Bioorganic & Medicinal Chemistry 20 (2012) 5720–5729

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Studies on synthesis and activation mechanism of mitomycin dimers connected by 1,2-dithiolane and diol linkers

Hyoung Rae Kim, Jae Jin Kim, Jung Jae Park, Sang Hyup Lee *

College of Pharmacy, Duksung Women's University, 419 Ssangmun-dong, Dobong-gu, Seoul 132-714, Republic of Korea

ARTICLE INFO

Article history:
Received 13 July 2012
Revised 8 August 2012
Accepted 9 August 2012
Available online 16 August 2012

Keywords:
Disulfide mitomycin
Mitomycin dimer
1,2-Ditholane
Nucleophilic activation
DNA interstrand cross-link

ABSTRACT

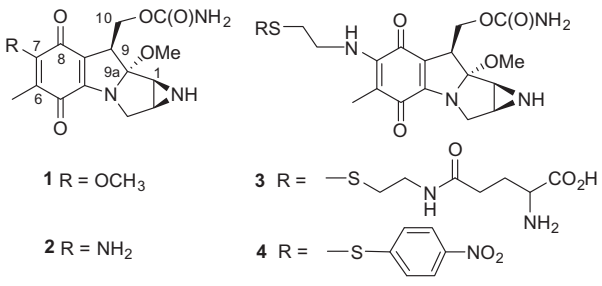
We report the synthetic and mechanistic studies on a new cyclic disulfide mitomycin dimer, 7-*N*,7′-*N*′-(1″,2″-dithiolanyl-3″,5″-dimethylenyl)bismitomycin C (**8**), and a diol mitomycin dimer, 7-*N*,7′-*N*′-(2″,4″-dihydroxy-1″,5″-pentanediyl)bismitomycin C (**9**). Mitomycin **8** is a dimer connected by a 1,2-dithiolane (a five-membered cyclic disulfide) linker, and was specifically designed to undergo nucleophilic activation and double DNA alkylations leading to efficient production of DNA interstrand cross-link (DNA ISC) adducts. Disulfide cleavage in **8** would generate two thiol groups that could serve as probes to activate two mitomycin rings. At first, the target mitomycin **8** was synthesized using mitomycin A (**1**) and the key intermediate, cyclic disulfide (**10**), which was prepared through a seven-step synthetic sequence. Diol mitomycin **9** was also synthesized from **1** and diamine salt **13**. Next, kinetic studies using solvolysis reaction revealed that the activation rates of **8** were much higher than those of **9** and mitomycin C (**2**) under nucleophilic conditions provided by Et_3P presumably due to the presence of a cyclic disulfide unit in **8**. These findings led us to propose a nucleophilic activation pathway for **8**. Then, DNA ISC experiments further revealed that the levels of DNA ISC caused by **8** in the presence of Et_3P were much higher (97%) than those by **9** (5%) and **2** (4%). More importantly, mitomycin **8** underwent much faster activation and produced slightly higher levels of DNA ISC than the previously reported mitomycins **5**–**7**. Overall, we concluded that **8** was highly efficient for both nucleophilic activation and corresponding DNA ISC formation, and that this differentiation came from the crucial function of the cyclic disulfide unit in **8**.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Mitomycins are DNA alkylating agents that constitute a class of potent antitumor agents. Although mitomycin A (MMA, **1**)[1] was the first compound in this series, mitomycin C (MMC, **2**) is considered a prototype compound and has been used as a potent agent of clinical significance.[1,2] Mitomycins must be converted to good electrophiles through proper activation processes in order to exhibit biological activities. Studies on activation mechanism revealed that the reduction of the quinone ring initiates activation leading to the generation of electrophilic C(1) and C(10) sites that react with DNA,[3] which was reductive activation. Generally, the C(1) site is 10–100 times more reactive than the C(10) site.[4] Among plausible DNA alkylation of mitomycins, the DNA interstrand cross-link (DNA ISC) adducts by bis-alkylation are the most lethal, and in particular, the DNA ISC adducts of **2** display ~60 times greater lethality than the corresponding monoadducts of **2**.[5] This clearly implies the significance of DNA ISC adducts in this series of compounds.

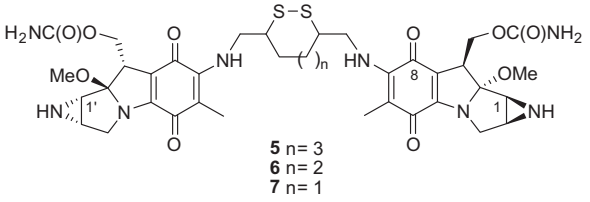
However, long-term administration of **2** has been found to induce severe drug resistance and side effects.[2] Therefore, subsequent efforts have focused on finding mitomycin derivatives that could be activated by a different activation mechanism. As a result, disulfide mitomycins **3** (KW-2149)[6] and **4** (BMS-181174)[7] were developed. Significantly, the disulfide mitomycins **3** and **4** contained, in particular, a C(7) disulfide ethylamino unit instead of the C(7) amino unit in **2**, and were believed to work by a different activation mechanism than **2** based on enhanced pharmacological activities in **2**-resistant tumor cell lines and in non-hypoxic cells.[8–10] Interestingly, at the core of the activation mechanism lies the disulfide group.[6,7,10] Disulfide cleavage in **3** and **4** provides corresponding thiols that could trigger the activation of the mitomycin ring by intramolecular cyclization to the quinone ring,[11,12] and induce the corresponding DNA adduction.[11–13] These processes are considered a nontraditional nucleophilic activation mechanism that differs from those of **2**.

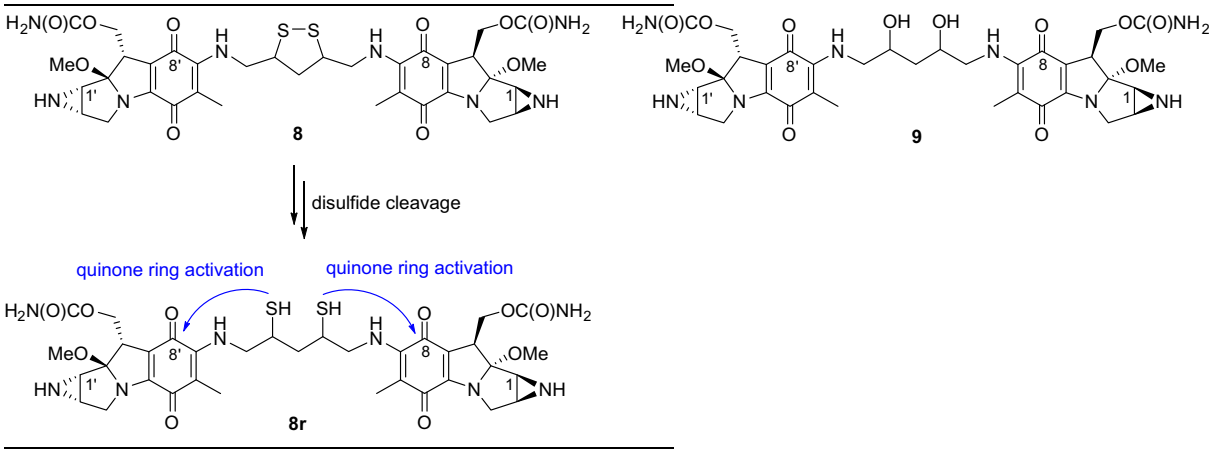


* Corresponding author. Tel.: +82 2 901 8393; fax: +82 2 901 8386.
E-mail address: sanghyup@duksung.ac.kr (S.H. Lee).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.08.015

In addition, considering the significance of DNA ISC, we were interested in dimerization of mitomycin units to enhance the formation of DNA ISC. Mitomycin dimers would provide two C(1) sites of higher reactivity and, as a result, would be expected to produce higher levels of DNA ISC by bis-alkylation at the two distal C(1) sites. We had previously started a program to investigate disulfide mitomycin dimers that contained a cyclic disulfide as a linker. From the initial results of this program, we have already reported the studies on the mitomycin dimers, **5**,[14] **6**,[15,16] and **7**.[17,18] The structural differences among these compounds were the ring size of the cyclic disulfide linkers. Comparing the three compounds **5**–**7** each other, we found that all compounds displayed high levels of DNA ISC formation ($\geqslant$83%), and in detail, the levels of DNA ISC were similar or slightly decreased (e.g., DNA ISC by Et_3P: 94% → 86% → 83%) as the ring size of the cyclic disulfide linker decreased (**5** → **6** → **7**, respectively). However, the activation rates for these compounds in the presence of nucleophiles (e.g., Et_3P or L-dithiothreitol (L-DTT)) showed different patterns. For example, the activation rates were decreased by almost half (e.g., k_{obs} by Et_3P 10 equiv: 8.7 d^{-1} → 4.6 d^{-1} → 2.7 d^{-1}) as the ring size of the cyclic disulfide linker decreased (**5** → **6** → **7**, respectively). These differences between the activation rates and DNA ISC formation according to the ring size of the linkers seemed to be quite meaningful and led us to focus on the influence of the ring size of the linker on the activation and DNA ISC formation.





2. Results and discussion

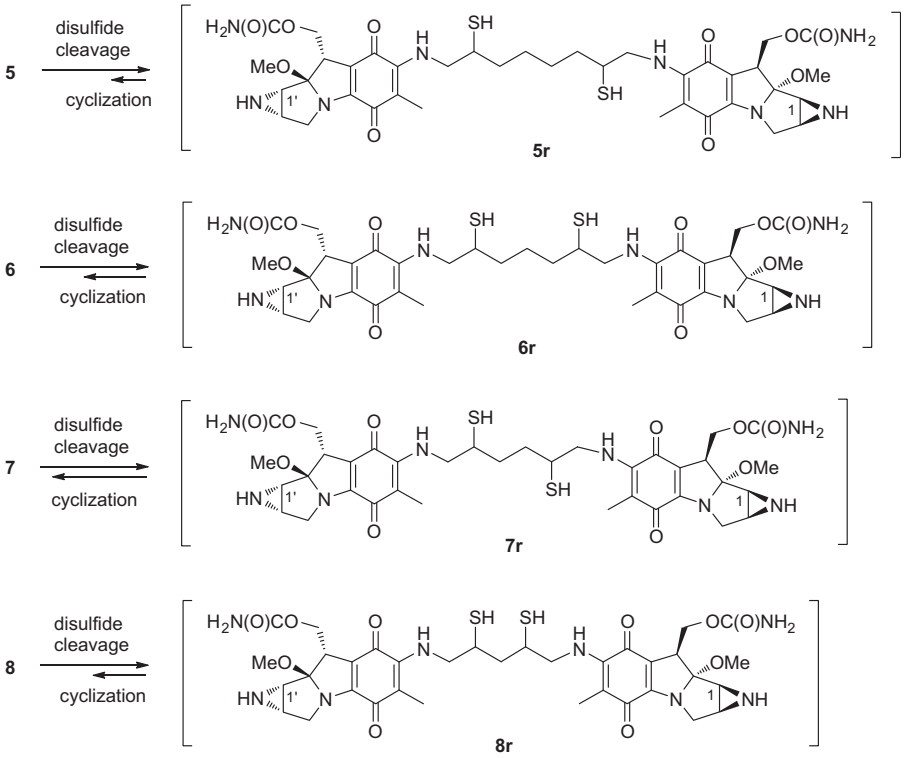
2.1. Design and structural features of 8 and 9

The results above prompted us to examine the influence of the ring size of the cyclic disulfide linker on mitomycin activation. We report herein the studies on new mitomycin dimers **8** and **9** and the comparison of these compounds with the precedent examples (e.g., **5**–**7**). Compound **8** contains a 1,2-dithiolane (a five-membered cyclic disulfide) and was designed to undergo efficient nucleophilic activation and corresponding facile DNA adduction. Compound **9** contains a diol linker instead of a cyclic disulfide linker based on the same carbon skeleton as **8**, and could therefore serve as a good reference to identify the net effect of the disulfide unit in **8**. The objectives of this work were to study the design and synthesis, activation mechanism, and the DNA ISC formation for **8** compared to **9**, to compare **8** with **5**–**7** and to elucidate the linker effect. Considering that **8** and **9** retained all the structure of mitomycin C (**2**), we believed that **8** and **9** would undergo corresponding activations under reductive[3] and acidic conditions[19] and that these mitomycin dimers would display basic levels of cytotoxicity. Furthermore, since we were mainly interested in the activation mechanism of these peculiar mitomycins, we did not include the cytotoxicity data of **8** and **9** in the scope of our studies.

As mentioned above, compound **8** was designed to combine two effects: a disulfide effect and a dimerization effect. The disulfide effect represents nontraditional nucleophilic activation by the disulfide unit and the dimerization effect represents double C(1) activations at two C(1) sites of higher reactivity provided by a dimeric structure, which could eventually induce enhanced DNA ISC formation. The disulfide cleavage or reduction in **8** may provide a crucial dithiol species (**8r**: reduced form of **8**) that contains two thiols in the linker part. The two thiols in **8r** were both expected to work as good activation probes to activate two mitomycin rings in a dimer by intramolecular cyclization to quinone ring and subsequent activation processes,[17,20] leading to the corresponding DNA adduction.[21] Unfortunately, the thiol that is required for activation was very unstable[12] due to the presence of adjacent quinone ring. Thus, the disulfide unit might be an appropriate, stable precursor for the required thiol, which was a key aspect in our design. Despite comprehensive investigations on mitomycin activation, much is still unclear about the reasonable structure of activated products when reacting with DNA.

When comparing compound **8** with **5**–**7**, the only difference is the ring size of the linker. Disulfide cleavage of compounds **5**–**8** provides the corresponding dithiol species **5r**–**8r**, respectively, which induce activation of mitomycin rings. Therefore, we assumed that the formation and lifetime of thiol(s) in dithiol species, and the flexibility and distance between the two C(1) sites would be crucial for the activation and subsequent DNA adduction. In order to verify this assumption, we investigated the effect of the ring size of the linker using **8** and compared the results with those for **5**–**7**. The distance between the two C(1) sites in **8** (or **8r**) may be slightly shorter than those in **5**–**7**. The maximum distances in **8** were 23∼24 Å depending on their conformations (Sybyl 6.0,

HyperChem 7.1) and differed from those in **5**–**7**. In addition, the lifetimes of corresponding thiol(s) in dithiol species would be different, which may directly affect the activation of mitomycins. Based on the previous studies[22,23] regarding thiol-disulfide interchange and their stabilities, it was believed that the cyclization tendency of dithiol species would be **7r**>>**6r**⩾**8r**>>**5r**.

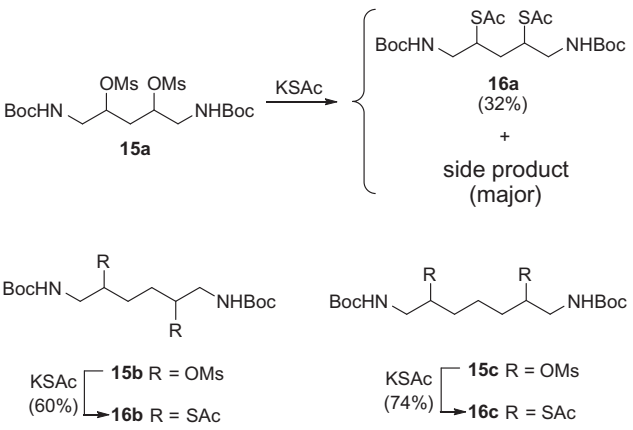


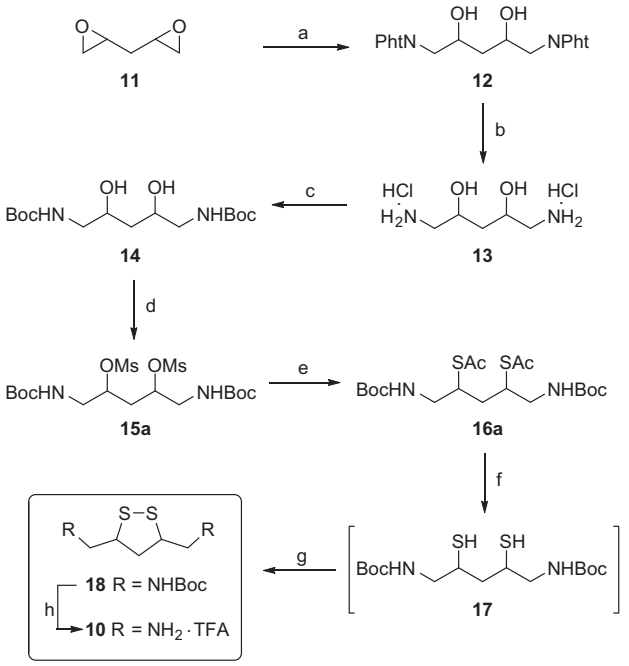
2.2. Chemistry

2.2.1. Synthesis of cyclic disulfide 10

In order to synthesize the target mitomycin **8**, we first synthesized the required key intermediate, cyclic disulfide **10**, as shown in Scheme 1. Compound **10** and all of the related precursors shown in Scheme 1 contain a symmetrical structure with two stereocenters and could therefore exist as three stereoisomers (*meso*-(*R*,*S*) and enantiomeric pair, *threo*-(*R*,*R*) and *threo*-(*S*,*S*)). Thus, we analyzed the diastereomeric ratio (dr) of compounds by ^{13}C NMR.

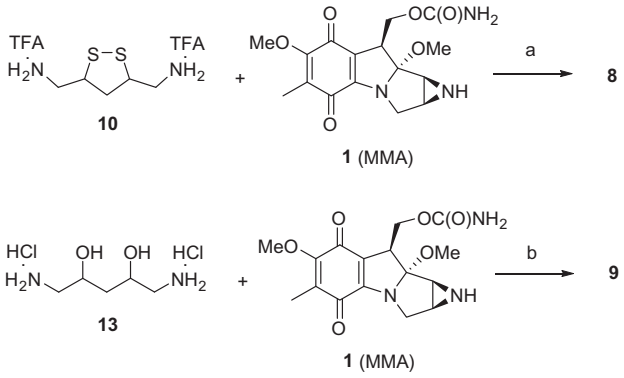
At first, we prepared diepoxide **11** in good yield (90%, dr = 1.1:1) according to previous procedures.[24] Then, treatment of **11** with phthalimide (PhtNH) afforded diphthalimide derivative **12** in relatively low yield (30–40%) with a varying ratio of diastereomers (1:1–3:1). We applied stepwise temperature control in order to avoid loss of the starting diepoxide through facile evaporation, and careful work-up including precipitation using diethyl ether (Et_2O) to provide **12** in good yield (76%, dr = 1.4:1). We further attempted to improve the diastereomeric ratio by recrystallization in DMF-Et_2O and consequently obtained **12** in relatively low yield (21%) but in high diastereomeric ratio (dr = 4:1). Subsequent deprotection of phthalimide groups in **12** using hydrazine hydrate, followed by treatment of hydrochloric acid, provided amine salt **13** (dr = 5:1) in 80% yield. Treatment of amines in **13** with di-*t*-butyl dicarbonate (Boc_2O) afforded **14** (dr: an apparent single set of signals in ^{13}C NMR) in 70% yield. We then reacted **14** with methanesulfonyl chloride (MsCl) to obtain the dimesyl derivative **15a** in 93% yield. Replacement of the mesylate units with acetylthio groups to give diacetylthio derivative **16a** through the use of potassium thioacetate (KSAc) in DMF was problematic. Surprisingly, in this reaction we observed formation of a major unknown side product that showed a higher R_f value (0.53) than that (0.50) of the desired product **16a**. In our previous studies,[15,17] we conducted similar reactions of **15b** and **15c** using KSAc and obtained the diacetylthio derivatives **16b** and **16c** in reasonably good yields, 60% and 74%, respectively. Considering these results, the reaction of only **15a** with KSAc might undergo some side reaction or overreaction. The side product contained only one acetylthio group and no mesylate group relative to two Boc groups based on the NMR analysis. We suggest that the Boc-amino group probably participates in removing the mesylate or acetylthio group. We also found that the side product might be in equilibrium with **16a** since we observed that treatment of the side product with KSAc partly produced **16a**. Unfortunately, intensive efforts to clarify the reaction and identify the structure of the side product were inconclusive. Further attempts to improve the yield of the desired product **16a** were conducted by modulating the reaction conditions such as amount of KSAc, temperature, and reaction time, finally affording **16a** in 32% yield.





Scheme 1. Synthesis of cyclic disulfide **10**. Reagents and conditions: (a) PhtNH, DMF, 100 °C /1 h → 115 °C /1 h → 135 °C /1 h, 76%; then, recrystallization in DMF, 21%; (b) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux, 3.5 h; then HCl, reflux, 1 h, 80%; (c) Boc_2O, DMF–H_2O (1:1), room temperature, 1 d, 70%; (d) MsCl, Et_3N, CH_2Cl_2, 0 °C, 5 h, 93%; (e) KSAc, DMF, 60 °C, 8 h, 32%; (f) K_2CO_3, MeOH-H_2O (5:1), room temperature, 1 h; (g) I_2, Et_3N, $CHCl_3$, room temperature, 3 h, 69% (for two steps); (h) TFA, room temperature, 1 h, 100%.

Hydrolysis of **16a** using potassium carbonate (K_2CO_3) was conducted to give dithiol derivative **17** that was directly used for the next reaction. Considering the instability of the dithiol species, we performed the two reactions, hydrolysis and oxidation, in situ without isolation of dithiol **17**. At first, intramolecular cyclization of dithiol units was achieved by oxidation using I_2/Et_3N in varying concentrations to afford cyclic disulfide **18**. In order to avoid the formation of unwanted side products through intermolecular disulfide formation,[14] we then applied reaction conditions of high dilution (~5 mM concentration) and obtained product **18** in relatively good yield. However, we believed that the intramolecular cyclization of 1,3-dithiol might be more favorable than that of 1,6-dithiol. We further investigated the reaction conditions of higher concentrations and eventually established the reaction condition of 50 mM concentration to give the desired product **18** in good yield (69%, for two steps). The product **18** was identified by NMR (^1H and ^{13}C), Mass, and IR, and more significantly, **18** was verified by reasonable R_f value (0.53, EtOAc/hexanes = 1:2) on TLC by comparison with the values of 3,6-bis(*tert*-butyloxycarbonylaminomethyl)-1,2-dithiane (0.55),[17] 3,7-bis(*tert*-butyloxycarbonylaminomethyl)-1,2-dithiepane (0.56)[15] and 3,8-bis(*tert*-butyloxycarbonylaminomethyl)-1,2-dithiocane (0.56).[14] Then, deprotection of Boc groups in **18** using trifluoroacetic acid (TFA) was executed to afford amine·TFA salt **10** in near quantitative yield. Interestingly, compounds **14**, **15a**, **16a**, **17**, **18** and **10** showed an apparent single set of signals in ^{13}C NMR and therefore, we believed that, among three possible stereoisomers, these compounds including **10** existed as either a meso-isomer or an enantiomeric pair, not as diastereomeric mixtures. Consequently, the key intermediate, cyclic disulfide **10** was synthesized from **11** through a seven-step synthetic sequence in 2.4% overall yield.



Scheme 2. Synthesis of mitomycins **8** and **9**. Reagents and conditions: (a) Et_3N, MeOH, room temperature, 2 d, 66%; (b) Et_3N, MeOH, room temperature, 2 d, 67%.

2.2.2. Synthesis of mitomycins 8 and 9

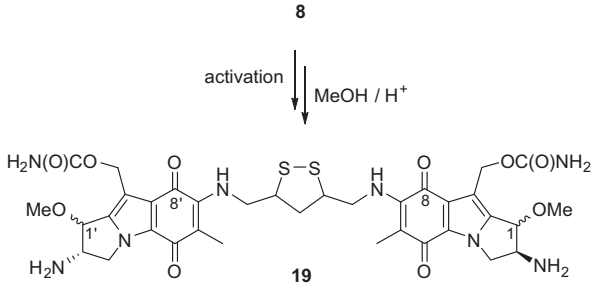
The target mitomycins **8** and **9** were synthesized using mitomycin A (MMA, **1**, KyowaHakko Kirin Co.) and the corresponding intermediates **10** and **13**, respectively, as shown in Scheme 2. Treatment of **1** with intermediate **10** in MeOH provided the target mitomycin **8** in 66% yield, and it was found that **8** was a mixture of diastereomers (dr = 1.2:1) by analysis of ^{13}C NMR signals. In addition, treatment of **1** with amine salt **13** afforded diol mitomycin **9** in 67% yield, and **9** was also found to be a mixture of diastereomers (dr = 1.1:1) by analysis of ^{13}C NMR signals. Mitomycin **9** was used as a reference one for the studies on **8**.

2.3. Activation studies on mitomycins 8 and 9

2.3.1. Methanolysis of mitomycin 8 to give mitosene product 19

We employed a methanolysis reaction of mitomycin as a chemical model system to mimic the activation and subsequent reaction with nucleophiles in biological systems. Methanolysis of **8** was conducted for ~2 d under acidic conditions (MeOH–$CHCl_3$ (1:1) solution at nonaqueous effective pH ('pH') 2.5–3.0) to afford the activated mitosene product, C(1) methoxymitosene **19**. As the reaction proceeded, starting mitomycin **8** disappeared and monoactivated intermediate was generated; the intermediate was eventually converted to diactivated mitosene product **19**. However, the diactivated mitosene product was highly polar and unstable, even at room temperature, and displayed long tailing on TLC. Accordingly, it was very difficult to isolate and purify the product. However, careful monitoring on TLC and HPLC enabled us to identify the progress of the reaction. We purified the product by preparative thin layer chromatography (PTLC) with poor results (45% yield). Further efforts to improve the results were inconclusive. We believe that, as mentioned above, the mitosene product is highly polar due to the presence of two newly generated primary amine groups and is composed of a mixture of many diastereomers due to the uncontrolled stereochemistry at two C(1) sites, without considering the stereochemistry of cyclic disulfide linker. Despite these complications, we succeeded in partially characterizing **19** using HPLC, UV–vis, ^1H NMR, and mass spectroscopy. The HPLC chromatogram showed three peaks (t_R = 30.1, 31.3, 32.5 min, ~1:2:1) that might indicate the formation of corresponding diastereomers of two unidentified stereocenters at C(1) and C(1′). More conclusively, the UV–vis spectra confirmed the activation of mitomycin leading to the generation of a mitosene product based on absorption pattern.[19,25] We observed an absorption maximum at ~313 nm for the mitosene product **19**, not at ~376 nm for starting mitomycin **8**. Furthermore, we observed the expected resonance (δ 3.52) for the C(1) methoxy units and the downfield resonance

and specific coupling pattern (δ 5.73 and 5.78, 1/2ABq) for the C(10) methylene protons in the ^{1}H NMR spectra for **19**. These signals were known to be characteristic for the formation of C(1) methoxymitosenes.[26,27] Taken together, the mitosene product **19** was believed to be appropriately identified and was then employed as an authentic sample for subsequent kinetic experiments.



2.3.2. Kinetic studies on nucleophilic activation of 8 and 9

The rates of methanolysis were believed to directly represent the efficiency of activation and adduction of mitomycins. Therefore, kinetic studies on nucleophilic activations were conducted by measuring the rate of methanolysis of mitomycins in the presence of appropriate nucleophiles. We wished to see if the target mitomycin **8** undergoes efficient nucleophilic activation compared with reference diol mitomycin **9**, and particularly, to verify the critical function of the disulfide group in **8**. We used Et_3P, L-DTT and glutathione (GSH) as proper nucleophiles to induce disulfide cleavage and subsequent activation. The activations were induced in buffered methanolic solutions (0.1 M Tris·HCl, 'pH' 7.4) at room temperature and monitored by UV–vis spectroscopy (200–500 nm) for more than two half-lives. The absorption was monitored at ~373 nm for starting mitomycins and ~313 nm for mitosene products (activated products). At the end of the reactions, the solutions were analyzed by HPLC and TLC by comparison with authentic samples of **8**, **9**, and mitosene product **19**. Generally, the reactions well followed pseudo first-order kinetics, and the k_{obs} (d^{-1}) and $t_{1/2}$ (d) were calculated. The reactions were run in duplicate, and the results averaged.

As shown in Table 1, we measured the methanolysis rates of **8** and **9** in the absence of nucleophile and observed no distinct decrease (less than 10% of original amount) of starting mitomycins in 5 d, which means there was no appreciable activation without nucleophile. Next, we measured the effect of thiol nucleophiles such as L-DTT and GSH on the activation of **8** and **9**. When we used 10 and 20 equiv of L-DTT, and 20 equiv of GSH, we observed no distinct decrease (less than 10% of original amount) of starting mitomycins **8** and **9** in 3 d, which indicated that neither L-DTT nor GSH significantly induced the nucleophilic activation of mitomycin dimers **8** and **9**.

Then, we investigated the effect of Et_3P as a phosphine nucleophile on activation of **8** compared with **9**. We found that 5–50 equiv of Et_3P significantly induced a decrease of disulfide mitomycin **8**, which implies that **8** underwent rapid nucleophilic activation by Et_3P. The half-lives ($t_{1/2}$) of the activation reactions ranged from 0.075 d (5 equiv) to 0.011 d (50 equiv), and the activation rates were roughly proportional to the amount of Et_3P. However, we found that Et_3P (until 50 equiv) did not induce an appreciable decrease of diol mitomycin **9** in 3 d, which indicated that **9** did not undergo appreciable activation by Et_3P. We believed that the rapid nucleophilic activation for only **8** but not **9** by Et_3P must be induced by the presence of the disulfide unit in **8**. So, Et_3P was expected to attack the disulfide unit in **8** leading to the generation of thiol (or thiolate) that could trigger the activation by attacking the quinone ring of mitomycin. When we monitored the activation reactions by Et_3P using UV–vis spectroscopy, we observed a gradual decrease of starting mitomycin **8** at 373 nm and a subsequent increase of mitosene products at 313 nm, which is in accord with the known absorption patterns.[19,25] The HPLC chromatograms for these activation reactions displayed multiple peaks between ~30 and ~33 min. All these peaks showed absorption maxima at 313 nm which again confirmed the formation of mitosene products. However, identification of all these peaks by coinjection with the authentic sample **19** was inconclusive. In reality, the peak pattern was complex probably due to the many diastereomers of mitosene products. In addition, we had to consider the low concentrations of reaction solutions (~0.03 mM) and the possibility of precipitation of polar mitosene products during the reaction period. Further efforts did not lead to successful results.

According to the information obtained above we propose a plausible mechanism for nucleophilic activation of **8** by Et_3P as shown in Scheme 3. Mitomycin **8** undergoes disulfide cleavage by Et_3P[28] and subsequent decomposition of thiophosphonium group by H_2O (or MeOH)[28] to provide a dithiol species **8r** that has two thiols. As mentioned above, the generation of thiol is significant since the generated thiol group would serve as a key probe to initiate mitomycin activation. Both of the thiols in **8r** are expected to attack the C(8) carbon of the quinone ring by intramolecular cyclization to give hemi-thioketal **20**. Once **20** is formed the stabilization of the nonbonding electron pair at N(4) through N(4)–C(4a)–C(8a)–C(8)–O conjugated system may be disturbed due to loss of the C(8) carbonyl group. This destabilization would facilitate elimination of methoxide at C(9a) to give an iminium ion at N(4)–C(9a) that may undergo subsequent shift of the double bond to C(9)–C(9a) leading to the generation of mitosene structure **21**. The structure **21** may be slightly unstable and easily undergo aziridine ring opening. Consequently, the C(1) site becomes highly electrophilic and reacts with nucleophile (e.g., MeOH or DNA) to afford **22**,[21] which eventually represents C(1)–C(1′) double activations and adductions. Then, the facile decomposition of the hemi-thioketal structure in **22** would give dithiol and finally disulfide **23** by proper oxidation processes. Thus, through the action of Et_3P, mitomycin **8** underwent double activations and adductions at the two distal C(1) and C(1′) sites, which might constitute a kind of nucleophilic activation mechanism that differs from traditional mechanisms.

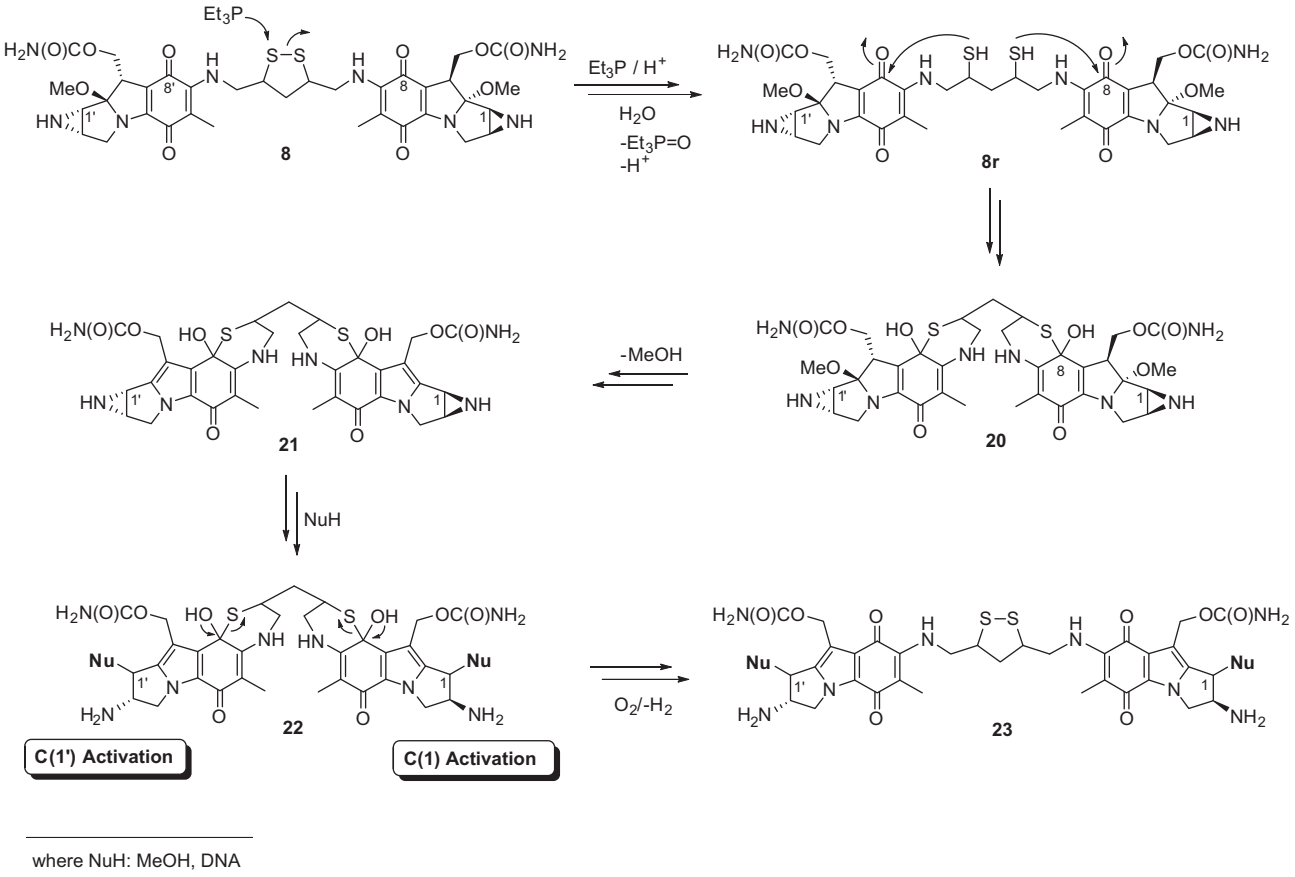
Table 1
Activation rates for **8** and **9** at 'pH' 7.4[a]

Reagents		8		9	
Nu	equiv	k_{obs}(d^{-1})	$t_{1/2}$(d)	k_{obs}(d^{-1})	$t_{1/2}$(d)
No Nu		[b]	[b]	[b]	[b]
L-DTT	10	[c]	[c]	[c]	[c]
	20	[c]	[c]	[c]	[c]
GSH	20	[c]	[c]	[c]	[c]
Et_3P	2	[c]	[c]	—	—
	5	9.2	0.075	—	—
	10	16	0.043	[c]	[c]
	20	23	0.030	[c]	[c]
	50	63	0.011	[c]	[c]

[a] Reactions were run in buffered methanolic solution (0.1 M Tris·HCl, 'pH' 7.4) at 25 °C. The reactions were run in duplicate and the values averaged. The results were obtained using a Shimatzu UV-1800 spectrophotometer and the reactions monitored at ~373 nm unless otherwise indicated. The concentration of the mitomycin was 0.030 mM.
[b] No appreciable change in 5 d (less than 10% of the original amount).
[c] No appreciable change in 3 d (less than 10% of the original amount).

2.4. Studies on the formation of DNA ISC for 8 and 9

We next examined the ability of mitomycin dimer **8** to produce DNA ISC under nucleophilic activation conditions at pH 7.4 compared to reference **9** in order to verify and highlight the



Scheme 3. Proposed mechanism of nucleophilic activation of **8** by Et_3P.

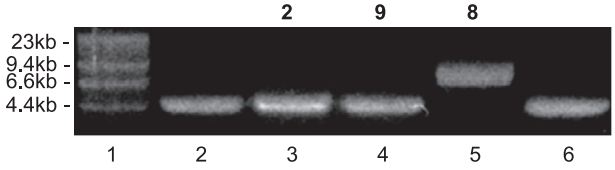


Figure 1. Denaturing 1.2% Alkaline Agarose Gel for **2**, **9** and **8** using Et_3P (5 equiv). DNA cross-linking experiments using 0.1 mM concentration of mitomycins except for **2** (0.2 mM) and *Eco*RI-linearized pBR322 plasmid DNA with Et_3P (5 equiv). All reactions were incubated at room temperature (2 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: control (only linearized pBR322). Lane 3: **2** + Et_3P. Lane 4: **9** + Et_3P. Lane 5: **8** + Et_3P. Lane 6: only Et_3P.

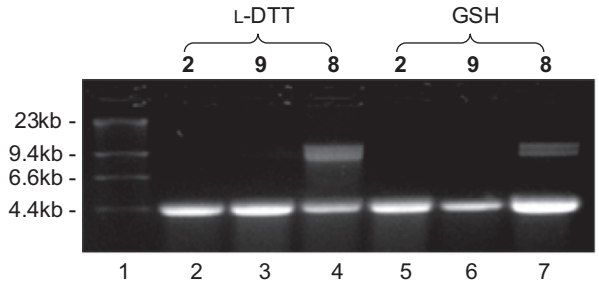


Figure 2. Denaturing 1.2% Alkaline Agarose Gel for **2**, **9** and **8** using L-DTT and GSH (5 equiv). DNA cross-linking experiments using 0.1 mM concentration of mitomycins except for **2** (0.2 mM) and *Eco*RI-linearized pBR322 plasmid DNA with L-DTT (5 equiv) and GSH (5 equiv). All reactions were incubated at room temperature (2 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: **2** + L-DTT. Lane 3: **9** + L-DTT. Lane 4: **8** + L-DTT. Lane 5: **2** + GSH. Lane 6: **9** + GSH. Lane 7: **8** + GSH.

function of the disulfide in **8**. For this purpose we employed the methods by Cech,[29] and Tepe and Williams[30] with minor modifications. Based on these methods pBR322 plasmid DNA was linearized using *Eco*RI enzyme. The linearized pBR322 DNA was treated with mitomycins under nucleophilic activation conditions and applied to denaturing alkaline agarose gel electrophoresis to analyze the formation of DNA ISC along with λ DNA digested with *Hin*dIII as a molecular weight marker. Based on the information obtained from our previous studies with **7**[17] we selected appropriate conditions such that the reactions were run at 0.1 mM concentrations of mitomycin dimers with 5 equiv of nucleophiles for 2 h at room temperature to efficiently differentiate the ability to form DNA ISC. To keep the concentrations of mitomycin units per experiment the same, we used 0.2 mM concentration of **2** since it is a monomeric structure not a dimeric structure.

First, we checked the formation of DNA ISC for **2**, **9**, and **8** in the presence of Et_3P (5 equiv), and the results were shown in Figure 1.

Remarkably, mitomycin **8** produced much higher levels of DNA ISC (97%) under these conditions compared to references **9** (5%) and **2** (4%). As expected, mitomycin **8** underwent fast activation by the function of Et_3P on the disulfide unit. Accordingly, we concluded that target mitomycin **8** was highly efficient for nucleophilic activation and DNA ISC formation compared to references **9** and **2**. In addition, we compared the results for DNA ISC formation with the rate data of kinetic studies (Table 1). Among the mitomycins tested, only mitomycin **8** underwent facile activation by Et_3P (5 equiv: t_R = 0.27 d) while references **9** and **2** did not, which enabled us to confirm a good correlation.

We then checked the formation of DNA ISC for **2**, **9**, and **8** by L-DTT and GSH as thiol nucleophiles, and the results were shown

Table 2
Comparison of **8** with **5**–**7** in activation rates and DNA ISC formations using Et_3P

Compounds	**5**[c]	**6**[d]	**7**[e]	**8**
Activation rates[a] k_{obs} (d^{-1})	8.7	4.6	2.7	16
DNA ISC[b] (%)	94	86	83	97

[a] Activation rates with 10 equiv of Et_3P.
[b] DNA ISC formations with 5 equiv of Et_3P.
[c] Ref. [14].
[d] Ref. [16].
[e] Ref. [17].

in Figure 2. By L-DTT(5 equiv), **8** produced medium levels (45%) of DNA ISC while **9** and **2** produced trace amount of DNA ISC (5% and 3%, respectively). By GSH (5 equiv), **8** produced low levels (15%) of DNA ISC while **9** and **2** produced trace amount of DNA ISC (less than 5%). These results implied that **8** underwent moderate or low activation by L-DTT and GSH, and that thiol nucleophiles were not as efficient as the phosphine nucleophile for activation of mitomycin **8**. We also compared these results with kinetic data of **8** in the presence of thiols (Table 1). Neither L-DTT nor GSH induced apparent activation of **8** in 3 d, so the formation of medium levels of DNA ISC for **8** by L-DTT was not clearly understood. We believe that this discrepancy might originate from the different reaction conditions between the two sets of experiments and that the DNA experiments were more sensitive than the kinetic experiments.

2.5. Comparison of mitomycin 8 with mitomycins 5–7

As discussed above, one of our main interests lies in the effects of the ring size of the linkers in a dimer on the activation rates and the formation of DNA ISC. Therefore, we compared mitomycin **8** with mitomycins **5**–**7** connected through a different cyclic disulfide linker with respect to the rate and the DNA ISC data as shown in Table 2. We used the rate constants (k_{obs}) instead of the half-lives for activation rates in order to more easily discern the differences. In general, all mitomycins displayed high activation rates and DNA ISC formation in the presence of Et_3P through nucleophilic activation pathways. When we compared the two sets of data we found that the DNA ISC data reasonably correlated with the rate data. However, when we compared the two sets of data according to the corresponding compounds we found unexpected results for mitomycin **8**. For mitomycins **5**–**7**, as the ring size of the cyclic disulfide linker decreased from an eight-membered ring (mitomycin **5**) to a seven-membered ring (mitomycin **6**) and then to a six-membered ring (mitomycin **7**), the activation rates decreased (k_{obs}: 8.66 → 4.62 → 2.67 d^{-1}, respectively) and the levels of DNA ISC slightly decreased (DNA ISC: 94 → 86 → 83%, respectively),[14,16,17] which seemed to be consistent with the ring size of the linkers in dimers. Nevertheless, when we compared the activation rate data of **8** with **5**–**7**, the activation rate of **8** was suddenly increased (k_{obs}: 16 d^{-1}), which was almost six- and two-fold higher than those of **7** and **5**, respectively. The sudden increase in activation rates for **8** was surprising and we could not clearly understand it. We previously suggested that the lifetime of generated thiol (e.g., **5r**–**7r**) upon disulfide cleavage would be significant since the thiol group itself was known to initiate the activation.[14] The lifetime of thiol would be inversely proportional to the rate of disulfide formation (cyclization), thus, as mentioned above, the lifetimes of thiols would be **7r**<<**6r**⩽**8r**<<**5r**.[22,23] These results reasonably explained the trend for the activations of mitomycins **5**–**7**, but not for activation of mitomycin **8**. The lifetime of thiol in **8r** would be in between those of **6r** and **5r**. If this factor mainly governs the activations, mitomycin **8** should have shown faster activation than **6**, and slower activation than **5**. However, **8** did undergo much faster activation than all three mitomycins **5**–**7**, which seemed to be unexpected, but interesting. Furthermore, the levels of DNA ISC for **8** were also higher than those for **5**–**7**. Despite the trend for **5**–**7**, mitomycin **8** that has the shortest distance between the two C(1) sites provided the highest level of DNA ISC (97%). This fact might imply that shorter distance between the two reaction sites, in a limited range, could provide better condition for the formation of DNA ISC, which could constitute an important piece of information on DNA ISC. However, we believed that much is still unknown and many other factors might be involved in controlling the activation and DNA adduction, and so, the working mechanism needs to be better elucidated by further studies. Despite these complexities, mitomycin **8**, taken together, was found to be more efficient than **5**–**7** for both nucleophilic activation and DNA ISC formation.

3. Conclusions

We report the studies on the synthesis and modes of action for mitomycins **8** and **9**, and the comparison of **8** with previously reported **5**–**7**. Mitomycin **8** is a dimer tethered through a 1,2-dithiolane (a five-membered cyclic disulfide) linker, which may provide the facile nontraditional nucleophilic activation and the two C(1) activations by the dimeric structure, leading to efficient formation of DNA ISC.

First, we established an efficient seven-step synthetic sequence to prepare the key intermediates, a cyclic disulfide (**10**) and a diol intermediate (**13**). Then the new target mitomycin **8** and the diol mitomycin **9** were synthesized using the obtained intermediates. Next, we performed mechanistic studies by measuring the activation rates of **8** by appropriate nucleophiles compared with the reference **9**, and found that the target mitomycin **8** underwent facile activation by Et_3P (5–50 equiv) while **9** did not. This indicated that **8** underwent initial activation by the instantly generated thiol group upon disulfide cleavage and advanced to subsequent activation. As a result, we propose a nucleophilic activation pathway for **8**. Then, we evaluated the ability of mitomycin **8** to produce DNA ISC by proper nucleophiles, and found that **8** displayed high efficiency in forming DNA ISC (98%) compared to **9** (5%) and **2** (4%) in the presence of Et_3P. We therefore concluded that the target mitomycin **8** is more efficient for both nucleophilic activation and the corresponding DNA ISC formation than references **9** and **2**. This finding highlighted the key function of the disulfide group on mitomycin activation. More significantly, mitomycin **8** underwent much faster activation and provided slightly higher levels of DNA ISC than previously reported mitomycins **5**–**7**.

4. Experimental

4.1. General

Melting points were measured in open capillary tubes using Buchi B-545 melting point apparatus and are uncorrected. FT–IR spectra were obtained by a Perkin–Elmer Spectrum GX spectrometer. ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Bruker DRX 300 spectrometer. Mass spectra were obtained by CI, EI or FAB ionization method. UV–vis spectra were obtained by Shimatzu UV-1800 Spectrophotometer. The pH of aqueous solutions were measured using an IQ Scientific Instruments IQ-240 meter. The nonaqueous effective pH ('pH') of the buffered methanolic solutions was similarly measured. Thin layer chromatography was run on silica gel plates (20 × 20 cm; Aldrich No. Z12272-6). HPLC analyses were conducted using the following Waters Associate Units: 515 A pump, 515 B pump, dual λ absorbance 2487 detector, 717 plus autosampler, and Hypersil ODS column (4.6 × 300 mm). The product analyses were performed using linear gradient

condition: 90% A (aqueous 0.025 M triethylammonium acetate, pH 6.5), 10% B (acetonitrile) isocratic for 5 min, then from 90% A, 10% B to 45% A, 55% B in 30 min. The flow rate was 1 mL/min, and the eluent was monitored from 200 to 400 nm. The HPLC solvents were filtered (aqueous solution with Millipore HVLP, 0.45 mm; acetonitrile with Millipore HV, 0.45 mm) and degassed before utilization.

4.2. 1,5-Bis(phthalimido)-2,4-pentanediol (12)

To a DMF solution (1.2 mL) of phthalimide (2.1 g, 14 mmol) was added 1,4-pentadiene diepoxide (**11**, 0.70 g, 7.0 mmol) with vigorous stirring. The reaction mixture was heated (100 °C /1 h → 115 °C/1 h → 135 °C /1 h) and then cooled. The precipitate was filtered and washed with Et_2O (50 mL) to give the crude title compound (2.1 g, 76%, dr = 1.4:1, ^{13}C NMR analysis) as a white solid. Recrystallization of crude product in DMF–Et_2O provided the title compound of higher diastereomeric ratio (0.55 g, 21%, dr = 4:1, ^{13}C NMR analysis). Mp 229–231 °C. R_f 0.25 (2:1 EtOAc/hexanes). IR (KBr) 3437, 2940, 1724, 1391, 1187, 1052, 889, 802, 720, 640, 533 cm^{-1}. ^1H NMR (300 MHz; DMSO-d_6) δ: 1.33–1.45 (m, 2H, CH_2CH), 3.44–3.56 (m, 4H, CH_2N), 3.91–4.06 (m, 2H, C*H*OH), 4.86 (d, J = 5.7 Hz, 2H, CHO*H*), 7.79–7.88 (m, 8H, Pht). ^{13}C NMR (75 MHz; DMSO-d_6) δ: 35.8 (*C*H_2CH), 44.5 (CH_2N), 64.1 (CHOH), 122.9 (Pht, *C*HCHC), 131.7 (Pht, C), 134.2 (Pht, *C*HC), 168.0 (NCO), for the minor diastereomer δ: 122.8 (Pht, *C*HCHC), 131.8 (Pht, C), 134.1 (Pht, *C*HC), 168.1 (NCO). MS m/z 395 $[M+H]^+$.

4.3. 1,5-Diamino-2,4-pentanediol dihydrochloride (13)

A mixture of 1,5-bis(phthalimido)-2,4-pentanediol (**12**, 1.4 g, 3.6 mmol), EtOH (32 mL) and $NH_2NH_2{\cdot}H_2O$ (0.44 mL, 8.9 mmol) was heated under reflux (3.5 h). After cooling to room temperature, the solvent was removed in vacuo. H_2O (33 mL) and concentrated hydrochloric acid (17 mL) were added to the residue and the mixture was heated under reflux (1 h). After cooling to 0 °C, the precipitate was filtered off and the filtrate was concentrated in vacuo. The residue was dissolved in H_2O (50 mL) and the insoluble matter was removed by filtration. The clear filtrate was concentrated in vacuo to give the title compound (595 mg, 80%, dr = 5:1, ^{13}C NMR analysis) as a white solid. Mp 175–180 °C. R_f 0.10 (4:6 MeOH/$CHCl_3$). IR (KBr) 3233, 1709, 1504, 1465, 1396, 1280, 1117, 1044, 891, 835, 799, 724, 628, 532 cm^{-1}. ^1H NMR (300 MHz; CD_3OD) δ: 1.52–1.82 (m, 2H, CH_2CH), 2.70–2.98 (m, 2H, C*H*H'N), 3.00–3.20 (m, 2H, CH*H*'N), 3.96–4.20 (m, 2H, C*H*OH). ^{13}C NMR (75 MHz; CD_3OD) δ: 40.6 (*C*H_2CH), 46.4 (CH_2N), 65.5 (CHOH), for the minor diastereomer δ: 40.3 (*C*H_2CH), 45.8 (CH_2N), 66.0 (CHOH). MS m/z 135 $[M-2HCl+H]^+$. HRMS (+FAB) calcd for $C_5H_{15}N_2O_2$ $[M-2HCl+H]^+$: 135.1135; found 135.1134.

4.4. 1,5-Bis(*tert*-butyloxycarbonylamino)-2,4-pentanediol (14)

To a stirred solution of 1,5-diamino-2,4-pentanediol dihydrochloride (**13**, 468 mg, 2.3 mmol) and Et_3N (1.9 mL, 14 mmol) in H_2O–DMF (1:1, 40 mL) was added a solution of di-*tert*-butyl dicarbonate (Boc_2O) (1.2 g, 5.7 mmol) in DMF (4 mL). After stirring at room temperature (1 d), the solvent was removed in vacuo. The remaining residue was treated with H_2O (80 mL) and then the mixture was extracted with EtOAc (2 × 80 mL). The combined organic layers were successively washed with aqueous 0.1 N HCl (80 mL), saturated aqueous $NaHCO_3$ (80 mL) and H_2O (80 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:1 → 2:1 EtOAc/hexanes) afforded the title compound (535 mg, 70%, dr: an apparent single set of signals in ^{13}C NMR) as a white solid. Mp 93–94 °C. R_f 0.35 (2:1 EtOAc/hexanes). IR (KBr) 3748, 2977, 1693, 1524, 1455, 1366, 1251, 1170, 1042, 892, 781, 737, 672, 616 cm^{-1}. ^1H NMR (300 MHz; $CDCl_3$) δ: 1.37 (s, 18H, OC(CH_3)$_3$), 1.50–1.60 (m, 2H, CH_2CH), 3.00–3.13 (m, 2H, C*H*H'N), 3.15–3.28 (m, 2H, CH*H*'N), 3.65 (br s, 2H, CHO*H*), 3.91 (br s, 2H, C*H*OH), 4.99 (br s, 2H, NHCO). ^{13}C NMR (75 MHz; $CDCl_3$) δ: 28.6 (OC(*C*H_3)$_3$), 37.9 (*C*H_2CH), 47.1 (CH_2N), 69.5 (CHOH), 80.1 (O*C*(CH$_3$)$_3$), 157.4 (NHCO). MS m/z 335 $[M+H]^+$. HRMS (+FAB) calcd for $C_{15}H_{31}N_2O_6$ $[M+H]^+$: 335.2182; found 335.2182.

4.5. 1,5-Bis(*tert*-butyloxycarbonylamino)-2,4-pentanediol dimethanesulfonate (15a)

To a cooled (0 °C) solution of 1,5-bis(*tert*-butyloxycarbonylamino)-2,4-pentanediol (**14**, 262 mg, 0.78 mmol) in CH_2Cl_2 (4 mL) was added Et_3N (0.3 mL, 2.4 mmol) and methanesulfonylchloride (MsCl) (0.1 mL, 1.9 mmol). After stirring at the same temperature (5 h) the solvent was removed in vacuo. H_2O (50 mL) was added to the residue and then the mixture was extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (50 mL) and H_2O (50 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. Crystallization of crude product in EtOAc/hexanes mixture afforded the title compound (355 mg, 93%, dr: an apparent single set of signals in ^{13}C NMR) as a white solid. Mp 88–90 °C. R_f 0.6 (2:1 EtOAc/hexanes). IR (KBr) 3748, 2978, 1713, 1520, 1455, 1353, 1251, 1172, 909, 787, 526 cm^{-1}. ^1H NMR (300 MHz; $CDCl_3$) δ: 1.38 (s, 18H, OC(CH$_3$)$_3$), 1.87–1.97 (m, 2H, CH_2CH), 3.08 (s, 6H, OMs), 3.21–3.40 (m, 2H, C*H*H'N), 3.41–3.55 (m, 2H, CH*H*'N), 4.75–4.90 (m, 2H, C*H*OMs), 4.97 (br s, 2H, NHCO). ^{13}C NMR (75 MHz; $CDCl_3$) δ: 28.5 (OC(*C*H_3)$_3$), 34.6 (*C*H_2CH), 38.8 (OMs), 44.6 (CH_2N), 77.7(*C*HOMs), 80.3 (O*C*(CH$_3$)$_3$), 156.3 (NHCO). MS m/z 491 $[M+H]^+$; HRMS (+FAB) calcd for $C_{17}H_{35}N_2O_{10}S_2$ $[M+H]^+$: 491.1733; found 491.1730.

4.6. 1,5-Bis(*tert*-butyloxycarbonylamino)-2,4-bis(acetylthio)pentane (16a)

To a stirred solution of 1,5-bis(*tert*-butyloxycarbonylamino)-2,4-pentanediol dimethanesulfonate (**15a**, 100 mg, 0.20 mmol) in DMF (2 mL) was added KSAc (57 mg, 0.50 mmol). After warming to 60 °C, stirring was continued (8 h) and then the solvent was removed in vacuo. The residue was treated with H_2O (50 mL) and the resulting mixture was extracted with EtOAc (2 × 50 mL). The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:4 → 1:3 EtOAc/hexanes) afforded the title compound (29 mg, 32%) as a brown solid. Mp 75–76 °C. R_f 0.50 (1:2 EtOAc/hexanes). IR (KBr) 3748, 2927, 1698, 1511, 1455, 1366, 1251, 1168, 955, 872, 759, 632 cm^{-1}. ^1H NMR (300 MHz; $CDCl_3$) δ: 1.43 (s, 18H, OC(CH_3)$_3$), 1.77–1.90 (m, 2H, CH_2CH), 2.32 (s, 6H, SAc), 3.16–3.34 (m, 2H, C*H*H'N), 3.35–3.47 (m, 2H, CH*H*'N), 3.65–3.82 (m, 2H, C*H*SAc), 4.85 (br s, 2H, NHCO). ^{13}C NMR (75 MHz; $CDCl_3$) δ: 28.3 (OC(*C*H_3)$_3$), 30.7 (CO*C*H$_3$), 33.4 (*C*H_2CH), 42.9 (CH_2N), 44.7 (*C*HSAc), 79.6 (O*C*(CH$_3$)$_3$), 155.9 (NHCO), 195.2 (*C*OCH$_3$). MS m/z 451 $[M+H]^+$. HRMS (+FAB) calcd for $C_{19}H_{35}N_2O_6S_2$ $[M+H]^+$: 451.1936; found 451.1937.

4.7. 3,5-Bis(*tert*-butyloxycarbonylaminomethyl)-1,2-dithiolane (18)

To a stirred solution of 1,5-bis(*tert*-butyloxycarbonylamino)-2,4-bis(acetylthio)-pentane (**16a**, 20 mg, 0.040 mmol) in MeOH–H_2O (5:1, 0.4 mL, c = 100 mM) was added K_2CO_3 (36 mg, 0.25 mmol). After stirring at room temperature (1 h), Et_3N (13 μL, 0.10 mmol) was added. A saturated $CHCl_3$ solution of iodine (0.4 mL) was then added dropwise at room temperature until slight excess of iodine was evidenced by its color. The resulting solution (c = 50 mM) was stirred at room temperature (3 h) and then treated with saturated aqueous $Na_2S_2O_3$ (50 mL). The mixture

was extracted with EtOAc (2 × 40 mL), and the combined organic layers were washed with saturated aqueous $NaHCO_3$ (40 mL) and H_2O (40 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:3 EtOAc/hexanes) afforded the title compound (10 mg, 69%) as a yellow solid. Mp 128–129°C. R_f 0.53 (1:2 EtOAc/hexanes). IR (KBr) 3748, 2927, 1698, 1511, 1455, 1366, 1251, 1168, 955, 872, 759, 632 cm^{-1}. ^1H NMR (300 MHz; $CDCl_3$) δ: 1.38 (s, 18H, OC(CH_3)$_3$), 1.98–2.09 (m, 2H, CH_2CH), 3.03–3.18 (m, 2H, CHH'N), 3.29–3.42 (m, 2H, CHH'N), 3.69–3.83 (m, 2H, CHS), 4.90 (br s, 2H, NHCO). ^{13}C NMR (75 MHz; $CDCl_3$) δ: 28.3 (OC(CH_3)$_3$), 39.9 (CH_2CH), 43.2 (CH_2N), 56.1 (CHS), 79.7 (OC(CH_3)$_3$), 155.8 (NHCO). MS *m*/*z* 364 $[M]^+$. HRMS (+FAB) calcd for $C_{15}H_{28}N_2O_4S_2$ $[M]^+$: 364.1490; found 364.1491.

4.8. 3,5-Bis(aminomethyl)-1,2-dithiolane·2TFA (10)

3,5-Bis(tert-butyloxycarbonylaminomethyl)-1,2-dithiolane (18, 4.1 mg, 0.010 mmol) was dissolved in trifluoroacetic acid (TFA) (0.50 mL) and stirring was continued at room temperature (1 h). The reaction mixture was concentrated in vacuo to give the title compound (4.4 mg, ~100%) as a brown solid. Mp 154–156 °C. R_f 0.13 (4:6 MeOH/$CHCl_3$). IR (KBr) 3433, 1675, 1519, 1433, 1390, 1342, 1201, 837, 799, 722, 597, 519 cm^{-1}. ^1H NMR (300 MHz; CD_3OD) δ: 2.07–2.23 (m, 2H, CH_2CH), 2.97–3.13 (m, 4H, CH_2 N), 3.85–3.98 (m, 2H, CHS). ^{13}C NMR (75 MHz; CD_3OD) δ: 41.6 (CH_2CH), 42.5 (CH_2 N), 54.4 (CHS). MS *m*/*z* 165 [M-2TFA+H]+. HRMS (+FAB) calcd for $C_5H_{13}N_2S_2$ [M-2TFA+H]+: 165.0520, found 165.0520

4.9. 7-*N*,7′-*N*′-(1″,2″-Dithiolanyl-3″,5″-dimethylenyl)bismitomycin C (8)

To a solution of 3,5-bis(aminomethyl)-1,2-dithiolane·2TFA (**10**, 2.5 mg, 6.4 μmol) and Et_3N (4.9 μL, 35 μmol) in MeOH (0.4 mL) was added MMA (**1**, 4.1 mg, 12 μmol). The reaction solution was stirred at room temperature (2 d) and then the solvent was removed in vacuo. Purification of the reaction mixture by PTLC (2:8 MeOH/$CHCl_3$) afforded the title compound (3.3 mg, 66%, dr = 1.2:1, ^{13}C NMR analysis) as a dark blue solid. R_f 0.65 (3:7 MeOH/$CHCl_3$). HPLC t_R 27.2 min. UV–vis (MeOH) λ_{max}: 223, 373 nm. ^1H NMR (300 MHz; pyridine-d_5) δ: 2.11 (s, 6H, C(6)CH_3), 2.21–2.29 (m, 2H, C(4'')H_2), 2.75 (d, *J* = 3.6 Hz, 2H, C(2)H), 3.15 (d, *J* = 4.2 Hz, 2H, C(1)H), 3.25 (s, 6H, C(9a)OCH_3), 3.61 (d, *J* = 12.6 Hz, 2H, C(3)*H*H'), 3.76–3.95 (m, 4H, C(7)NHCH_2), 3.97–4.12 (m, 4H, C(9)H, C(3'')H), 4.54 (d, *J* = 12.6 Hz, 2H, C(3)H*H'*), 5.09–5.16 (m, 2H, C(10)*H*H'), 5.36–5.48 (m, 2H, C(10)H*H'*), 7.25–7.40 (m, 2H, C(7)NH), the signals for the N(1a)H and C(10)OC(O)NH_2 protons were not detected and are believed to overlap with the observed peaks. ^{13}C NMR (75 MHz; pyridine-d_5) δ: 10.0 (C(6)CH_3), 32.6 (C(2)), 36.7 (C(1)), 40.0 (C(4'')), 44.3 (C(9)), 47.5 (C(7)NHCH_2), 49.6 (C(9a)OCH_3), 50.9 (C(3)), 57.2 (C(3'')), 62.4 (C(10)), 104.7 (C(6)), 106.9 (C(9a)), 110.9 (C(8a)), 146.9 (C(7)), 155.7 (C(5a)), 158.1 (C(10a)), 176.9 (C(8)), 179.3(C(5)), for the minor diastereomer δ: 10.1 (C(6)CH_3), 44.4 (C(9)), 57.1 (C(3'')), 147.0 (C(7)). MS *m*/*z* 799 $[M+H]^+$. HRMS (+FAB) calcd for $C_{35}H_{43}N_8O_{10}S_2$ $[M+H]^+$: 799.2543, found 799.2544.

4.10. 7-*N*,7′-*N*′-(2″,4″-Dihydroxy-1″,5″-pentanediyl)bismitomycin C (9)

To a solution of 1,5-diamino-2,4-pentanediol dihydrochloride (**13**, 0.90 mg, 4.3 μmol) and Et_3N (3.6 μL, 25 μmol) in MeOH (0.3 mL) was added MMA (**1**, 3.0 mg, 8.6 μmol). The reaction solution was stirred at room temperature (2 d) and then the solvent was removed in vacuo. Purification of the reaction mixture by PTLC (3:7 MeOH/$CHCl_3$) afforded the title compound (2.2 mg, 67%, dr = 1.1:1, ^{13}C NMR analysis) as a dark blue solid. R_f 0.38 (3:7 MeOH/$CHCl_3$). HPLC t_R 21.1 min. UV–vis (MeOH) λ_{max}: 222, 370 nm. ^1H NMR (300 MHz; pyridine-d_5) δ: 1.48–1.82 (m, 2H, C(3'')H_2), 2.18 (br s, 6H, C(6)CH_3), 2.74 (d, *J* = 3.6 Hz, 2H, C(2)H), 3.13 (d, *J* = 4.5 Hz, 2H, C(1)H), 3.19 (br s, 6H, C(9a)OCH_3), 3.52–3.64 (m, 2H, C(3)*H*H'), 3.69–3.82 (m, 2H, C(1'')*H*H'), 3.83–3.96 (m, 6H, C(1'')H*H'*, C(2'')H, C(9)H), 4.54 (dd, *J* = 12.9, 3.6 Hz, 2H, C(3)H*H'*), 5.00–5.10 (m, 2H, C(10)*H*H'), 5.26–5.45 (m, 2H, C(10)H*H'*), 7.62–7.90 (m, 2H, C(7)NH), the signals for the N(1a) H, C(10)OC(O)NH_2 and C(2'')OH protons were not detected and are believed to overlap with the observed peaks. ^{13}C NMR (75 MHz; pyridine-d_5) δ: 11.8 (C(6)CH_3), 31.2 (C(3'')), 33.7 (C(2)), 38.3 (C(1)), 45.8 (C(9)), 51.2 (C(9a)OCH_3), 52.2 (C(3)), 53.3 (C(1'')), 64.0 (C(10)), 69.1 (C(2'')), 104.8 (C(6)), 108.5 (C(9a)), 112.0 (C(8a)), 149.3 (C(7)), 157.2 (C(5a)), 159.8 (C(10a)), 178.3 (C(8)), 181.0 (C(5)), for the minor diastereomer δ: 38.2 (C(1)). MS *m*/*z* 791 $[M+Na]^+$.

4.11. C(1) Methoxymitosenes (19)

Mitomycin **8** (2.0 mg, 2.5 μmol) was dissolved in MeOH–$CHCl_3$ (1:1, 2 mL) and then the 'pH' was adjusted to 2.5–3.0 with a methanolic 0.02 M HCl solution. The reaction solution was stirred at room temperature (2 d) along with continual adjustment of pH and then the solvent was removed under reduced pressure. Purification of the reaction mixture by PTLC (3:7 MeOH/$CHCl_3$) afforded the title compound (0.9 mg, 45%) as a red solid. R_f 0.24 (3: 7 MeOH/$CHCl_3$). HPLC t_R 30.1, 31.3, 32.7 min (~1:2:1). UV–vis (MeOH) λ_{max}: 253, 313 nm. ^1H NMR (300 MHz; pyridine-d_5) δ: 2.13 (br s, 6H, C(6)CH_3), 2.16–2.26 (m, 2H, C(4″)H_2), 3.52 (br s, 6H, C(1)OCH_3), 3.72–4.04 (m, 8H, C(3″)H, C(7)NHCH_2, C(2)H), 4.09–4.45 (m, 4H, C(3)H_2), 4.52–4.74 (m, 2H, C(1)H), 5.73 (1/2ABq, *J* = 10.3 Hz, 2H, C(10)*H*H'), 5.78 (1/2ABq, *J* = 10.3 Hz, 2H, C(10)H*H'*), 6.78–6.95 (m, 2H, C(7)NH), the signals for the C(2)NH_2 and C(10)OC(O)NH_2 protons were not detected and are believed to overlap with the observed peaks. MS *m*/*z* 821 $[M+Na]^+$. HRMS (+FAB) calcd for $C_{35}H_{42}N_8NaO_{10}S_2$ $[M+Na]^+$: 821.2363, found 821.2326.

4.12. General procedure for the mitomycin activation studies

To a buffered methanolic solution (0.1 M Tris·HC l 'pH' 7.4) (final volume 1.5 mL) maintained at 25 °C containing the mitomycins (45 μL of 1.0 mM methanolic solution, final concentration 0.03 mM) was added a methanolic solution (18–113 μL) of the nucleophile of choice (stock solution: 5–20 mM, final nucleophile concentration 0.06–1.5 mM). The reaction was monitored by UV–vis spectroscopy (200–500 nm), and generally followed for more than two half-lives. The 'pH' of the solution was measured at the conclusion of the reaction and found to be within ±0.1 pH units of the original solution. The reaction solutions were analyzed by HPLC and unreacted staring materials and products (e.g., **8**, **9**, **19**) were determined by coinjection of authentic samples in the HPLC and cospotting of authentic samples in the TLC. The λ_{max} of mitomycin (~373 nm) was plotted versus time and found to decrease in a first-order decay (exponential decay) process. Nonlinear regression analysis by SigmaPlot Program (SigmaPlot, 2001) was used to fit the observed exponential decay and yielded pseudo-first-order rate constants (k_{obs}) and half-lives ($t_{1/2}$). The reactions were done in duplicate and the results averaged.

4.13. General procedure for alkaline agarose gel electrophoresis[29,30]

The agarose gels were prepared by adding 1.20 g of agarose to 100 mL of an aqueous 100 mM NaCl and 2 mM EDTA solution

(pH 8.0). The suspension was heated in a microwave oven until all of the agarose was dissolved (1 min). The hot solution was poured and then allowed to cool and solidify at room temperature (1 h). The gel was soaked in an aqueous alkaline running buffer solution (50 mL) containing 40 mM NaOH and 1 mM EDTA (1 h) and then the comb was removed. The buffer solution was refreshed prior to electrophoresis.

To an aqueous solution of ~80 μL of H_2O (sterile) and 2.5 μL of 1 M Tris·HCl (pH 7.4) was added a solution of linearized pBR322 (5 μL, 5 μg) in 10 mM Tris solution containing 1 mM EDTA (pH 8.0). After deaeration with N_2 gas (15 min), the mitomycin (2–4 μL of 5 mM DMSO solution, final concentration 0.1–0.2 mM) and the nucleophile (2–10 μL of 5–25 mM DMSO solution, final concentration 0.5 mM) were added and the resulting solution (final volume 100 μL) was incubated at room temperature (2 h). The solution was washed with 1:1 PhOH/$CHCl_3$ (100 μL) and $CHCl_3$ (2 × 100 μL), and precipitated (12.1 μL of 3 M NaOAc and 250 μL of EtOH, −70 °C (10 min)). The mixture was centrifuged at 0 °C (15 min), and the EtOH was decanted off and evaporated in vacuo. The remaining DNA was dissolved in 25 μL of aqueous 10 mM Tris solution containing 1 mM EDTA (pH 8.0).

Agarose loading dye (5 μL) was added to the sample (5 μL) and the samples were loaded onto the wells. The gel was run at 75 mA/25 V (30 min) and then at 145 mA/38 V (3–4 h). The gel was then neutralized for 45 min in an aqueous 100 mM Tris pH 7.0 buffer solution containing 150 mM NaCl, which was refreshed every 15 min. The gel was stained with an aqueous 100 mM Tris pH 7.5 buffer solution (100 mL) containing ethidium bromide (20 μL of an aqueous ethidium bromide stock solution (10 mg/10 mL)) and 150 mM NaCl for 20 min. The background staining was then removed by soaking the gel in aqueous 50 mM NH_4OAc and 10 mM β-mercaptoethanol solution (3 h). The gel was analyzed with a Bio-Rad Smartspec™ 3000 and/or UV Trans Illuminator with a digital camera, and quantitative analyses of each band were also performed.

Acknowledgments

This work was supported by a National Research Foundation of Korea Grant funded by the Korean Government (2009–0066477).

References and notes

1. Hata, T.; Hoshi, T.; Kanamori, K.; Matsumae, A.; Sano, Y.; Shima, T.; Sugawara, R. *J. Antibiot.* **1956**, *9*, 141.
2. Mitomycin, C. In *Current Status and New Developments*; Carter, S. K., Crooke, S. T., Eds.; Academic Press: New York, 1979.
3. (a) Iyer, V. N.; Szybalski, W. *Science* **1964**, *145*, 45; (b) Szybalski, W.; Iyer, V. N. In *Antibiotics: Mechanism of Action*; Gottlieb, D., Shaw, P. D., Eds.; Springer: New York, 1967; Vol. 1, pp 211–245; (c) Moore, H. W.; Czerniak, R. *Med. Res. Rev.* **1981**, *1*, 249.
4. Hornemann, U.; Keller, P. J.; Kozlowski, J. F. *J. Am. Chem. Soc.* **1979**, *101*, 7121.
5. (a) Keyes, S. R.; Loomis, R.; DiGiovanna, M. P.; Pritsos, C. A.; Rockwee, S.; Sartorelli, A. C. *Cancer Commun.* **1991**, *3*, 351; (b) Ramos, L. A.; Lipman, R.; Tomasz, M.; Basu, A. K. *Proc. Am. Assoc. Cancer Res.* **1997**, *38*, 182; (c) Tomasz, M.; Palom, Y. *Pharmacol. Ther.* **1997**, *76*, 73.
6. Kono, M.; Saitoh, Y.; Kasai, M.; Sato, A.; Shirahata, K.; Morimoto, M.; Ashizawa, T. *Chem. Pharm. Bull.* **1989**, *37*, 1128.
7. Vyas, D. M.; Chiang, Y.; Benigni, D.; Rose, W. C.; Brander, W. T. In *Recent Advances in Chemotherapy. Anticancer Section*; Tshigami, J., Ed.; University of Tokyo Press: Tokyo, 1985; p 485.
8. Tsuruo, T.; Sudo, Y.; Asami, N.; Inaba, M.; Morimoto, M. *Cancer Chemother. Pharmacol.* **1990**, *27*, 89.
9. Morimoto, M.; Ashizawa, T.; Ohno, H.; Azuma, M.; Kobayashi, E.; Okabe, M.; Gomi, K.; Kono, M.; Saitoh, Y.; Arai, H.; Sato, A.; Kasai, M.; Tsuruo, T. *Cancer Res.* **1991**, *51*, 110.
10. Kobayashi, E.; Okabe, M.; Kono, M.; Arai, H.; Kasai, M.; Gomi, K.; Lee, J.-H.; Inaba, M.; Tsuruo, T. *Cancer Chemother. Pharmacol.* **1993**, *32*, 20.
11. He, Q.-Y.; Maruenda, H.; Tomasz, M. *J. Am. Chem. Soc.* **1994**, *116*, 9349.
12. Wang, S.; Kohn, H. *J. Med. Chem.* **1999**, *42*, 788.
13. (a) Masters, J. R. W.; Know, R. J.; Hartley, J. A.; Kelland, L. R.; Hendricks, H. R.; Conners, T. *Biochem. Pharmacol.* **1997**, *53*, 279; (b) McAdam, S. R.; Knox, R. J.; Hartley, J. A.; Masters, J. R. W. *Biochem. Pharmacol.* **1998**, *55*, 1777.
14. Park, H. J.; Kim, J. J.; Kim, H. R.; Lee, E. K.; Kim, E. S.; Jeong, C. S.; Moon, A.; Lee, S. H. *Bioorg. Med. Chem.* **2011**, *19*, 4004.
15. Kim, J. J.; Kim, H. R.; Arai, H.; Lee, S. H. *Arch. Pharm. Res.* **2012**, *35*, 1413.
16. Kim, J. J.; Kim, H. R.; Lee, S. H., *Arch. Pharm. Res.* **2012**, *35*, in press.
17. Lee, S. H.; Kohn, H. *Org. Biomol. Chem.* **2005**, *3*, 471.
18. Lee, S. H. *Synthesis and Mechanistic Studies of Novel Mitomycins*, Ph.D. Thesis, University of North Carolina, North Carolina, August 2003.
19. (a) Stevens, C. L.; Taylor, K. G.; Munk, M. E.; Marshall, W. S.; Noll, K.; Shah, G. D.; Shah, L. G.; Uzu, K. *J. Med. Chem.* **1965**, *8*, 1; (b) Tomasz, M.; Lipman, R. *J. Am. Chem. Soc.* **1979**, *101*, 6063; (c) McClelland, R. A.; Lam, K. *J. Am. Chem. Soc.* **1985**, *107*, 5182.
20. Lee, S. H.; Kohn, H. *J. Am. Chem. Soc.* **2004**, *126*, 4281.
21. Wang, S.; Kohn, H. *J. Org. Chem.* **1997**, *62*, 5404.
22. Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 6825.
23. Burns, J. A.; Whitesides, G. M. *J. Am. Chem. Soc.* **1990**, *112*, 6296.
24. Baylon, C.; Heck, M.-P.; Mioskowski, C. *J. Org. Chem.* **1999**, *64*, 3354.
25. Tomasz, M.; Lipman, R. *Biochemistry* **1981**, *20*, 5056.
26. Hong, Y. P.; Kohn, H. *J. Am. Chem. Soc.* **1991**, *113*, 4634.
27. Han, I.; Kohn, H. *J. Org. Chem.* **1991**, *56*, 4648.
28. Lee, S. H.; Kohn, H. *Heterocycles* **2003**, *60*, 47.
29. Cech, T. R. *Biochemistry* **1981**, *20*, 1431.
30. Tepe, J. J.; Williams, R. M. *J. Am. Chem. Soc.* **1999**, *121*, 2951.