Bioorganic & Medicinal Chemistry 19 (2011) 4004–4013

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Synthesis and mechanistic studies of a mitomycin dimer containing an eight-membered cyclic disulfide

Hyun Jung Park, Jae Jin Kim, Hyoung Rae Kim, Eun Kyung Lee, Eun Sook Kim, Choon Sik Jeong, Aree Moon, 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 12 April 2011
Revised 12 May 2011
Accepted 13 May 2011
Available online 19 May 2011

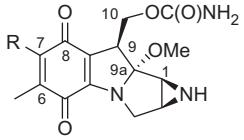
Keywords:
Mitomycin dimer
Cyclic disulfide
Nucleophilic activation
DNA interstrand cross-link

ABSTRACT

Dimeric DNA alkylating agents have drawn significant interest because these compounds are expected to provide at least two reactive sites and as a result, generate enhanced levels of DNA interstrand cross-link (DNA ISC) adducts compared to their monomeric agents. We report the synthesis and mechanistic studies of a novel mitomycin dimer, 7-*N*,7′-*N*′-(1″,2″-dithiocanyl-3″,8″-dimethylenyl)bismitomycin C (**8**) connected by an eight-membered cyclic disulfide. Mitomycins require prior activation (i.e., transformation to a good electrophile) for DNA adduction and therefore, **8** was aimed to undergo facile nucleophilic activation and produce enhanced levels of DNA ISC. At the core of this function lies a cyclic disulfide in **8**. It was expected that disulfide cleavage by an appropriate nucleophile would successively produce two thiols that may trigger activation of two mitomycin rings in a dimer through intramolecular cyclization to quinine rings. Compound **8** was synthesized from mitomycin A (**1**) and the key intermediate, cyclic disulfide (**11**), along with the reference diol mitomycin 7-*N*,7′-*N*′-(2″,7″-dihydroxy-1″,8″-octanediyl)bismitomycin C (**23**) which does not contain the disulfide unit. We found that **8** underwent significantly enhanced nucleophilic activation in the presence of Et_3P compared with **23**, and that the disulfide unit in **8** played a key role for the nucleophilic activation. Based on these findings, we proposed a mechanism for nucleophilic activation of **8**. We further demonstrated that **8** generated much higher levels of DNA ISC (94%) compared with **23** (4%) and **2** (3%) in the presence of Et_3P (and L-DTT) leading to the conclusion that **8** is more efficient for DNA ISC processes than **23** and **2** due to the role of disulfide unit.

1. Introduction

The mitomycins are a family of potent antitumor antibiotics that induce DNA alkylations. Among them, mitomycin A[1] (MMA, **1**) was isolated first and subsequently, mitomycin C (MMC, **2**) was reported as another example and has been used as a potent antitumor agent of clinical importance.[2] Mitomycins require prior activation leading to the generation of a reactive electrophile which is responsible for its biological activities. It was shown by mode of action studies that the activation is triggered upon quinone *reduction* and leads to the generation of electrophilic sites at C(1) and C(10) that induce DNA modification.[3] Under reductive conditions, the reactivity of the C(1) site was estimated to be 10–100 times higher than that of the C(10) site.[4] As a result, the reaction of mitomycin C and DNA generates both mono- and bis-alkylation adducts,[3] and among them DNA interstrand cross-link (DNA ISC) products have been considered to be the most effective.[5] For example, DNA ISC adducts by **2** have been found ~60 times more lethal than the corresponding monoadducts.[5] These DNA ISC adducts by **2** are believed to retard DNA replication and subsequent cell proliferation.



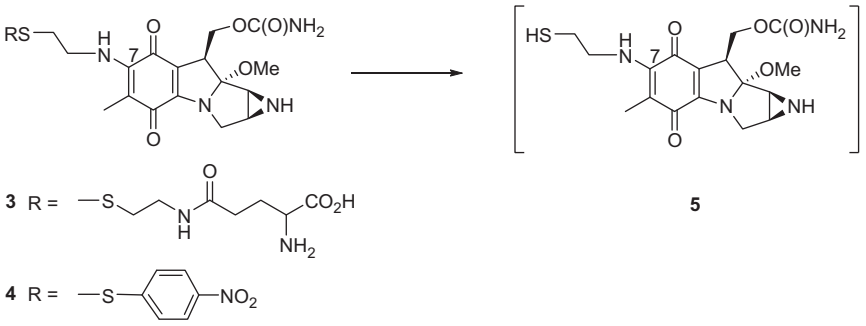
1 R = OCH_3
2 R = NH_2

However, MMC causes severe side effects and drug resistance following long-term administration.[2] Extensive studies to improve the pharmacological properties have led to the discovery of disulfide mitomycins that contain a disulfide bond, represented by **3** (KW-2149)[6] and **4** (BMS-181174).[7] Both **3** and **4** contain an aminoethylene disulfide unit at C(7) instead of an amino unit in **2** and displayed excellent pharmacological properties including activities both in **2**-resistant tumor cell lines and in non-hypoxic cells.[8–10] Interestingly, it was found that the improved pharmacological properties were attributed partly to the disulfide unit.[6,7,10]

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

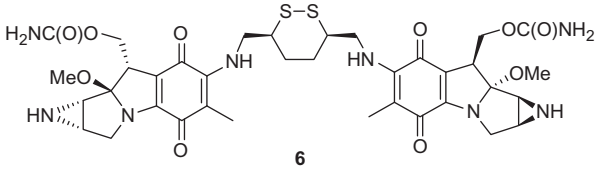
0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.05.020

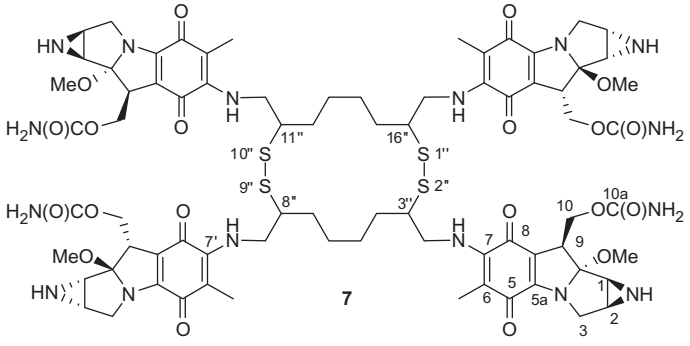
According to these findings, it was suggested that **3** and **4** might undergo activation by a different activation mechanism than **2** and further studies showed that **3** and **4** can undergo activation under nucleophilic conditions as nonreductive conditions. Mechanistic studies further implied that disulfide cleavage of **3** and **4** by thiol (e.g., glutathione (GSH) or L-dithiothreitol (L-DTT)) generated a key intermediate, C(7)-aminoethylene thiol **5** that could activate the mitomycin ring.[11–14] Additional studies by the Tomasz and Kohn groups reported that the thiol in **5** generated in situ undergoes intramolecular cyclization by attacking the quinone ring, followed by mitomycin activation and DNA adduction.[11,13–15]



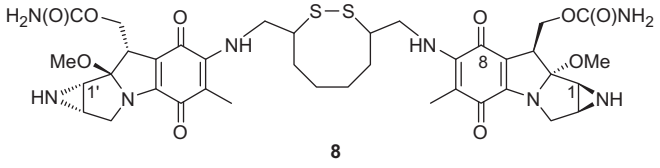
Another interest in these studies lies in dimerization of monofunctional or even bifunctional DNA damaging agents[16,17] due to the expected advantages in generating DNA ISC. Since the number of reactive sites is doubled by dimerization, dimeric alkylating agents are expected to induce DNA ISC more efficiently than monomeric agents. Nevertheless, few dimeric mitomycins have been synthesized taking advantages of the two C(1) sites with enhanced reactivity and the corresponding high probability for DNA ISC formation. As supporting examples, the dimeric mitomycins that are connected by carbon, ether or amine linkage at C(7) were reported.[18,19] Although they do not contain a disulfide unit, it was found that under proper activation conditions these compounds afforded enhanced levels of DNA ISC adducts over **2**, presumably by bis-alkylation at the two distal C(1) sites. These observations seem to support the effect of dimerization of mitomycins with an appropriate linker.

Based on the results above, we previously began a program studying cyclic disulfide mitomycin dimers, in which the two effects of disulfide unit and the dimerization are combined to enhance the abilities to generate DNA ISC. In this program the first example that we reported was the mitomycin dimer **6** that contained a six-membered cyclic disulfide unit.[17] Subsequently, we reported a second example, mitomycin tetramer **7** that has a 16-membered cyclic bis-disulfide unit.[20,21] Significantly, we observed that **6** and **7** underwent faster activation and generated higher levels of DNA ISC adducts under nucleophilic conditions than did **2**. Compound **6** also displayed higher efficiency for DNA ISC than **7** despite the fact that **6** underwent activation that was about three times slower than **7**.[17,21,22] This discrepancy in the activation studies and DNA ISC formation was not clearly understood and further studies are required for clarification. In general, it was suggested that the high activation rate and the improved efficiency of DNA ISC for **6** and **7** under nucleophilic conditions were attributed partly to the cyclic disulfide unit.





These significant findings on **6** and **7** led us to design, synthesize and evaluate the third example in this program, the cyclic disulfide mitomycin dimer **8** that contains an eight-membered cyclic disulfide linker. Compound **8** was aimed to undergo facile activation at two distal C(1) sites leading to the generation of DNA-ISC adducts by nucleophilic disulfide cleavage processes.

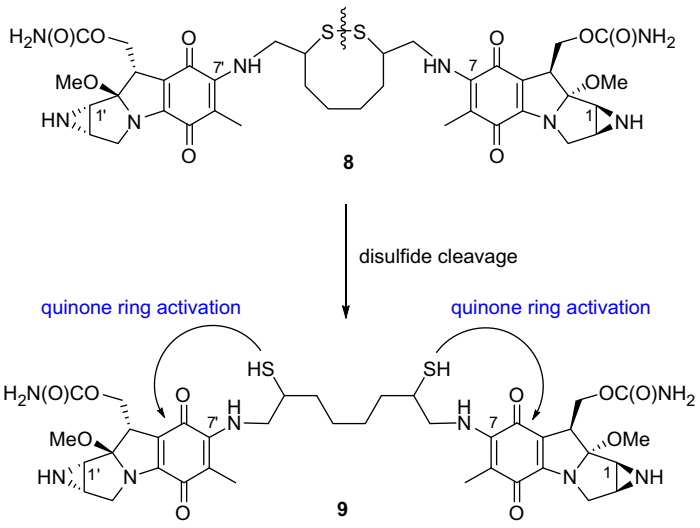


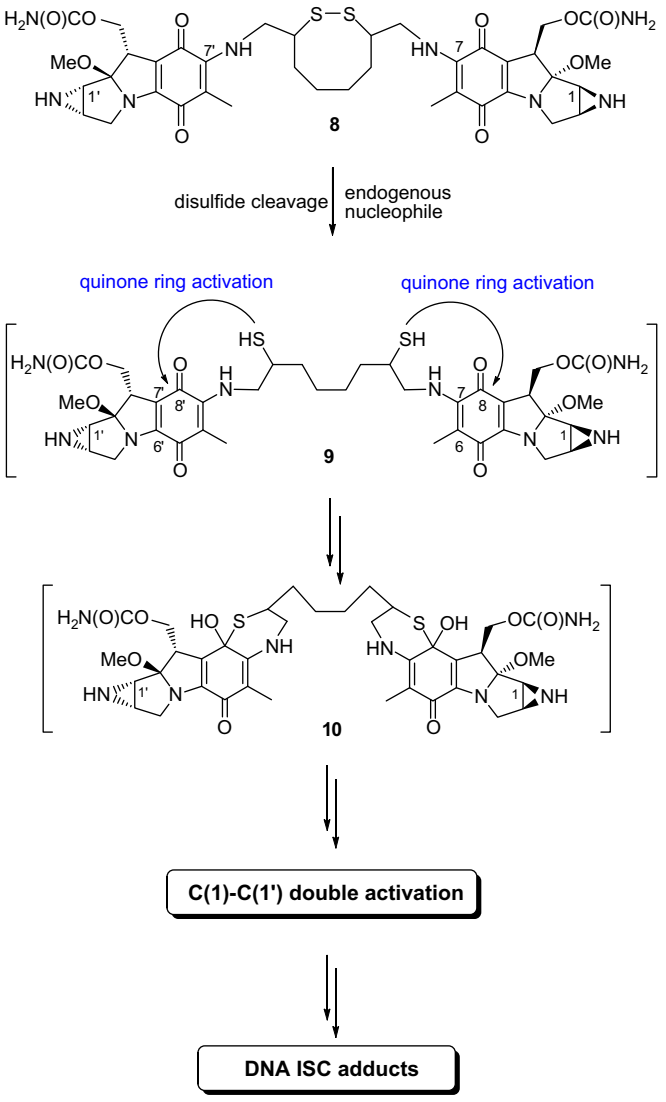
2. Results and discussion

2.1. Design of mitomycin dimer 8

As discussed above, the main focus lies in both nonreductive activation and double C(1) site activation in a dimeric structure. Compound **8** was strategically designed to meet these requirements and expected to undergo efficient nucleophilic activation at two C(1) sites. Compound **8** differs from **6** in the ring size of the cyclic disulfide linker and also differs from **7** in the ring size and the number of disulfide units of the linker and in the number of mitomycin rings per molecule. In addition, **8** still retained the key structural components found in **2**, and therefore, we envisioned that **8** would also be activated under reductive[3] and acidic conditions.[23] Compound **8** was designed to contain several key structural moieties. First, the disulfide unit in **8** was intentionally placed three atoms away from the C(7) position in mitomycin, which ensures the best condition for the generated thiol to undergo facile intramolecular cyclization to the quinine ring and subsequent mitomycin ring activation.[14,17,24] Second, **8** is a dimer that provides two reactive C(1) sites, which is aimed to enhance the formation of DNA ISC. Third, the linker is composed of an eight-membered cyclic disulfide, which may provide two thiols in a molecule (e.g., **9**) upon disulfide cleavage. Two thiols are preferred to activate two mitomycin rings in a dimer. Two thiols would be provided upon disulfide cleavage only when it is a cyclic disulfide, which is crucial in our design. Fourth, two mitomycin units are still tethered by an 8-carbon flexible linker even after disulfide cleavage. We measured the maximum distance between the two C(1) sites in **9** to be ~28 Å depending on their conformation (Sybyl 6.0, HyperChem 7.1) and therefore **9** could target a range of nucleophilic sites within the dsDNA. This distance was found longer than that predicted for dimer **6** (or the 1,4-dithiol structure generated by disulfide cleavage in **6**) (~25 Å),[17] which differentiated **9** (or **8**) from **6**.

Consequently, the objectives of this study were design and synthesis, documentation of activation mechanisms and evaluation of the efficiency to generate DNA ISC for target mitomycin **8**. Since we were mainly interested in the mode of action of mitomycins, the cytotoxicity data of **8** were not included in our assessment of these mechanistically-designed mitomycins. We presumed that these mitomycins would display at least basic levels of cytotoxicity since these compounds already contain mitomycin units.



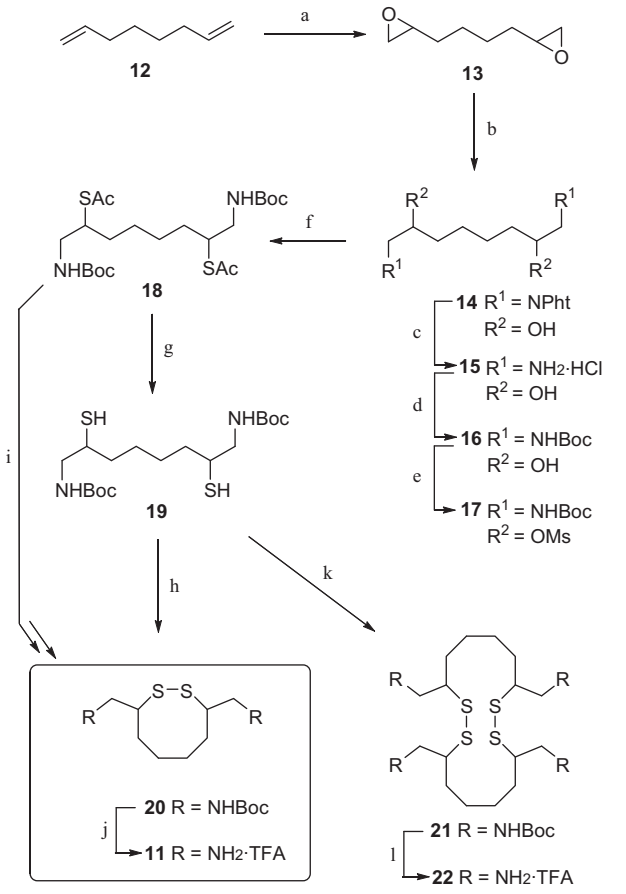


Scheme 1. Proposed activation pathway for **8**.

According to the information obtained from the previous studies, we may perceive the activation mechanism for **8**. The activation would be triggered through disulfide cleavage[11,13–15,25,26] by some endogenous nucleophiles such as an intracellular thiol or serum albumin to give dithiol **9**, followed by intramolecular cyclization of the thiol toward the quinine ring to provide **10** (Scheme 1). Although we considered the C(8) position as the intramolecular cyclization site, the C(7) and C(6)[11,14,24] positions may also be the working site to initiate the mitomycin activation process. Once compound **10** is formed, the stabilization of N(4) nonbonding electron pair through the N(4)–C(5a)–C(8a)–C(8)–O conjugated system is disturbed, which sequentially facilitates the rapid loss of MeOH from C(9) and C(9a) to form a double bond (mitosene structure), the double activation at C(1) and C(1′), and the formation of DNA adducts.[12,14,27] If the two nucleophiles in DNA come from complementary strands of the DNA, this results in the formation of DNA ISC adducts.

2.2. Synthesis

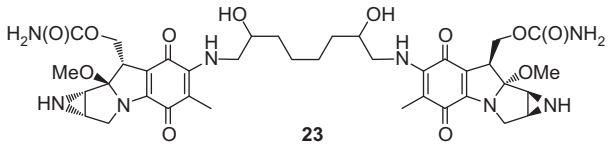
In order to prepare mitomycin **8**, cyclic disulfide **11** as a key intermediate was synthesized as shown in Scheme 2. According to our previous procedures[20] we prepared acetylthio derivative



Scheme 2. Synthesis of cyclic disulfide **11**. Reagents and conditions: (a) *m*-CPBA, CH_2Cl_2, 0 °C→room temperature, 1 d, 94%; (b) PhtH, DMF, 100 °C/1 h→115 °C/1 h→135 °C/1 h, 52%; (c) $NH_2{\cdot}NH_2{\cdot}H_2O$, EtOH, reflux, 3.5 h; then HCl, reflux, 1 h, 91%; (d) Boc_2O, DMF–H_2O (1:1), room temperature, 4 h, 65%; (e) MsCl, Et_3N, CH_2Cl_2, 0 °C, 1 h, 89%; (f) KSAc, DMF, 60 °C, 6 h, 79%; (g) K_2CO_3, MeOH–H_2O (5:1), room temperature, 1 h, 33%; (h) I_2, Et_3N, $CHCl_3$, room temperature, 3 h, 66%; (i) K_2CO_3, MeOH–H_2O (5:1), room temperature, 1 h; then I_2, Et_3N, MeOH–H_2O–$CHCl_3$ (5:1:5), room temperature, 3 h, 50%; (j) TFA, room temperature, 1 h, 100%; (k) O_2, KOH, room temperature, 3 d, 60%; (l) TFA, room temperature, 1 h, 100%.

18, in which we modified a few steps to obtain better results. At first, 1,7-octadiene (**12**) was treated with *m*-chloroperbenzoic acid (*m*-CPBA) to give diepoxide **13**[20,28] (diastereomeric ratio (dr) = 1:1, ^{13}C NMR analysis) in 94% yield. Next, **13** was reacted with phthalimide (PhtH) to afford the diphthalimide derivative **14**[20] in 30% yield. Since the yield of this step was too low, we attempted to modify the reaction and work-up procedures, and as a result, obtained **14** in higher yield (52%). Deprotection of the phthalimide group in **14** using hydrazine hydrate gave the amine salt **15**[20] (dr = 3.2:1, ^{13}C NMR analysis) in 91% yield, and subsequent Boc-protection provided **16**[20] (dr = 3.5:1, ^{13}C NMR analysis) in 65% yield. Then, we applied MsCl/pyridine condition to convert **16** to **17**,[20] leading to unsuccessful results due to practical problems in the work-up and isolation procedures. Therefore, we employed $MsCl/Et_3N/CH_2Cl_2$ condition and obtained the dimesyl derivative **17** in 89% yield. We then substituted the mesylate units with acetylthio units by treating potassium thioacetate (KSAc) in DMF leading to the generation of **18**[20] in 79% yield. Compounds **13**–**18** contain two stereocenters with a symmetrical structure and as a result, exist as three stereoisomers (*meso*-(*R*,*S*) and enantiomeric pair, *threo*-(*R*,*R*) and *threo*-(*S*,*S*)). Amine salt **15** and Boc-protected compound **16** were identified as mixtures of diastereomers by ^{13}C NMR analysis (two closely-spaced signals for C(1) in **15** (CD_3OD) and for C(4) in **16** ($CDCl_3$)). However, we could not determine the ratio of diastereomers for **14**, **17** and **18** because these compounds displayed only a single set of ^{13}C NMR resonances. Then, hydrolysis of **18** using potassium carbonate (K_2CO_3) was achieved to generate dithiol derivative **19** (33% yield) that was found relatively unstable. Again, **19** was obtained as a mixture of diastereomers (dr = 3.5:1, ^{13}C NMR analysis). Subsequent intramolecular cyclization (oxidation) of the dithiol units in **19** was found problematic due to easy oxidations and unwanted polymerizations. We already recognized and confirmed that oxidation of **19** using O_2/KOH in regular concentrations gave the dimeric compound **21** (16-membered cyclic bis-disulfide)[20] not the desired compound **20** (eight-membered cyclic disulfide). Even at very low concentrations (10–1 mM) the major product was identified as compound **21**. So, we explored other oxidation reactions and finally established an appropriate condition using I_2/Et_3N. Employing this method we obtained the desired compound **20** as a major product at around 10 mM concentrations (66% yield). In addition, two steps, namely hydrolysis and cyclization, were conducted in situ without isolation of dithiol **19** to afford **20** in higher yield (50%). Compound **20** was identified by NMR (^1H and ^{13}C), Mass, and IR, and more importantly, **20** was clearly differentiated from **21** by its R_f value on TLC. The R_f value (0.56) for **20** was higher than that of **21** (0.45) in EtOAc/hexanes = 1:2 mixture. Then **20** was treated with trifluoroacetic acid (TFA) to give **11** in quantitative yield. Unfortunately, we could not determine the ratio of diastereomers of **20** and **11** by ^1H and ^{13}C NMR analysis. Consequently, the key intermediate **11** was prepared from **12** through a nine-step synthetic sequence in 10% overall yield.

Then, we treated the key intermediate **11** obtained above with mitomycin A (**1**, Kyowa Hakko Kirin Co.) in MeOH to afford the target mitomycin dimer **8** in 90% yield, and ^{13}C NMR analysis indicated that **8** existed as a 1.7:1 mixture of diastereomers. The reference diol mitomycin **23**[20] was also prepared, from 1,8-diamino-2,7-octanediol dihydrochloride (**15**, dr = 3.2:1) and MMA in 66% yield. Again, ^{13}C NMR analysis showed that **23** existed as a 2.2:1 mixture of diastereomers. Mitomycin **23** retained the same carbon skeleton found in **8** (and **9**), except the two thiol units in **9** were replaced by two hydroxy groups.

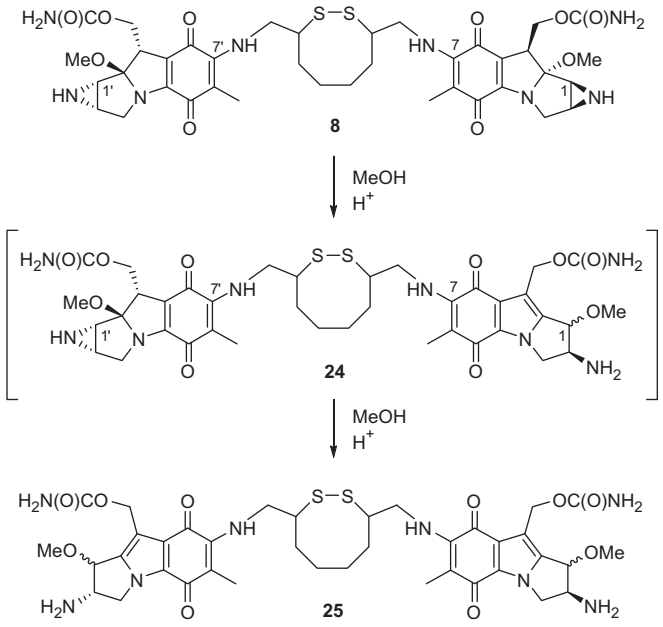


23
(2.2:1 diastereomeric mixture)

2.3. Methanolysis of 8

Methanolysis reaction of **8** in the acidic media (MeOH–$CHCl_3$ (1:1) solution at effective 'pH' 3) was performed (2 d) for acid-mediated activation leading to the generation of C(1) methoxymitosene **25** (di-activated product), probably through the intermediate, mono-activated product **24**. Although the intermediate **24** was not fully identified, it was clearly detected on TLC as an intermediate and clarified by HPLC profile and UV–vis absorption pattern.[17] Purification using preparative thin layer chromatography (PTLC) afforded **25** in 52% yield. We characterized **25** using HPLC, UV–vis, ^1H NMR and mass spectroscopy. The HPLC chromatogram showed apparently four peaks (t_R = 31.8, 32.4, 32.6, 33.3 min) of near equal amount that might designate the corresponding diastereomers. Compound **25** has two unidentified stereocenters at C(1) and C(1′) that can lead to four possible diastereomers not considering the stereochemistry in the cyclic disulfide linker. The

UV–vis spectra showed an appropriate absorption pattern consistent with mitosene production. In particular, we observed an absorption maximum at ~313 nm for the mitosene unit but no peak at ~373 nm associated with the starting material, mitomycin **8.** In the ^{1}H NMR spectra for **25** we found the expected resonance (δ 3.52) for the C(1) methoxy units and the downfield resonance (δ 5.64–5.80) for the C(10) methylene protons. These signals are characteristics for the formation of C(1) methoxy-mitosenes.[29,30] Compound **25** served as an authentic sample for our activation studies.



2.4. Activation studies for mitomycin dimers 8 and 23

We conducted the activation studies by measuring the rate of methanolysis of **8** and **23** in the absence and presence of nucleophiles to see if **8** is efficiently activated under nucleophilic conditions and to determine the effect of the disulfide group in **8**. Therefore, the diol mitomycin **23** was used as a reference. For this study we chose Et_3P, L-DTT and GSH as appropriate nucleophiles. The activations were conducted in buffered methanolic solutions (0.1 M Tris·HCl, effective 'pH' 7.4) at 25 °C and monitored by UV–vis spectroscopy (200–600 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).[23] At the conclusion of the reactions, the mixtures were analyzed using HPLC and TLC. Authentic samples of **8**, **23** and the mitosene product **25** were co-injected with the HPLC samples and co-spotted with the TLC samples. In case of no appreciable changes in starting mitomycins, reactions were monitored for more than 3–6 d and the unreacted mitomycins were identified using HPLC and TLC. The reactions 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 were averaged.

The results for **8** and **23** activations were shown in Table 1 and the data obtained for **23** were consistent with our previous results[21] despite the difference in the ratio of diastereomers. At first, we measured the methanolysis rates of **8** and **23** in the absence of nucleophile and as expected, found no appreciable decrease (less than 10% of the original amount) of starting mitomycins after at least 5 d. Next, we investigated the effect of L-DTT on the activation of **8** and **23**. When 10 and 20 equiv of L-DTT were used, we still observed no appreciable decrease (less than 10% of the original amount) of starting mitomycins after 3 d. Similarly, when 20 equiv of GSH was employed for **8** and **23**, no significant decrease of starting mitomycins after 3 d was observed. Based on these results we concluded that both L-DTT and GSH did not significantly contribute to the activation of disulfide mitomycin dimer **8** and reference **23**.

Then, we checked the effect of Et_3P as a nucleophile for the activation of **8** compared with **23**. When 2 equiv of Et_3P was used for **8**, no detectable decrease was observed, and when 5–50 equiv were employed, the rates of activation were remarkably increased as shown in Table 1 ($t_{1/2}$ = 0.23 d for 5 equiv, and 0.020 d for 50 equiv). Interestingly, the activation rate is generally proportional to the amount of Et_3P used. However, we observed no appreciable decrease in diol mitomycin **23** after 5 d in the presence of Et_3P. As a result, we attribute the remarkable rate increase for only **8** but not for **23** in the presence of Et_3P possibly to the function of disulfide unit in **8**. More concretely, Et_3P is believed to attack the disulfide unit in **8** leading to the generation of thiol which then attacks the quinone ring and triggers the activation of the mitomycin ring. Significantly, when we followed the reactions at ~313 nm we observed a gradual increase in the ~313 nm signal with time, which is diagnostic for the formation of mitosene products.[23] The HPLC chromatograms for the Et_3P activation experiments showed

Table 1
Activation rates for **8** and **23** at effective 'pH' 7.4[a]

Reagents		**8**		**23**[b]	
Nu	Equiv	k_{obs} (d^{-1})	$t_{1/2}$ (d)	k_{obs} (d^{-1})	$t_{1/2}$ (d)
No Nu		[c]	[c]	[c]	[c]
L-DTT	10	[d]	[d]	[c]	[c]
	20	[d]	[d]	[c]	[c]
GSH	20	[d]	[d]	[d]	[d]
Et_3P	2	[c]	[c]	—	—
	5	3.01	0.23	—	—
	10	8.66	0.080	[d]	[d]
	20	13.9	0.050	[d]	[d]
	50	34.7	0.020	[d]	[d]

[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] The data for **23** were similar to those found in Ref. 21.
[c] No appreciable change in at least 5 d (less than 10% of the original amount).
[d] No appreciable change in 3 d (less than 10% of the original amount).

multiple peaks between ~31 and ~34 min, which displayed absorption maxima at ~313 nm. Intensive efforts to identify the Et_3P reaction products from **8** by using HPLC were inconclusive. Although coinjection of authentic sample **25** (four peaks, t_R = 31.8, 32.4, 32.6, 33.3 min) with these reaction mixtures did not apparently change the peak pattern, it was very difficult to clearly identify all the peaks observed in the reaction mixtures. We attributed the complex HPLC pattern to the many plausible diastereomeric mixtures of mitosene products. Interestingly, we compared the rate data of **8** with those previously reported for mitomycin dimer **6** and mitomycin tetramer **7**. We found that **8** was consumed about three times faster than **6** ($t_{1/2}$ values of **6** with Et_3P: 0.26 d (10 equiv); 0.13 d (20 equiv)).[17] The only difference between **8** and **6** lies in the ring size of the linker (an eight- vs six-membered ring) and we presumed that 1,4-dithiol generated from **6** could revert back to the disulfide more easily[17,24] than 1,6-dithiol generated from **8** and therefore, the activation rate of **6** might be retarded. We also found that **8** was consumed at a similar rate with **7** ($t_{1/2}$ values of **7** with Et_3P: 0.083 d (20 equiv); 0.042 d (40 equiv)).[21] Although the apparent difference lies in the ring size and the number of disulfide units, we also needed to compare the structures of compounds generated by disulfide cleavage. Double disulfide cleavage of **7** would give compound **9**, which could be also generated from **8** by disulfide cleavage. Thus, it is not surprising that two compounds **8** and **7** displayed a similar activation rate in spite of the difference in structure of the linker.

Based on the results obtained above, we propose a nucleophilic activation mechanism for **8** with Et_3P as shown in Scheme 3. Et_3P induces disulfide cleavage[31] by attacking one of two sulfur atoms leading to the generation of thiol **26**, which serves as a key probe in the activation pathway. The thiol in **26** is expected to attack the quinine ring by intramolecular cyclization to give the hemi-thioketal **27**, thereby disrupting the stabilization of a nonbonding electron pair at the N(4) through a N(4)–C(4a)–C(8a)–C(8)–O conjugated system. This disruption induces the N(4)-mediated loss of the methoxide unit at C(9a) leading to the generation of a double bond at C(9) and C(9a), which is a mitosene structure capable of undergoing an aziridine ring opening. Through these sequential transformations, the C(1) site becomes highly electrophilic and reacts with nuleophile (e.g., MeOH or DNA) to give **28**,[12,14,27] which represents C(1) activation and adduction. Then, a second round of activation would start by the following decomposition of thiophosphonium **28** with MeOH[31] to give another thiol **29**. The thiol in **29** would trigger similar sequential transformations (**29**→**30**→**31**) to afford **31**, which represents C(1′) activation and adduction. The hemi-thioketal **31** would revert back to dithiol **33** and finally to disulfide **34** upon equilibrium and air oxidation processes. As a result, the two distal C(1) and C(1′) sites have been activated and modified. This proposed pathway seems consistent with our previous studies which indicated that phosphines contain high reactivity with the disulfide bond in *cis*- and *trans*-4,5-dihydroxy-1,2-dithanes,[31] and that Et_3P activated **6**[17] and **7**.[21]

2.5. DNA bonding profiles for 8 and 23

Since **8** was aimed to activate under nonreductive, nucleophilic conditions, we attempted to determine the ability of **8** to generate DNA ISC under nucleophilic conditions at pH 7.4 and furthermore, see if **8** is more efficient than the reference **23**. For this purpose, we employed the method by Cech,[32] and Tepe and Williams.[33] We compared **8** with references **23** and **2** in generating DNA ISC with *Eco*RI-linearized pBR322 DNA under nucleophilic conditions and denaturing alkaline agarose gel electrophoresis. The size of the DNA product(s) was estimated using λ DNA digested with *Hin*dIII as a molecular weight marker. Notably, we used a twofold higher concentration of **2** (monomeric structure) than those of **8** and **23** (dimeric structures) to ensure the same concentration of mitomycin unit per experiment.

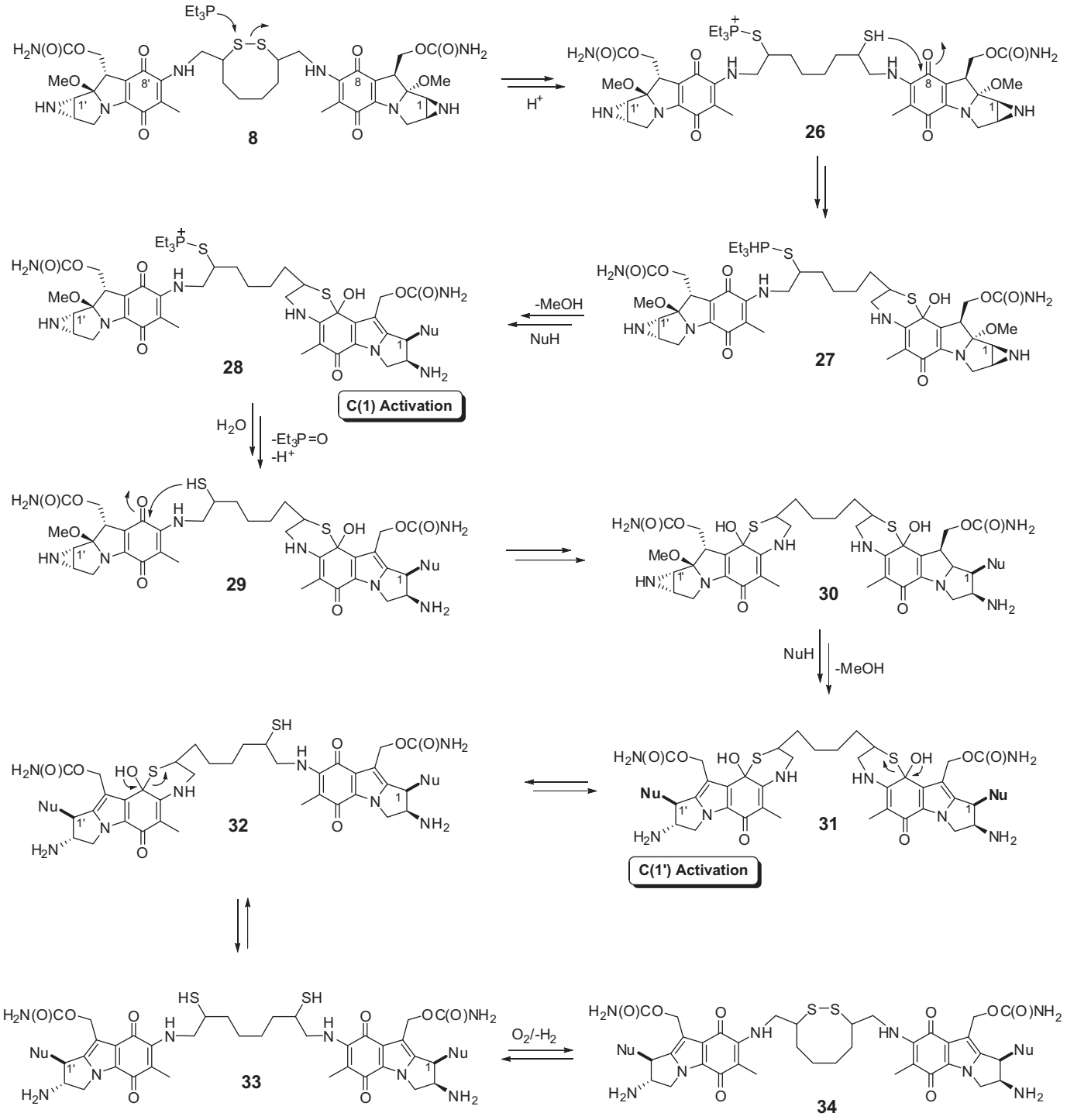
We first needed to determine the appropriate concentration of mitomycin so that the ability of each compound to make DNA ISC could be sufficiently differentiated. Based on the information of our previous studies on **6** and **7**, we examined the extent of DNA ISC at room temperature for 2 h in the presence of Et_3P (5 equiv) with varying concentration of **8** (0.025–0.5 mM) and found that substantial amounts of DNA ISC were observed at 0.05–0.25 mM concentrations, among which we chose 0.1 mM concentration for our study.

Then, we conducted the experiments with Et_3P (5 equiv) as a nucleophile for **8**, **23** and **2**, and the results were shown in Figure 1. We found that in Et_3P-mediated conditions, compound **8** efficiently generated DNA ISC (94%) while compound **23** and **2** generated only trace amounts of DNA ISC (4% and 3%, respectively). These results apparently differentiate **8** from **23** and **2**. Accordingly, we concluded that target mitomycin **8** is more efficient for DNA ISC formation than references **23** and **2** that do not contain a disulfide group, and that this differentiation originates from the presence of a disulfide unit in **8**. Significantly, these results seem to correlate well with the rate data of activation studies. In the presence of Et_3P (5–20 equiv) significant consumption and activation were observed for only **8** but not for **23** (Table 1). Interestingly, when we compared **8** with the previously reported **6** and **7** in the presence of Et_3P, we found that the levels of DNA ISC of **8** (94%) is similar to that of **6** (83%)[17] and much higher than that of **7** (35%, despite of the differences in reaction conditions (10 equiv of Et_3P and 6 min)).[21]

We also investigated the effects of L-DTT as a nucleophile on DNA ISC processes for **2**, **23** and **8**. We reacted the linearized DNA with the mitomycins using L-DTT (5 equiv) at room temperature for 2 h, and the results were shown in Figure 2. In these experiments, we observed that in the presence of L-DTT, compound **8** also provided substantial levels of DNA ISC (95%) whereas **23** and **2** produced small amounts of DNA ISC (6% and 2%, respectively). We again confirmed that **8** was more efficient in generating DNA ISC than **23** and **2** in the presence of L-DTT, which implied that L-DTT activated the mitomycin **8** by cleaving the disulfide bond. When we compared **8** with **6** using L-DTT we recognized that the levels of DNA ISC of **8** (95%) is slightly higher than that of **6** (77%).[17]

Interestingly, when we compared the DNA ISC results and kinetic data for L-DTT, we found some discrepancies. In the kinetic studies, 10 and 20 equiv of L-DTT scarcely induced mitomycin activation (less than 10% of the original amount in 3 d), but the levels of DNA ISC in the presence of L-DTT (5 equiv) were found to be substantially high (95%). Of course, the two sets of data (activation rate and DNA ISC data) were obtained through different experiments using different reaction conditions. Nevertheless, it is still surprising. Notably, in the DNA experiments even a single cross-link of DNA will produce a DNA ISC product visible in the gel while in the kinetic experiments the activations of whole mitomycin units in solution were measured. Therefore, in the DNA experiments, the activation of small amount of mitomycin (even less than 10% of the original amount) may lead to the generation of substantial levels of DNA ISC. In this regard, it is suggested that DNA experiments may be more sensitive than kinetic experiments.

We then examined the effects of GSH as a nucleophile on DNA ISC processes for **2**, **23**, and **8**. As shown in Figure 3, we observed that GSH (5 equiv) provided moderate levels of DNA ISC adducts for **8** (21%) while the extent of DNA ISC formation for **23** and **2** were 7% and 2%, respectively. This implied that the GSH may not be an appropriate nucleophile for nucleophilic activation of disulfide mitomycins. These results seem consistent with the rate data where GSH scarcely induced mitomycin activation (less than 10% of the original amount in 3 d).



where NuH: MeOH, DNA

Scheme 3. Proposed nucleophilic activation mechanism for **8** with Et_3P.

As shown above, when we compared the results of DNA ISC between **8** and **6** we observed that **8** generated slightly higher DNA ISC in the presence of Et_3P and L-DTT than **6**. Notably, the only difference is the ring size of the disulfide linker (eight- vs six-membered ring). We presumed that **8** (or 1,6-dithiol **9**) provided a slightly higher flexibility and a little longer distance between the two C(1) sites than **6** (or 1,4-dithiol generated from **6** upon disulfide cleavage), and that these features for **8** (or **9**) might improve the efficiency of DNA ISC. Further studies will be required in order to obtain advanced information on these observations.

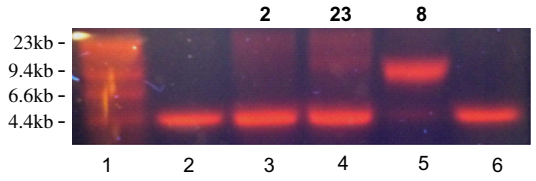


Figure 1. Denaturing 1.2% alkaline agarose gel for **2**, **23** and **8** using Et_3P (5 equiv). DNA cross-linking experiments using 0.1 mM concentrations except for **2** (0.2 mM) and *Eco*RI-linearized pBR322 plasmid DNA with Et_3P (5 equiv). All reactions were incubated at rt (2 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: control (only linearized pBR322). Lane 3: **2** + Et_3P (5 equiv). Lane 4: **23** + Et_3P (5 equiv). Lane 5: **8** + Et_3P (5 equiv). Lane 6: only Et_3P (5 equiv).

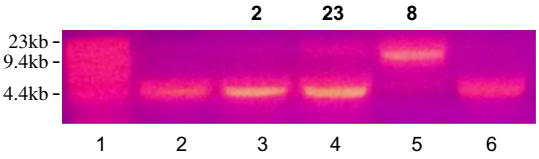


Figure 2. Denaturing 1.2% alkaline agarose gel for **2**, **23** and **8** using L-DTT (5 equiv). DNA cross-linking experiments using 0.1 mM concentrations except for **2** (0.2 mM) and *Eco*RI-linearized pBR322 plasmid DNA with L-DTT (5 equiv). All reactions were incubated at rt (2 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: control (only linearized pBR322). Lane 3: **2** + L-DTT (5 equiv). Lane 4: **23** + L-DTT (5 equiv). Lane 5: **8** + L-DTT (5 equiv). Lane 6: only L-DTT (5 equiv).

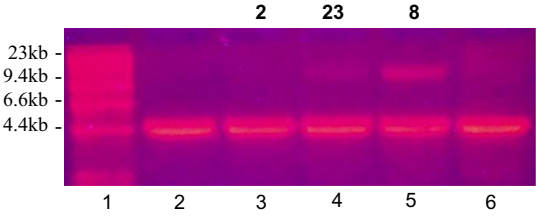


Figure 3. Denaturing 1.2% alkaline agarose gel for **2**, **23** and **8** using GSH (5 equiv). DNA cross-linking experiments using 0.1 mM concentrations except for **2** (0.2 mM) and *Eco*RI-linearized pBR322 plasmid DNA with GSH (5 equiv). All reactions were incubated at rt (2 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: control (only linearized pBR322). Lane 3: **2** + GSH (5 equiv). Lane 4: **23** + GSH (5 equiv). Lane 5: **8** + GSH (5 equiv). Lane 6: only GSH (5 equiv).

3. Conclusions

In this study, we report the synthesis and mechanistic studies of a novel cyclic disulfide mitomycin dimer **8**. Compound **8** was strategically designed and expected to undergo nucleophilic activation and generate DNA ISC efficiently. The linker in **8** is composed of an eight-membered cyclic disulfide, which is a crucial unit for mitomycin activation and generation of DNA ISC.

We achieved the synthesis of **8** using a key intermediate, cyclic disulfide (**11**) that was prepared through a nine-step synthetic sequence, along with the preparation of diol mitomycin **23**. We investigated the rate of activation of **8** compared with reference **23** in the presence of nucleophiles, and found that Et_3P as a nucleophile markedly enhanced the activation rate of **8** but not of **23**. This finding implicated that **8** underwent facile activation under nucleophilic conditions and that the disulfide unit in **8** played a key role in the activation. As expected, disulfide cleavage by phosphine provided thiol(s) that attacked the quinone ring through intramolecular cyclization leading to the activation of mitomycins. These findings led us to propose a mechanism of nucleophilic activation of **8**.

We then investigated the ability of **8** to generate DNA ISC in the presence of nucleophiles. Compound **8** efficiently generated DNA ISC (94%) while **23** and **2** generated only trace amounts of DNA ISC in the presence of Et_3P. Accordingly, we concluded that **8** is more efficient for DNA ISC processes than references **23** and **2** that do not contain a disulfide unit, and that this efficiency of **8** originates from the role of disulfide unit. These results also corresponded with the rate data of solvolysis studies.

4. Experimental

4.1. General

^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Bruker DRX 300 spectrometer. Mass spectra were obtained by EI, FAB or CI ionization methods. FT-IR spectra were run on a Perkin-Elmer Spectrum GX spectrometer. Melting points were determined in open capillary tubes using Buchi B-545 melting point apparatus and are uncorrected. UV–vis spectra were obtained by a Shimatzu UV-1800 Spectrophotometer. pH Measurements of aqueous solutions were determined on a IQ Scientific Instruments IQ-240 pH meter. The effective 'pH' of the buffered methanolic solutions was similarly determined. Thin layer chromatography was run on general purpose silica gel plates (20 × 20 cm; Aldrich No. Z12272-6). HPLC analyses were conducted with 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 conducted using linear gradient conditions: 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. 1,8-Bis(*tert*-butyloxycarbonylamino)-2,7-dimercaptooctane (19)

To a stirred solution of 1,8-bis(*tert*-butyloxycarbonylamino)-2,7-bis(acetylthio)octane (**18**,[20] 70 mg, 0.14 mmol) in MeOH–H_2O (5:1, 16 mL) was added K_2CO_3 (0.12 g, 0.85 mmol). After stirring at room temperature (1 h), H_2O (50 mL) was added to the residue. The mixture was extracted with EtOAc (2 × 80 mL). The combined organic layers were successively washed with H_2O (80 mL), dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:4 EtOAc/hexanes) afforded the title compound (23 mg, 39%, dr = 3.5:1, ^{13}C NMR analysis) as a white solid. Mp 101–104 °C. R_f 0.54 (1:2 EtOAc/hexanes). IR (KBr) 3350, 2930, 1694, 1514, 1454, 1391, 1365, 1250, 1169, 860, 780 cm^{-1}. ^1H NMR (300 MHz; $CDCl_3$) δ: 1.32 (br s, 2H, SH), 1.40-1.78 (m, 26H, CH_2CH_2CH, $OC(CH_3)_3$), 2.84–2.88 (m, 2H, CHSH), 3.00-3.09 (m, 2H, CHH′N), 3.38–3.46 (m, 2H, CHH′N), 5.01 (br s, 2H, NHCO). ^{13}C NMR (75 MHz; $CDCl_3$) δ: 26.7 (CH_2CH_2CH), 28.3 ($OC(CH_3)_3$), 35.7 (CH_2CH_2CH), 41.4 (CHSH), 47.9 (CH_2N), 79.5 ($OC(CH_3)_3$), 155.8 (NHCO), for the minor diastereomer δ: 26.5 (CH_2CH_2CH). MS *m*/*z* 409 $[M+H]^+$. HRMS (+FAB) calcd for $C_{18}H_{37}N_2O_4S_2$ $[M+H]^+$: 409.2194; found 409.2190.

4.3. 3,8-Bis(*tert*-butyloxycarbonylaminomethyl)-1,2-dithiocane (20)

Method A: To a stirred solution of 1,8-bis(*tert*-butyloxycarbonylamino)-2,7-dimercaptooctane (**19**, 50 mg, 0.12 mmol) in $CHCl_3$ (122 mL) was added Et_3N (36 μL, 0.26 mmol). Iodine was added

dropwise to the resulting solution at room temperature until a slight excess of iodine was evidenced by its color. The solvent was removed in vacuo and saturated aqueous $Na_2S_2O_3$ (50 mL) was added to the residue. The mixture was extracted with EtOAc (2 × 80 mL) and the combined organic layers were washed with 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 (1:4 EtOAc/hexanes) afforded the title compound (32 mg, 66% from **19**) as a white solid. *Method B:* To a stirred solution of 1,8-bis(*tert*-butyloxycarbonylamino)-2,7-bis(acetylthio)octane (**18**, 70 mg, 0.14 mmol) in MeOH–H_2O (5:1, 16 mL) was added K_2CO_3 (0.12 g, 0.85 mmol). After stirring at room temperature (1 h), Et_3N (41 μL, 0.30 mmol) was added. A saturated $CHCl_3$ solution of iodine was then added dropwise with stirring at room temperature until a slight excess of iodine was evidenced by its color. The solvent was removed in vacuo and saturated aqueous $Na_2S_2O_3$ (50 mL) was added to the residue. The mixture was extracted with EtOAc (2 × 80 mL) and the combined organic layers were washed with 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 (1:4 EtOAc/hexanes) afforded the title compound (29 mg, 50% from **18**) as a white solid. Mp 62–65 °C. R_f 0.56 (1:2 EtOAc/hexanes). IR (KBr) 3368, 2976, 2927, 1693, 1519, 1451, 1390, 1365, 1250, 1169 cm^{-1}. ^1H NMR (300 MHz; $CDCl_3$) δ: 1.44 (s, 18H, OC$(CH_3)_3$), 1.65–1.90 (m, 8H, CH_2CH_2CH), 2.78 (br s, 2H, CHS), 2.98–3.03 (m, 2H, CHH'N), 3.29–3.38 (m, 2H, CHH'N), 4.93 (br s, 2H, NHCO). ^{13}C NMR (75 MHz; $CDCl_3$) δ: 24.6 (*C*H_2CH$_2$CH), 28.3 (OC(*C*$H_3)_3$), 29.7 (CH$_2$*C*H$_2$CH), 45.5 (CH$_2$N), 51.7 (CHS), 79.5 (O*C*$(CH_3)_3$), 155.7 (NHCO). MS *m*/*z* 407 $[M+H]^+$. HRMS (+FAB) calcd for $C_{18}H_{35}N_2O_4S_2$ $[M+H]^+$: 407.2038; found 407.2038.

4.4. 3,8-Bis(aminomethyl)-1,2-dithiocane·2TFA (11)

3,8-Bis(*tert*-butyloxy-carbonylaminomethyl)-1,2-dithiocane (**20**, 4.4 mg, 0.011 mmol) was dissolved in trifluoroacetic acid (0.43 mL) and stirring was continued at room temperature (1 h). The reaction solution was concentrated in vacuo to afford the title compound (4.3 mg, 100%) as a brown solid. Mp 115–120 °C. IR (KBr) 2928, 1676, 1523, 1458, 1432, 1384, 1202, 1135, 838, 798, 723 cm^{-1}. ^1H NMR (300 MHz; D_2O) δ: 1.75–1.94 (m, 8H, CH_2CH_2CH), 2.97–3.18 (m, 6H, NCH$_2$CH). ^{13}C NMR (75 MHz; D_2O) δ: 23.6 (*C*H$_2$CH$_2$CH), 28.8 (CH$_2$*C*H$_2$CH), 43.6 (CH$_2$N), 48.7 (CHS)). MS *m*/*z* 207 $[M-2TFA+1]^+$.

4.5. 7-*N*,7′-*N*′-(1″,2″-Dithiocanyl-3″,8″-dimethylenyl)-bismitomycin C (8)

To an anhydrous methanolic solution (0.3 mL) of 3,8-bis(aminomethyl)-1,2-dithiocane·2TFA (**11**, 1.7 mg, 4.3 μmol) and triethylamine (3.6 μL, 26 μmol) was added mitomycin A (**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 (15% MeOH–$CHCl_3$) afforded the title compound (3.3 mg, 90%, dr = 1.7:1, ^{13}C NMR analysis) as a dark blue solid. HPLC t_R 31.6 min. R_f 0.55 (20% MeOH-$CHCl_3$). UV–vis (MeOH) λ_{max}: 226, 373 nm. ^1H NMR (300 MHz; pyridine-d_5) δ: 1.43–1.92 (m, 8H, C(4″)H_2, C(5″)H_2), 2.07 (s, 6H, C(6)CH_3), 2.70 (d, *J* = 3.3 Hz, 2H, C(2)H), 3.10 (d, *J* = 4.2 Hz, 2H, C(1)H), 3.16 (s, 6H, C(9a)OCH_3), 3.56 (d, *J* = 12.7 Hz, 2H, C(3)*H*H′), 3.60–3.71 (m, 4H, C(7)NH–CH_2), 3.98 (dd, *J* = 11.2, 4.1 Hz, 2H, C(9)H), 4.50 (d, *J* = 12.7 Hz, 2H, C(3)H*H*′), 5.04 (t, *J* = 10.7 Hz, 2H, C(10)*H*H′), 5.38 (dd, *J* = 10.4, 4.1 Hz, 2H, C(10)H*H*′), 7.14–7.28 (m, 2H, C(7)NH), for the minor diastereomer δ: 3.17 (s, 6 H, C(9a)OCH_3), the signals for the N(1a)H, C(3″)H, C(10)OC(O)NH_2 protons were not detected and believed to overlap with the observed peaks. ^{13}C NMR (75 MHz; pyridine-d_5) δ: 10.4 (C(6)CH_3), 23.3 (C(5″)), 30.3 (C(4″)), 33.0 (C(2)), 37.0 (C(1)), 44.6 (C(9)), 50.0 (C(9a)OCH_3), 51.0 (C(7)NH–CH_2), 52.4 (C(3)), 62.7 (C(10)), 104.7 (C(6)), 107.2 (C(9a)), 111.2 (C(8a)), 147.5 (C(7)), 156.1 (C(5a)), 158.2 (C(10a)), 177.2 (C(5)), 179.7 (C(8)), for the minor diastereomer δ: 10.3 (C(6)CH_3), 29.9 (C(4″)), 32.4 (C(2)), 104.8 (C(6)), 111.1 (C(8a)), 147.3 (C(7)), 177.1 (C(5)), 179.5 (C(8)), the signal for the (C(3″)) carbon was not detected and believed to overlap with the observed peaks. MS *m*/*z* 841 $[M+H]^+$. HRMS (+FAB) calcd for $C_{38}H_{49}N_8O_{10}S_2$ $[M+H]^+$: 841.3013; found 841.3009.

4.6. Methanolysis of 8 to give C(1) methoxymitosenes (25)

Mitomycin dimer **8** (2.7 mg, 3.2 μmol) was dissolved in MeOH–$CHCl_3$ (1:1, 2.3 mL) and then the 'pH' was adjusted to ~3.0 with a methanolic 0.02 M HCl solution. The reaction solution was stirred at room temperature (2 d) and then the solvent was removed under reduced pressure. Purification of the reaction mixture by PTLC (25% MeOH–$CHCl_3$) afforded the title compound (1.4 mg, 52%) as a red solid. HPLC t_R 31.8, 32.4, 32.6, 33.3 min (4 peaks, ~1:1:1:1). UV–vis (MeOH) λ_{max}: 213, 253, 313 nm. R_f 0.20 (20% MeOH–$CHCl_3$). ^1H NMR (300 MHz; pyridine-d_5) δ: 1.61–1.85 (m, 8H, C(4″)H_2, C(5″)H_2), 2.13 (br s, 6H, C(6)CH_3), 3.06 (br s, 2H, C(3″)H), 3.52 (br s, 6H, C(1)OCH_3), 3.67–4.13 (m, 6H, C(7)NH-CH_2, C(2)*H*), 4.18–4.31 (m, 2H, C(3)*H*H′), 4.36–4.72 (m, 4H, C(3)H*H*′, C(1)*H*), 5.64–5.80 (m, 4H, C(10)H_2), 6.75–6.82 (m, 2H, C(7)NH), the signals for the C(10)OC(O)NH_2, C(2)NH_2 protons were not detected and believed to overlap with the observed peaks. MS *m*/*z* 841 $[M+H]^+$. HRMS (+FAB) calcd for $C_{38}H_{49}N_8O_{10}S_2$ $[M+H]^+$: 841.3013; found 841.3004.

4.7. General procedure for the mitomycin activation studies

To a buffered methanolic solution (0.1 M Tris·HCl '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 (200–600 nm), and generally followed for greater than two half-lives. The 'pH' of the solution was determined 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 starting materials and products (e.g., **8**, **23**, **25**) 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. The nonlinear regression analysis to fit the observed exponential decay by SigmaPlot Program (SigmaPlot, 2001) yielded pseudo-first-order rate constants (k_{obs}) and half-lives ($t_{1/2}$). The reactions were done in duplicate and the results averaged.

4.8. General procedure for alkaline agarose gel electrophoresis[32,33]

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 was 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) and the amount of DNA in the resulting solution was quantitatively analyzed, if needed.

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 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 an 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.

Acknowledgements

This work was supported by National Research Foundation of Korea Grant funded by the Korean Government (2009–0066477). We thank Drs. Etsuo Ohshima and Hitoshi Arai (Kyowa Hakko Kirin Co., Ltd., Shizuoka, Japan) for generously supplying mitomycin A.

References

1. Hata, T.; Hoshi, T.; Kanamori, K.; Matsumae, A.; Sano, Y.; Shima, T.; Sugawara, R. *J. Antibiot.* **1956**, *9*, 141.
2. *Mitomycin C: 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-Verlag: 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; pp 485–486.
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. Kohn, H.; Wang, S. *Tetrahedron Lett.* **1996**, *37*, 2337.
13. Wang, S.; Kohn, H. *J. Med. Chem.* **1999**, *42*, 788.
14. Na, Y.; Wang, S.; Kohn, H. *J. Am. Chem. Soc.* **2002**, *124*, 4666.
15. (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.
16. Rajaski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723.
17. Lee, S. H.; Kohn, H. *Org. Biomol. Chem.* **2005**, *3*, 471.
18. Na, Y.; Li, V.-S.; Nakanishi, Y.; Bastow, K. F.; Kohn, H. *J. Med. Chem.* **2001**, *44*, 3453.
19. Paz, M. M.; Kumar, G. S.; Glover, M.; Waring, M. J.; Tomasz, M. *J. Med. Chem.* **2004**, *47*, 3308.
20. Lee, S. H.; Brodnick, R. L.; Glish, G. L.; Kohn, H. *Tetrahedron* **2005**, *61*, 1749.
21. Lee, S. H.; Kohn, H. *Chem. Pharm. Bull.* **2009**, *57*, 149.
22. Lee, S. H. *Synthesis and Mechanistic Studies of Novel Mitomycins*, Ph.D. Thesis, University of North Carolina, North Carolina, August 2003.
23. (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.
24. Lee, S. H.; Kohn, H. *J. Am. Chem. Soc.* **2004**, *126*, 4281.
25. Kobayashi, S.; Ushiki, J.; Takai, K.; Okumura, S.; Kono, M.; Kasai, M.; Gomi, K.; Morimoto, M.; Ueno, H.; Hirata, T. *Cancer Chemother. Pharmacol.* **1993**, *32*, 143.
26. Yasuzawa, T.; Tomer, K. B. *Bioconjugate Chem.* **1997**, *8*, 391.
27. Wang, S.; Kohn, H. *J. Org. Chem.* **1997**, *62*, 5404.
28. Lee, S. H.; Kohn, H. *J. Org. Chem.* **2002**, *67*, 1692.
29. Hong, Y. P.; Kohn, H. *J. Am. Chem. Soc.* **1991**, *113*, 4634.
30. Han, I.; Kohn, H. *J. Org. Chem.* **1991**, *56*, 4648.
31. Lee, S. H.; Kohn, H. *Heterocycles* **2003**, *60*, 47.
32. Cech, T. R. *Biochemistry* **1981**, *20*, 1431.
33. Tepe, J. J.; Williams, R. M. *J. Am. Chem. Soc.* **1999**, *121*, 2951.