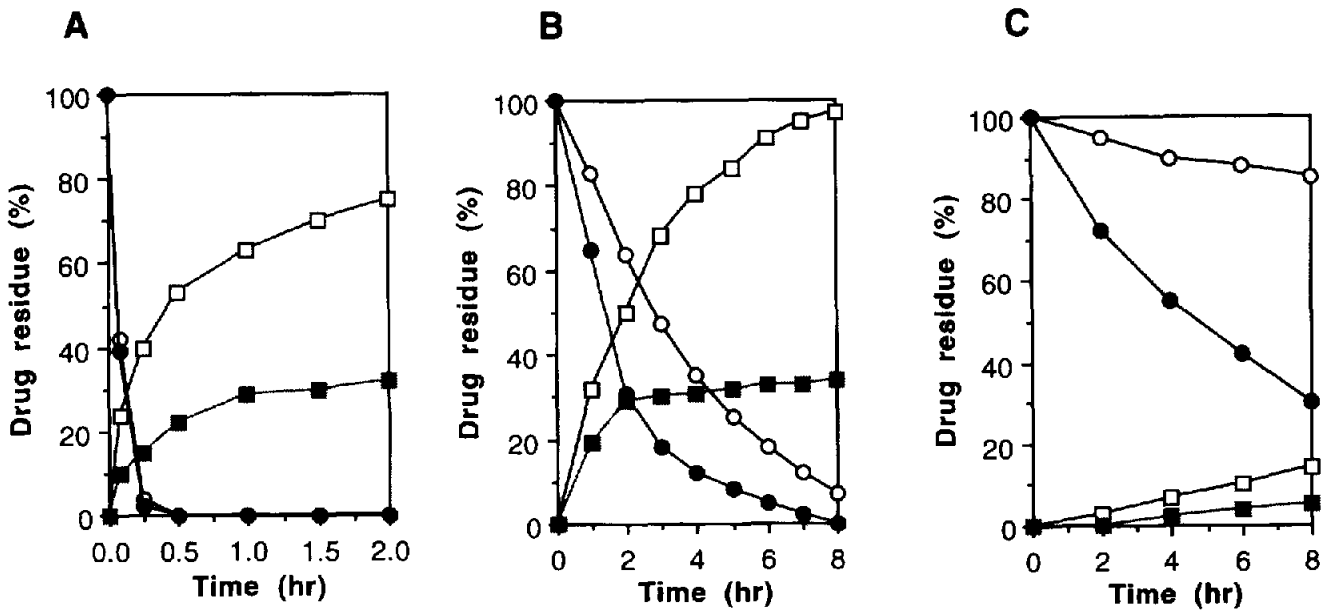


Figure 2. HPLC analysis of the DNA-binding by **1**, **3**, and **3a**. Assay conditions are described in Experimental. (A) DNA binding by **1** in the presence of 2-ME. Disappearance of **1** (circle) and generation of **2** (square) in the absence (open symbol) or presence (closed symbol) of DNA. (B) DNA binding by **3** in the absence of 2-ME. Disappearance of **3** (circle) and generation of **2** (square) in the absence (open symbol) or presence (closed symbol) of DNA. (C) DNA binding by **3a** in the absence of 2-ME. Disappearance of **3a** (circle) and generation of **2a** (square) in the absence (open symbol) or presence (closed symbol) of DNA.

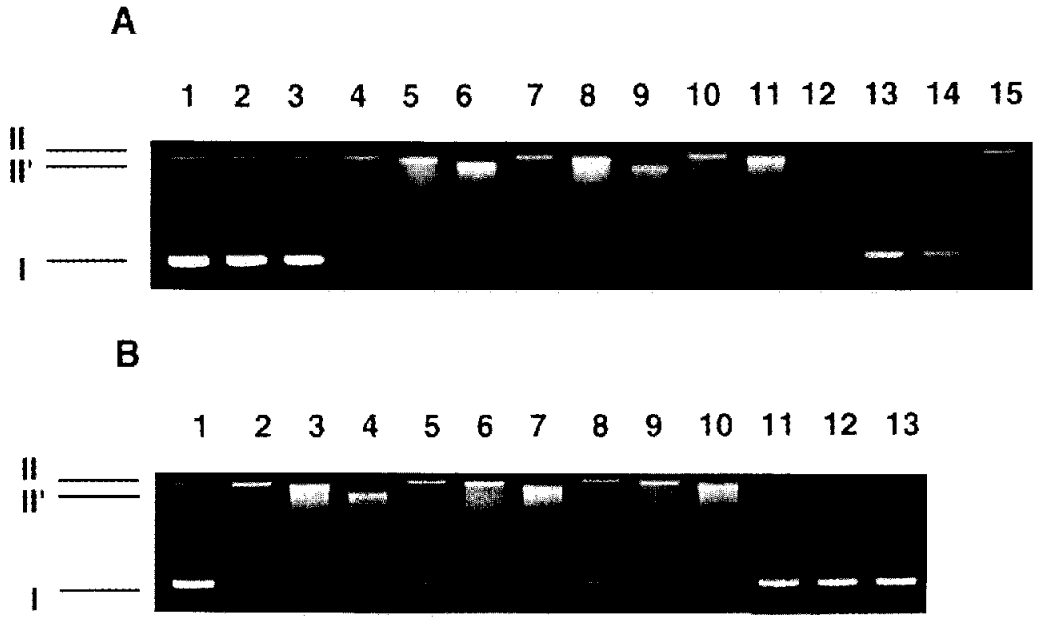


Figure 3. Agarose gel electrophoresis of pBR322 DNA treated with **1** and its related compounds. (A) Lane 1, DNA alone; lanes 2, 4–6 and 10–12, in the presence of 0.5 mM DTT; lanes 3, 7–9 and 13–15, in the absence of DTT; lanes 2 and 3, DNA plus **2** at 50 μM; lanes 4–6 and 7–9, DNA plus **3** at 3.1, 12.5, 50 μM, respectively; lanes 10–12 and 13–15, DNA plus **1** at 3.1, 12.5, 50 μM, respectively. (B) Lane 1, DNA alone; lanes 2–4, DNA plus **3** at 3.1, 12.5, 50 μM respectively; lanes 5–7, DNA plus **3a** at 3.1, 12.5, 50 μM respectively; lanes 8–10, DNA plus **3b** at 3.1, 12.5, 50 μM respectively; lanes 11–13, DNA plus **3c** at 3.1, 12.5, 50 μM respectively.

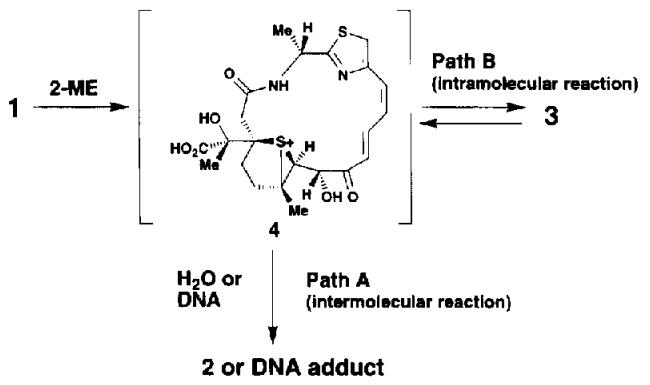
generation of **2** from **3** (data not shown). This observation indicates that the conversion of **3** to **2** is accompanied with the acid-catalyzed epoxy cleavage. The methyl ester **3a** gave a degradation product **2a**. **3b** also gave a degradation product with the same UV spectrum as that of **2** or **2a**. The degradation rates of these ester derivatives were slower than that of **3** (Figs 2B and C). **3** afforded **2** quantitatively in the absence of DNA, while in the presence of calf thymus DNA the rate of disappearance of **3** was accelerated without the quantitative production of **2** (Fig. 2B). No other degradation products were observed and further incubation induced the generation of the leinamycin–N7 guanine adduct. Ester derivatives **3a** or **3b** also afforded lower amounts of the degradation product respectively in the presence of DNA than in the absence of DNA (Fig. 2C for **2a**). On the other hand, sulfone derivative **3c** was stable and did not yield any degradation products in the absence or presence of DNA after incubation for 8 h. These results demonstrate that **3** and its ester derivatives possess DNA-alkylating activity. However, **2** and the stable sulfone **3c** did not show the activity. These results indicate that the 3,6-sulfide linkage apparent in **3** is necessary for generating the electrophilic species for the DNA alkylation by **3**.

DNA cleavage property

We have examined the DNA cleavage activity of isolated degradation products and their derivatives (Fig. 3). Incubation of supercoiled circular pBR322 (form I) DNA with **1** and dithiothreitol (DTT) induced the transformation to nicked circular (form II) DNA. The smear from the supercoiled to the open-circled position was observed at a lower concentration of **1** (3.1 μM) and the form II′ DNA was observed without linear (form III) DNA at higher concentrations of **1** (50 μM) as previously observed.[3] In the absence of DTT, though no change was observed at lower concentrations of **1**, the form I DNA disappeared with the generation of the smear by the treatment with higher concentrations of **1**. This observation indicates the noncovalent or covalent binding of **1** to DNA. Generation of small amounts of **2** was detected by HPLC analysis when **1** was incubated in the absence of thiol (data not shown). These results indicate that small amounts of the active species would be generated in the absence of thiol. More examination would be needed to understand the formation of the smear in the absence of thiol. Degradation product **2** did not cause the DNA cleavage nor any changes in electrophoretic mobility of DNA at 50 μM in the presence or absence of DTT. On the other hand, **3** caused DNA cleavage in the absence of thiol. The concentration that induces the DNA cleavage was approximately equal to that of **1** with thiol. (Fig. 3A) The form II′ DNA was also observed at 50 μM treatment. DNA-cleaving activity of **3** was pH dependent. Acidic conditions (pH 5) resulted in more efficient cleavage than basic conditions (pH 8) (data not shown). Ester derivatives **3a** and **3b** also cleaved DNA but these activities were weaker than **3** (Fig. 2B). The sulfone derivative **3c** did not induce any changes of DNA form. These results are consistent with the DNA-alkylating activity and also correlated with the stability in aqueous condition.

Discussion

1 afforded two main degradation products in the presence of thiol in aqueous buffered solution. One of them, **2**, was an inactive molecule with a 3,7-sulfide ring. Another product **3** contains 3,6-sulfide with 7,8-epoxide. In the absence of thiol, **3** causes DNA alkylation and subsequent strand cleavage. The potency of DNA cleavage activity of **3** was almost equal to that of leinamycin with thiol (Fig. 3A). Examination of the synthetic derivatives of **3** showed that the DNA cleavage activity correlated with the DNA-alkylation activity which is dependent on the electrophilic reactivity. These results indicate that the DNA alkylation is responsible for the DNA cleavage activity of leinamycin. We previously proposed the rate-determining formation of an electrophilic episulfonium **4**[6] in aqueous solution for the mechanism of DNA alkylation and subsequent DNA cleavage by thiol-activated **1** and by **3** (Scheme 3). No intermediates could be detected by HPLC analysis under the condition in which **3** was converted to **2**. Further efforts would be required for detecting or obtaining this presumed active species due to its high electrophilic reactivity. However, the generation of **2** and **3** from **1** in the presence of thiol, and the acid-catalyzed transposition of sulfide linkage of **3** from C-6 to C-7 carbon, which is accompanied with the cleavage of epoxide, strongly indicate the generation of **4** from **1** or **3** as an active species for DNA alkylation. The sulfone derivative **3c** was stable in aqueous condition and does not possess DNA-alkylating nor DNA cleavage activity. This observation indicates the electrophilicity of 3,6-sulfide enabling **3** to generate **4** and also supports the theory that the generation of **4** would be necessary for the DNA-damaging activity. From our results two pathways are considered to explain the generation of the products presented here from **4**. **4** would undergo intermolecular nucleophilic attack to generate the stable and inactive products with 3,7-sulfide (path A). On the other hand, **4** would undergo intramolecular nucleophilic attack of 8-hydroxyl group at C-7 carbon to generate **3**, saving its potency for DNA damaging (path B). Since there should be so many thiol functional groups in cells, both pathways presented in Scheme 3 could proceed in cells. However, though **3** possesses potent DNA cleavage activity in vitro, this compound showed less potent cytotoxicity than **1**. A synthetic derivative of **1** with protected hydroxyl group at C-8 afforded an inactive product with 3,7-sulfide in the presence of thiol, but no product with 3,6-sulfide was observed and further this derivative showed as potent a cytotoxicity as **1** (data not shown). These results suggest that the conversion of **3** from **4** via path B might be one of the inactivating processes rather than the activation of biological activity of leinamycin. However, if **3** would be generated around its target, DNA, inside cells it could cause the damage to DNA. As reported previously, leinamycin binds DNA through N7 guanine covalent adduct formation and this damage readily induces single-strand scission of DNA subsequently at 37 °C. These two kinds of damage could be generated in cells treated with leinamycin or epoxide **3**. Though kinds of damage on DNA could induce the cell-killing, which damage would be predominant in cells, or more important for the biological activity of leinamycin, is not yet elucidated. Further examination of the DNA damage induced by leinamycin in cells is underway.



Scheme 3. Presumed active species **4** generated from leinamycin.

Conclusions

1 afforded two major degradation products in the presence of thiol. **2** was an inactive product, while **3** caused DNA cleavage in the absence of thiol. The synthetic derivatives were prepared from **3**. The electrophilic reactivity of these molecules was correlated with both DNA alkylation and cleavage activity. These results indicate that the electrophilic episulfonium species would be generated from **1** or **3**, and the DNA alkylation by this species is responsible for the single-strand scission of DNA.

Experimental

Instrumentation and reagents

Leinamycin **1** was isolated from the culture broth of its producing organism, *Streptomyces* sp. Calf thymus DNA was purchased from Sigma. All of the NMR experiments were performed on a Bruker AM500 spectrometer operating at 500 MHz for proton and 125 for carbon observations. Fast atom bombardment mass spectra (FABMS) and fast atom bombardment high-resolution mass spectra (FABHRMS) were run on an HX110A. Infrared spectra (IR) were recorded on a JEOL JIR-RFX3001. Ultraviolet spectra (UV) were recorded on a Shimadzu UV-2200. For column chromatography, silica gel (SiO_2, Wako C-200) was used. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 plate (Merck). HPLC was done on an LC4A system (Shimadzu) with a column (Unisil Q C18 5 μm 20 × 250 mm , GL science) for preparative scale or on LCM10A system (Shimadzu) with a column (Unisil 5C18 250A, GL science) for analytical scale. Preparation and full characterization of compounds **2**, **2a**, **3** and **3a** was described in a previous report.[6]

Preparation of 3b

After 4 mg of **3** (0.0084 mmol) were dissolved in 0.2 mL of DMF, 8.4 μL of isopropyl iodide (0.084 mmol) and 4.2 mg of K_2CO_3 (0.030 mmol) were added to the solution by stirring. The mixture was stirred at 20 °C for 6 h and poured into saturated sodium bicarbonate solution and extracted with chloroform twice. The combined organic layers were washed with brine and dried (Na_2SO_4) and evaporated in vacuo, and the residue was purified by column chromatography (SiO_2, 50% ethyl acetate in hexane) to give **3b** (2.8 mg, 64%) as a white solid.

^{1}H NMR ($CDCl_3$) ppm: 9.52 (1H ddd J = 1.0, 11.4, 16.3 Hz), 7.62 (1H bd J = 7.1 Hz), 7.29 (1H s), 6.59 (1H d J = 11.4 Hz), 6.40 (1H br), 6.23 (1H dd J = 11.4, 11.4 Hz), 6.05 (1H d J = 16.3 Hz), 5.60 (1H dq J = 7.1, 7.1 Hz), 5.13 (1H dq J = 6.3, 6.3 Hz), 3.66 (1H dd J = 1.4, 2.0 Hz), 3.53 (1H d J = 2.0 Hz), 2.92 (1H d J = 14.3 Hz), 2.67 (1H d J = 14.3 Hz), 2.43 (1H ddd J = 7.2, 12.7, 12.7 Hz), 1.99 (1H ddd J = 1.8, 6.3, 12.7 Hz), 1.89 (1H ddd J = 6.3, 12.7, 12.7 Hz), 1.64 (3H s), 1.62 (1H ddd J = 1.8, 7.2, 12.7 Hz), 1.58 (3H d J = 7.1 Hz), 1.53 (3H s), 1.30 (6H d J = 6.3 Hz).

FABMS, m/z 521 $(M+H)^+$; FABHRMS, m/z 521.1774 ($C_{25}H_{32}O_6N_2S_2$ + H^+ requires 521.1780). $[\alpha]^{20}_D$ +26° (c 0.045, MeOH); IR (KBr) ν_{max} 3444, 2979, 1716, 1652, 1538, 1455, 1267, 1174, 1101 cm^{-1}; UV (MeOH) λ_{max} 203, 324 nm.

Preparation of 3c

4.3 mg of *m*-CPBA (0.025 mmol) was added to a solution of **3a** (5 mg, 0.01 mmol) in 0.5 mL of CH_2Cl_2 and stirred at 20 °C for 3 h. The mixture was poured into saturated sodium bicarbonate solution and extracted with chloroform twice. The combined organic layers were washed with brine and dried (Na_2SO_4) and evaporated in vacuo, and the residue was purified by column chromatography (SiO_2, 30% ethyl acetate in n-hexane) to give a sulfone derivative **3c** (3.2 mg, 60%) as a white solid.

^{1}H NMR ($CDCl_3$) ppm: 9.47 (1H ddd J = 0.9, 12.4, 16.3 Hz), 7.62 (1H bd J = 6.7 Hz), 7.34 (1H s), 6.58 (1H d J = 11.5 Hz), 6.22 (1H dd J = 11.5, 11.5 Hz), 6.10 (1H d J = 16.2 Hz), 6.06 (1H br), 5.49 (1H dq J = 6.7, 6.7 Hz), 3.82 (3H s), 3.74 (1H d J = 2.2 Hz), 3.57 (1H dd J = 1.1, 2.2 Hz), 3.22 (1H d J = 16.2 Hz), 2.78 (1H d J = 16.2 Hz), 2.46 (1H ddd J = 7.2, 13.9, 13.9 Hz), 2.28 (1H ddd J = 2.0, 7.2, 13.9 Hz), 1.75 (1H m), 1.62 (1H m), 1.79 (3H s), 1.57 (3H d J = 6.7 Hz), 1.50 (3H s). FABMS, m/z 525 $(M+H)^+$; FABHRMS, m/z 525.1370 ($C_{23}H_{28}O_8N_2S_2$ + H^+ requires 525.1365). $[\alpha]^{22}_D$ −52° (c 0.06, MeOH); IR (KBr) ν_{max} 3403, 2952, 1733, 1646, 1608, 1538, 1521, 1307, 1268, 1176, 1132 cm^{-1}; UV λ_{max} (MeOH) 203, 337 nm.

Stability and DNA alkylation activity

A sample (200 μM) was incubated with or without 2-ME (240 μM) in 500 μL of 10% MeOH/10 mM phosphate buffer (pH 7) at 37 °C. For analyzing the binding of drugs to DNA, a sample (200 μM) was mixed with calf thymus DNA (2 mM bp) and 2-ME (240 μM) was added to the solution when required. The amounts of residual drug or degradation products in the reaction mixture were estimated by HPLC analysis at intervals (wavelength, 320 nm; eluate, 40–60% MeOH/50 mM phosphate buffer (pH 5.9); flow rate, 1 mL min^{-1}).

DNA cleavage activity

The DNA cleavage activity was determined with purified pBR322 DNA. Typical reaction mixtures included 20 μL of 10 mM phosphate buffer (pH 7) and 0.3 μg of pBR322 DNA. In some studies, an excess amount of DTT was added (0.5 mM) after the addition of drugs. Reaction mixtures containing various amount of drugs were incubated at 37 °C for 60 min. After addition of 3.5 μL of 0.02% bromophenol blue and 50% sucrose, 20 μL of mixture was placed in the well of an agarose slab gel. Electrophoresis was carried out in 89 mM borate, pH 8.2–2 mM EDTA buffer containing 0.01% SDS at 50 mV for 12 h. Following electrophoresis, gels were stained with aqueous solution of ethidium bromide. DNA bands were visualized by transillumination with ultraviolet light (300 nm).

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