Nucleophilic Activation of a Tetra-Substituted Mitomycin Cyclic Bis-Disulfide

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The multimerization of functional DNA alkylating agents has drawn significant, recent interest because these compounds are expected to generate enhanced levels of DNA cross-linked adducts, compared with their monomeric agents. Here we report the evaluation of 7-*N*,7′-*N*′-(1″,2″,9″,10″-tetrathia-cyclohexadecanyl-3″,8″,11″,16″-tetramethylenyl)tetrakismitomycin C (8), in which four mitomycin units are attached to the novel bis-disulfide linker, 3,8,11,16-tetrakis(aminomethyl)-1,2,9,10-tetrathia-cyclohexadecane. Compound 8 was designed to undergo preferential C(1) mitomycin activation under nucleophilic as well as under acidic and reductive conditions. We anticipated that treating 8 with nucleophiles would lead to bis-disulfide cleavage thus producing two mitomycin dimers (9) capable of generating DNA interstrand cross-links (ISC). The mitomycin units in 9 are tethered by a stable carbon backbone linkage. According to the procedure reported by Lee and coworkers (*Tetrahedron*, 61, 1749–1754 (2005)), we synthesized 8 and the reference mitomycin dimer, 7-*N*,7′-*N*′-(2″,7″-dihydroxy-1″,8″-octanediyl)bismitomycin C (15). Compound 8 was activated under acidic conditions thereby generating mitosene product 16, in which all four mitomycin units within the 16-membered ring were activated. Using the nucleophile Et_3P, we found that 8 underwent significantly enhanced mitosene production compared with its reference compound 15. We further demonstrated that under nucleophilic activation conditions 8 generated higher levels of DNA ISC than either 1 or 15. The cytotoxicities of 8 and 15 in a select tumor cell line were evaluated and compared with mitomycin C (1).

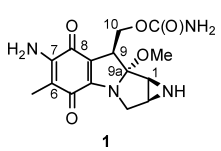
Key words mitomycin; disulfide; nucleophilic activation; DNA interstrand cross-link

Mitomycin C (MMC, **1**) is a potent antitumor antibiotic of clinical importance.[1)] Mechanistic studies have shown that drug activation is initiated upon quinone reduction and leads to the generation of electrophilic sites at C(1) and C(10) that permit DNA adduction.[2—4)] Under reductive conditions, the C(1) site has been estimated to be 10—100 times more reactive than C(10).[5)] Mitomycin C DNA adduction generates both mono- and bis-alkylation adducts,[2—4)] with the DNA interstrand cross-link (DNA ISC) products considered to be the most lethal.[6—8)] The mitomycin C ISC adducts are expected to inhibit DNA replication and subsequent cell proliferation.

Although **1** has found wide use in clinical chemotherapy, its long-term administration leads to unwanted side effects and drug resistance.[1)] Efforts to overcome these limitations led to studies focused on generating new mitomycins with improved pharmacological properties. Two mitomycin disulfides, **2** (KW-2149)[9)] and **3** (BMS-181174),[10)] emerged from these studies showing excellent pharmacological properties and were advanced to clinical trials. Both **2** and **3** contain C(7) aminoethylene disulfide units instead of the C(7) amino unit in **1**. Further studies showed that **2** and **3** were active in **1**-resistant tumor cell lines and in non-hypoxic cells[11,12)] and that the improved pharmacological properties were due, in part, to the disulfide unit.[9,10,13)] These findings suggested that **2** and **3** functioned, in part, by a different activation mechanism than **1**. Unlike **1**, mitomycins **2** and **3** can undergo activation under nucleophilic conditions. Mechanistic studies indicated that thiol-assisted (*e.g.*, glutathione (GSH)) disulfide cleavage of **2** and **3** provided C(7)-aminoethylene thiol **4** that was capable of activating the mitomycin.[14—17)] Support for this notion was provided by the Tomasz and Kohn groups who reported that thiol generation from either **2**, **3**, or structural analogs led to intramolecular cyclization of the quinone ring, thus permitting mitomycin activation and DNA adduction.[14,16—19)]

Recently, dimeric and multimeric agents that target DNA[20,21)] have drawn attention due to the presence of two or more reaction sites. These compounds are intercalating and alkylating agents. Special interest has been given to dimeric alkylating agents that induce DNA ISC, thereby disrupting DNA replication and cell division.

For **1**, DNA ISC adducts have been estimated to be *ca.* 60 times more lethal than the corresponding monoadducts.[6—8)] Surprisingly, few dimeric mitomycins have been designed to take advantage of the enhanced reactivity of the C(1) site and where DNA ISC adducts are produced by two successive C(1) alkylation processes. In 2001, Na and coworkers synthesized and evaluated the dimeric mitomycins **5**.[22)] They found that under reductive conditions these compounds provided enhanced levels of DNA ISC adducts over **1**, a finding



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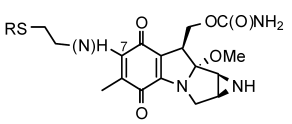

2 R = —$S(CH_2)_2N(H)C(O)(CH_2)_2C(H)(NH_2)CO_2H$

3 R = —S—NO_2

4 R = H

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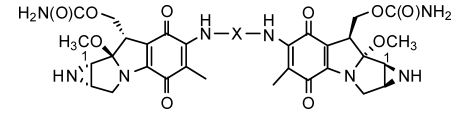
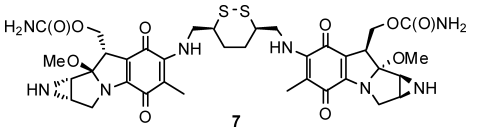


5 X=$(CH_2)_7$, $(CH_2)_nY(CH_2)_n$, where n= 2, 3; Y= O, NH

6 X=$(CH_2)_4$, $(CH_2)_{10}$, $(CH_2)_3N(CH_3)(CH_2)_3$



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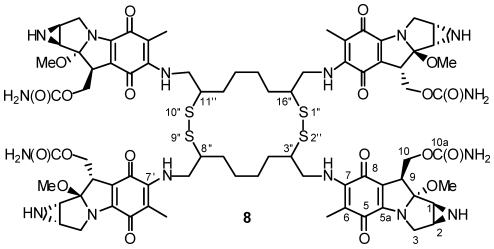


8

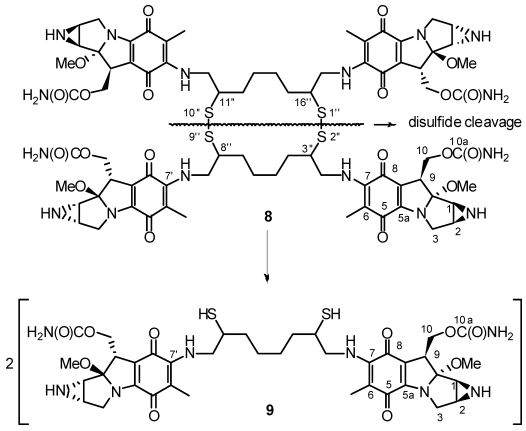
presumably due to DNA alkylation events that proceeded at the two distal C(1) sites. Similarly, compounds **6** were found to generate DNA ISC more efficiently than **1**.[23)]

Recently, we described the preparation and evaluation of the novel mitomycin dimer **7**, which contained a cyclic disulfide unit linking the two mitomycins.[21)] We observed that **7** underwent faster activation and generated higher levels of DNA ISC adducts under nucleophilic conditions than did **1**. The high activation rate and the efficiency of DNA ISC adduct formation for **7** under nucleophilic conditions was attributed to the nucleophile-mediated cleavage of the cyclic disulfide ring, thereby providing a substituted C(7) aminoethylene thiolate species capable of activating the mitomycin.

These findings led us to prepare **8**,[24)] a cyclic bis-disulfide linker that contains four strategically appended mitomycin units. Compound **8** was designed to undergo facile activation to generate DNA ISC adducts by two sequential nucleophile-assisted disulfide cleavage processes to generate two identical dimeric mitomycins, each of which contained a substituted C(7) aminoethylene thiol unit. We report herein that Et_3P significantly accelerated the activation and utilization of **8**, compared with **1**, and provided higher levels of DNA ISC adducts.

Results and Discussion

Compound Design Bis-disulfide **8** was designed to undergo efficient nucleophilic activation. Since **8** retained the key structural components found in **1**, we envisioned that this multimeric mitomycin would also be activated under reductive[2—4)] and acidic conditions.[25—27)] Several structural features distinguished **8** from most previous mitomycin analogs. First, the linker is composed of cyclic 16-membered bis-disulfide, permitting the attachment of four mitomycins. Second, **8** can be considered a 'dimeric dimer' and not simply a 'tetramer,' which upon successive bis-disulfide cleavage provides two equivalents of dimer **9**. The production of two activated dimeric mitomycins upon nucleophilic activation of **8** rather than the single dimeric species from **7** may lead to greater DNA modification and cellular cytotoxicity. Third,



the mitomycin units in dimer **9** contain an appended substituted C(7) aminoethylene thiol group positioned to undergo facile intramolecular cyclization and to allow mitomycin ring activation.[17,21,28)] Fourth, an 8-carbon flexible linker separated the two mitomycin units in cleaved **9**. We estimated that the maximum distance between the two C(1) sites in **9** to be *ca.* 28 Å (Sybyl 6.0, HyperChem 7.1) compared with *ca.* 25 Å for dimer **7**.[21)] Significantly, we have previously determined that the length and composition of the linker can impact the extent of DNA ISC adduct formation.[22)] The increased linker size in **9** is expected to permit this dimer to target more DNA ISC sites compared with **7**.

Proposed Nucleophilic Activation Pathway for 8 We envisioned that nucleophilic activation of **8** would be initiated by either intracellular thiol- or serum albumin (CSH)-mediated disulfide cleavage[14,16—19,29,30)] to give **10**, followed by intramolecular cyclization of released thiolate to provide **11** (Chart 1). Although we have designated the C(8) position as the intramolecular cyclization site, alternative sites (C(7), C(6))[14,17,28)] may also initiate the mitomycin activation process. Compound **11** can undergo mitomycin activation leading to C(1)-monoadducts or disulfide cleavage, giving thiol **12** and then **13**. The formation of **11** and **13** disrupts the N(4)–C(5a)–C(8a)–C(8)–O conjugated system thus allowing for the rapid loss of MeOH from C(9) and C(9a) and mitosene formation, and then mitomycin C(1) DNA adduction.[15,17,31)] If the DNA nucleophilic sites are on complementary strands of the DNA, **13** adduction leads to an ISC product.

Synthesis We have reported the synthesis of **8** as a mixture of diastereomers in 9 steps from commercially available 1,7-octadiene diepoxide (Sigma-Aldrich Co.) and mitomycin A[32)] (MMA, **14**, Kyowa-Hakko Co.) in 6.7% overall yield.[24)] Mass spectrometric analysis documented the 16-membered ring system formation. The reference mitomycin **15**[24)] was prepared from 1,8-diamino-2,7-octanediol dihydrochloride and MMA in 44% yield. Mitomycin **15** contained the same carbon backbone found in the **8** disulfide cleaved product **9**, except that the two thiol units in **9** have been replaced by alcohol groups. ^{13}C-NMR analysis indicated that **15** existed as a 1.1 : 1 mixture of diastereomers.[24)]

Methanolysis of 8 Dissolution of **8** in a MeOH–$CHCl_3$ (1 : 1) solution at "pH" 3 (2 d) led to acid-mediated activation and generation of methoxymitosene product **16** (86%). Compound **16** was purified as a diastereomeric mixture using preparative thin layer chromatography.

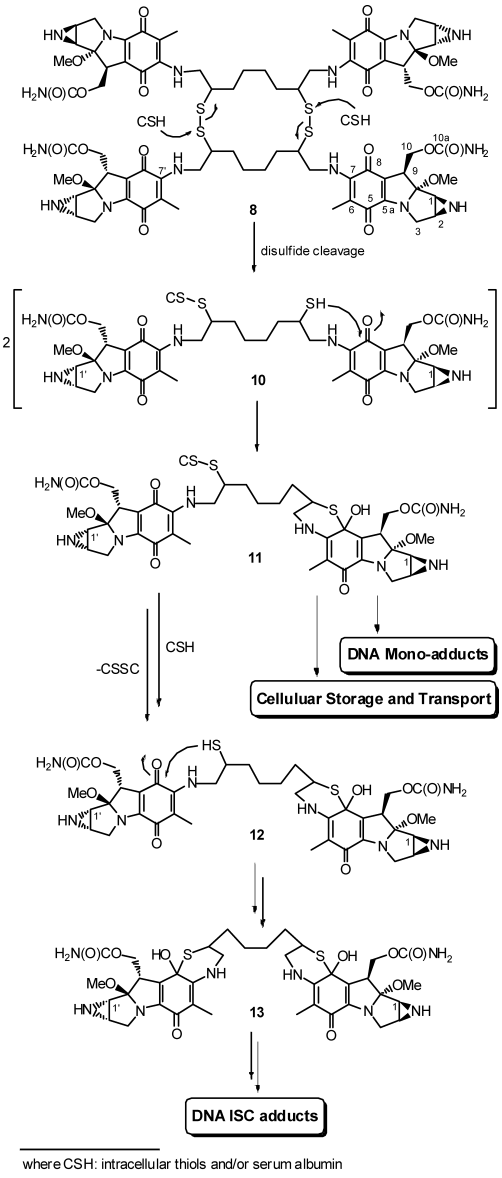
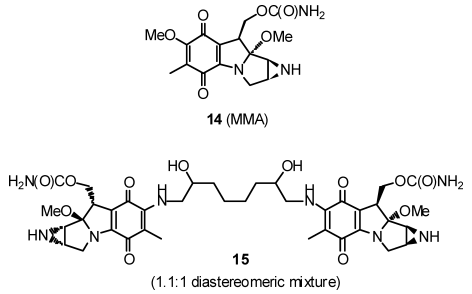
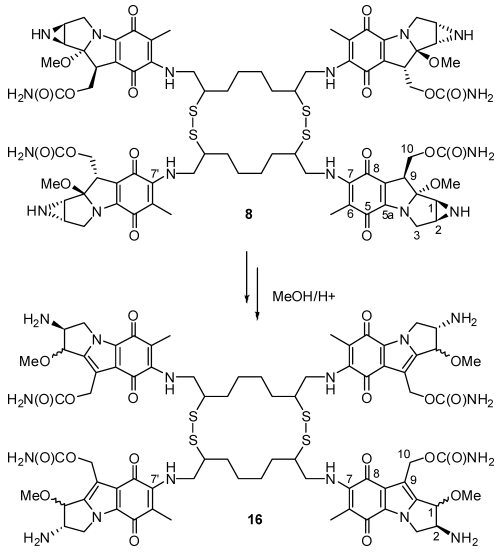


Chart 1. Proposed Nucleophile-Mediated Activation Pathway for **8**



We characterized **16** using HPLC, ultra violet–visible (UV–vis), ^{1}H-NMR, and mass spectroscopy. The HPLC chromatogram revealed a complex, overlapping peak pattern (t_R=33.8—36.5 min) that precluded our determining the precise number of diastereomers (Fig. 1a).[33] The UV–vis spectra for individual HPLC retention times within this array of peaks provided profiles consistent with mitosene production (Fig. 1b). In particular, we observed an absorption maximum at 313—317 nm for the mitosene unit but no 374 nm peak associated with the starting mitomycin **8**.[24] In the ^{1}H-NMR spectra for **16** we found the expected resonance (δ 3.49) for the C(1) methoxy units and the downfield resonance (δ 4.97) for the C(1) methine proton. These signals are diagnostic for mitosene formation and aziridine ring-opened C(1) methoxy-substituted products.[34,35] Finally, in the LC-MS (+ESI) a signal was observed at *m/z* 1703 $[M+Na]^+$ that was consistent with the fully solvolyzed mitosene **16**, along with a peak at *m/z* at 863 $[0.5M+Na]^+$ that corresponded with the bis-disulfide cleaved ion (Fig. 1c). A similar mass spectral fragmentation pattern was observed for **8**.[24] Mitosene **16** served as an authentic sample for our activation studies conducted in the absence of nucleophiles and for reactions that could lead to C(1) methoxy adducts.



Kinetic Studies for the Activation of 8 and 15 The activation studies for **8** and **15** were conducted in buffered "pH" 7.4 methanolic solutions (0.1 M Tris–HCl) at 25 °C. We included in our activation studies the nucleophiles L-dithiothreitol (L-DTT), GSH, and Et_3P. A concentration of **15** (0.3 mM) two-fold higher than **8** (0.15 mM) was used in the activation studies to insure the same concentration of mitomycin units per experiment. Correspondingly, twice as many nucleophile equivalents were used for **8** than for **15** per starting material.

The reactions were monitored using UV–vis spectroscopy (200—600 nm) for at least two half-lives for transformations in which $t_{1/2}$ values were less than 2 d and for at least 5—7 d for reactions with no appreciable loss of starting material. For those entries with no detectable change after 5—7 d, the unreacted mitomycin was 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. At the conclusion of the reactions, the mixtures were analyzed with HPLC and TLC. Authentic samples of **8** and **16** were co-injected with the HPLC samples and co-spotted with the TLC samples. The results for **8** and **15** activation are shown in Table 1.

In the absence of nucleophiles we detected no appreciable solvolysis of either **8** or **15** after 7 d. HPLC and TLC analysis showed only the presence of the starting mitomycin. We then investigated the effect of L-DTT on the activation of **8** and

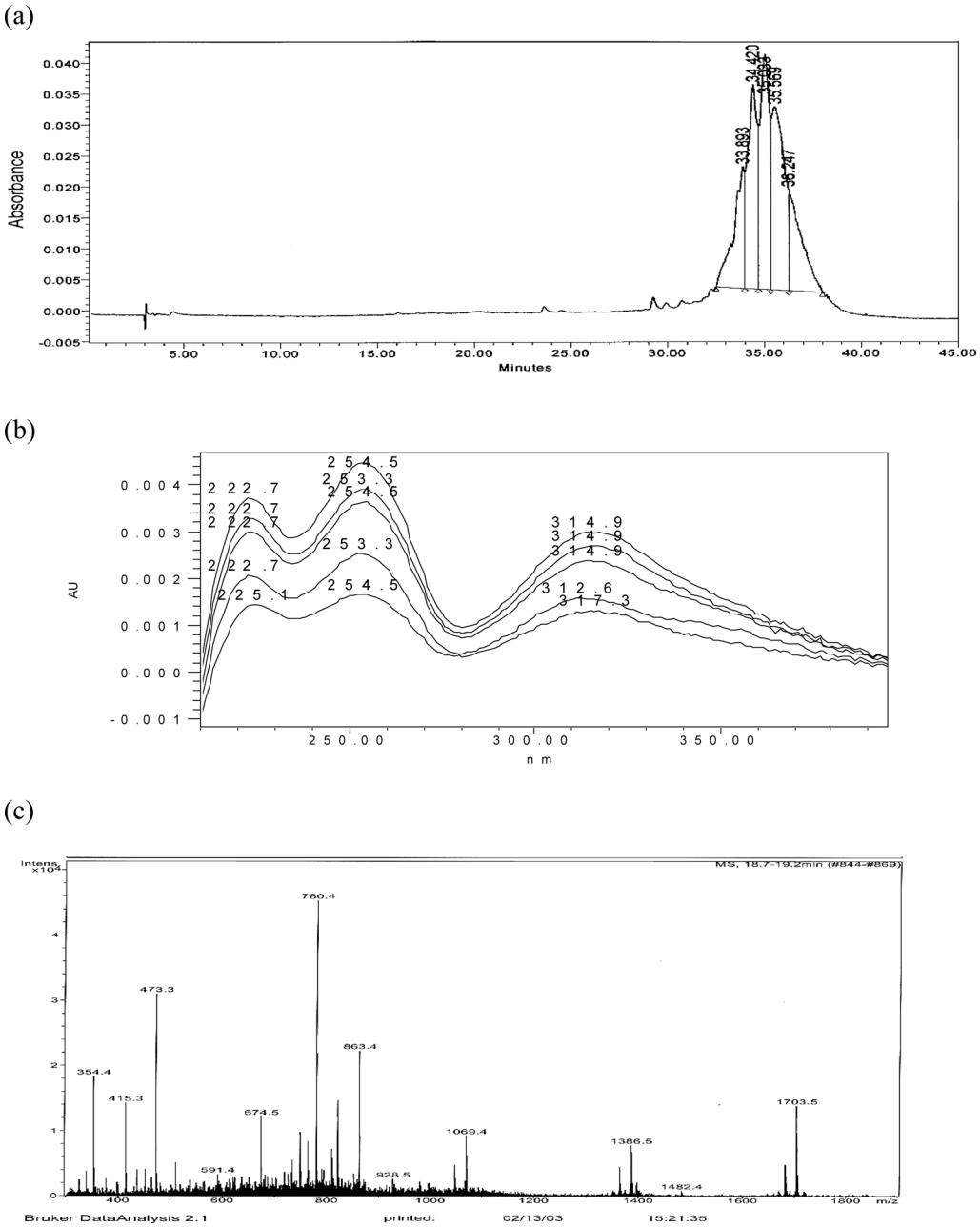


Fig. 1. HPLC Chromatogram (a), UV–Vis Profile (b), and Mass Spectrum (c) for **16**

Table 1. Methanolysis Rates for **8** and **15** at "pH" 7.4[a)]

Reagents		**8**		**15**	
Nu	Equivalent	k_{obs} (d^{-1})	$t_{1/2}$ (d)	k_{obs} (d^{-1})	$t_{1/2}$ (d)
A. No Nu		[b)]	[b)]	[b)]	[b)]
B. L-DTT	20 (**8**), 10 (**15**)	0.385	1.8	—	—
	40 (**8**), 20 (**15**)	1.03	0.67	[c)]	[c)]
C. GSH	40 (**8**), 20 (**15**)	[c)]	[c)]	[c)]	[c)]
D. Et_3P	20 (**8**), 10 (**15**)	8.35	0.083	[c)]	[c)]
	40 (**8**), 20 (**15**)	16.5	0.042	[c)]	[c)]

Nu: nucleophile. *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 data were obtained using a Cary 3Bio Varian UV–visible spectrophotometer and the reactions monitored at 374±3 nm. The concentration of the mitomycin was 0.015 mM for **8**, and 0.03 mM for **15**. *b*) No appreciable change after 7 d (<10% of original amount). *c*) No appreciable change after 5 d (<10% of original amount).

15. When 20 eq of L-DTT were employed per **8**, the $t_{1/2}$ value determined by UV–vis spectroscopy (374 nm) was 1.8 d, and when 40 eq of L-DTT were utilized, the $t_{1/2}$ decreased to 0.67 d. Significantly, when we followed the reactions at 313 nm we saw a concomitant increase in the 313 nm signal with time; a finding consistent with mitosene production.[26] Thus, adding L-DTT led to modest increases in **8** consumption rates, which depended upon L-DTT concentration. The HPLC chromatograms for the L-DTT activation experiments showed a complex pattern between *ca.* 33 and *ca.* 37 min.[36] By comparison, we observed little change when L-DTT (20 eq) was added to **15**, with the estimated loss of **15** being <10% after 5 d. When GSH was used as the nucleophile, solvolysis rates of **8** and **15** were not affected (<10% after 5 d) (Table 1).

We next measured the effect of Et_3P on the rate of activation of **8** and **15** using UV–vis spectroscopy (374 nm). The $t_{1/2}$ values for the activation of **8** were 0.083 d and 0.042 d when 20 and 40 eq, respectively, of Et_3P were added. The Et_3P effect for **8** was proportional with the Et_3P concentration. We again observed an increase in the UV–vis absorption at 313 nm (mitosene products) with time. Correspondingly, we detected no appreciable rate of **15** activation (<10% after 5 d) when Et_3P (10, 20 eq) was added. Accordingly, we concluded that Et_3P led to a large rate enhancement for **8** and have attributed this to the Et_3P-mediated cleavage of the disulfide bonds in **8** and the subsequent activation of the mitomycins units by the generated C(7) aminoethylene thiolate anions.

Interestingly, comparison of the Et_3P activation rate data of **8** with **7** showed that **8** was consumed 3.1-times faster than **7** ($t_{1/2}$ values of **7** with Et_3P: 0.26 d (10 eq); 0.13 d (20 eq)).[21] We have tentatively attributed this rate enhancement, in part, to the differences in regeneration rates of cyclic disulfide or bis-disulfide, respectively, after initial Et_3P cleavage of these mitomycins. The thiolate species produced from disulfide cleavage of **7** is expected to readily revert to the 6-membered cyclic disulfide due to the facile formation of 6-membered rings,[21,28] whereas the corresponding thiolate intermediate generated from **8** is expected to revert more slowly to the 16-membered bis-disulfide. Similarly, we expect slow rates of conversion of **9** to either **8** or the corresponding 8-membered cyclic disulfide.

Efforts to identify the Et_3P reaction products from **8** by using HPLC were unsuccessful. Again, we observed a complex pattern showing many overlapping peaks in the HPLC between t_R *ca.* 33 and *ca.* 37 min (data not shown).[36] We have attributed the HPLC pattern to the many plausible diastereomeric mitosene products that could be generated from **8** upon activation and C(1) substitution.

We propose the phosphine-mediated activation pathway for **8** shown in Chart 2. Et_3P-induced disulfide cleavage[37] generates thiol **17** and serves as a key step in the activation pathway. Intermediate **17** is expected to undergo intramolecular cyclization to afford hemi-thioketal **18**, disrupting the N(4)–C(4a)–C(8a)–C(8)–O conjugated system. This disruption facilitates the N(4)-mediated loss of MeOH at C(9) and C(9a) providing a mitosene capable of undergoing aziridine ring opening and then C(1) nucleophilic attack to give **19**.[15,17,31] A subsequent reaction of thiophosphonium **19** with MeOH[37] gave thiol **20** permitting a second round of activation to give **22** (**20**→**21**→**22**). Formation of **22** and then **24** allowed bis-disulfide **16** production or that of other C(1)-nucleophilic substituted mitosenes, as well as other cyclic and polymeric disulfides, upon air oxidation. The proposed pathway derives support from our previous studies that showed phosphines rapidly cleaved the disulfide bond in *cis*- and *trans*-4,5-dihydroxy-1,2-dithianes,[37] and that Et_3P activated **7**.[21]

DNA Bonding Profiles for Bis-Disulfide 8 The efficiency of mitomycin DNA ISC processes depend upon the mitomycin structure and activation conditions. Importantly, compound **8** was designed to activate under nonreductive, nucleophilic conditions thus leading to the generation of DNA products, including DNA ISC adducts. Thus, using the method of Cech,[38] and Tepe and Williams,[39] we determined whether **8**, reference compound **15**, and **1** cross-linked complementary strands of *Eco*RI-linearized pBR322 DNA under nucleophilic mediated activation conditions employing 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. Once again, the concentration used for **8** was one-half (0.05 mM) that of **15** (0.1 mM), so we could compare the effect of equivalent amounts of mitomycin units on DNA ISC production. Accordingly, we doubled the number of equivalents of nucleophiles (Et_3P, L-DTT and GSH) used for **8** over the number used for **15**. Finally, we used 0.1 mM **1** rather than 0.2 mM to compare the DNA ISC efficiencies of **8** with **7**.[21]

Treatment of **1**, **8**, and **15** with Et_3P led to varying levels of DNA ISC adducts (Table 2). Compound **8** gave moderate levels (35%), and **15** and **1** were 16% and 5%, respectively. The level for **8**-mediated DNA ISC was two-fold higher than reference compound **15**. The increased levels for **8** compared with **15** and **1** were in accord with the kinetic study results, wherein adding Et_3P (20 eq) led to significant consumption of **8**, but for **1** and **15** we saw no appreciable loss of starting material after 5 d (Table 1). When we compared **8** and **7** using Et_3P, we found that **8** provided lower levels of DNA ISC (**8**, 35%; **7**, 58%[21]) despite our finding that **8** was consumed faster than **7** ($t_{1/2}$ values: **8**, 0.083 d; **7**, 0.26 d[21]) in the kinetic studies. The reasons for these observations are unclear. We estimate that the distance between the two C(1) sites in **9** (28 Å) is larger than in the corresponding disulfide-cleaved **7** intermediate (*ca.* 25 Å), and we suspect that this increased conformational flexibility may entropically decrease the likelihood of generating DNA ISC adducts.

We also investigated the effects of L-DTT and GSH on DNA ISC processes for **1**, **8**, and **15** (**8**: 20 eq of nucleophile; **1** and **15**: 10 eq of nucleophile). As shown in Table 3, we observed that L-DTT provided moderate levels (30%) of DNA ISC adducts for **8** while the extent of DNA ISC for **15** and **1** were 10% and 3%, respectively. Thus, the 3-fold higher levels of DNA ISC for **8** over **15** provided by L-DTT supports the importance of the disulfide group in the **8** mitomycin activation process (Table 3). The L-DTT-mediated DNA ISC results paralleled the kinetic data in which L-DTT was found to accelerate the consumption of **8** ($t_{1/2}$=1.8 d, Table 1) but not **15**. Interestingly, the observed level of DNA ISC for **8** using L-DTT (30%) was only slightly lower than that found for Et_3P (35%) (Table 2). A similar level of DNA ISC adducts (30%) were observed for **7** with L-DTT (10 eq).[21] We are un-

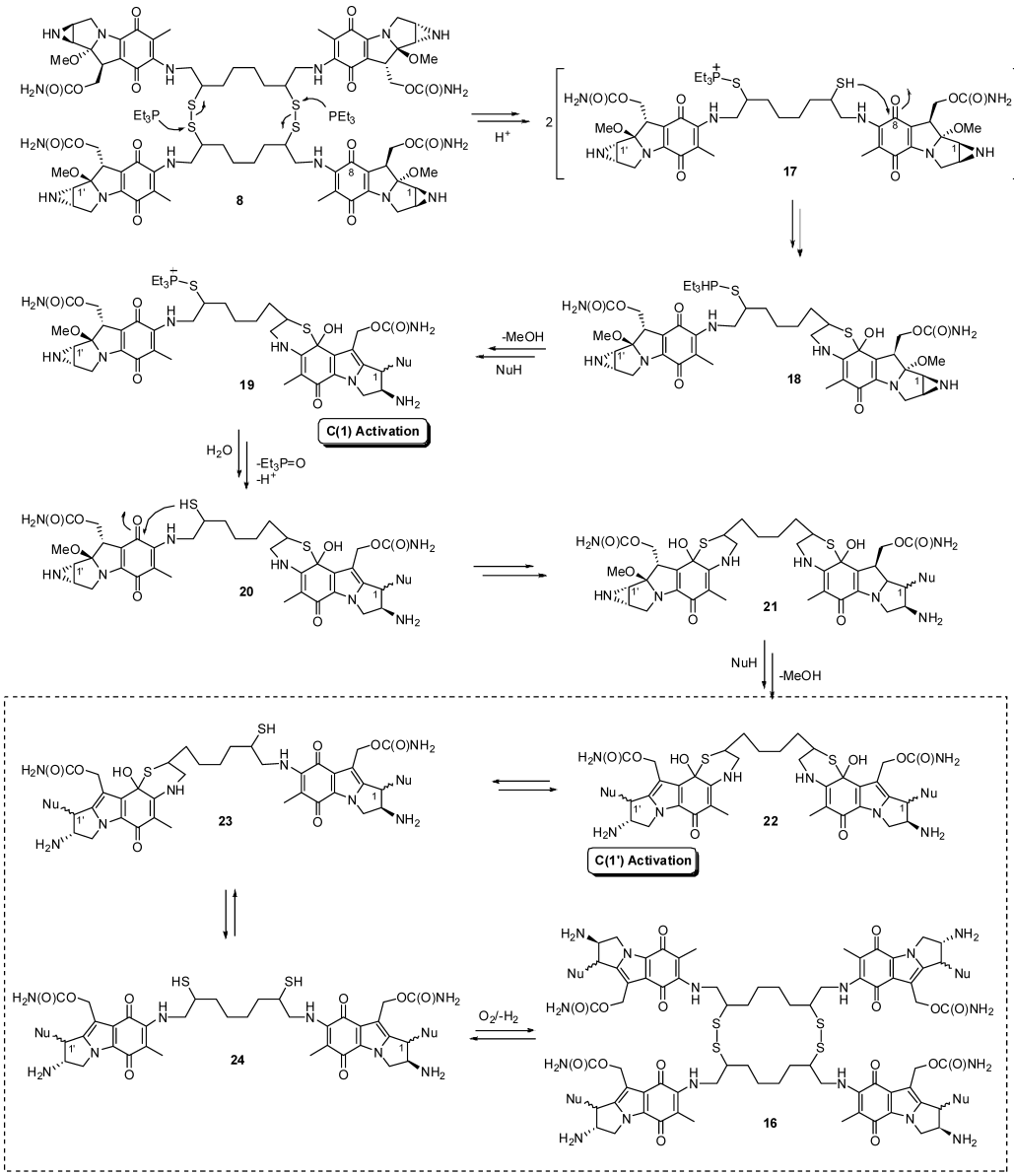


Table 2. Extent of DNA ISC for **1**, **15** and **8** Using Et_3P[a)]

Compound	**1**	**15**	**8**
DNA ISC	5%	16%	35%
ss-DNA	95%	84%	65%

a) DNA cross-linking experiments for **1** (0.1 mM), **15** (0.1 mM) and **8** (0.05 mM) using *Eco*RI-linearized pBR322 plasmid DNA and Et_3P (**1** and **15**: 10 eq; **8**: 20 eq). All reactions were incubated at room temperature (6 min). The percentage of DNA ISC was obtained by the quantitative analysis of DNA bands using a Storm™ 860 Phosphorimager and ImageQuant 5.0 software (Molecular Dynamics).

certain why the DNA ISC levels were similar for Et_3P and L-DTT since large differences were observed in the relative **8** consumption rates for these two nucleophiles (Table 1). There were important differences between these two experiments, which may account for these findings. First, the concentrations, reaction times, and solvents were different. Second, in the DNA experiments a single cross-linked adduct was sufficient to generate a DNA ISC product visible in the gel whereas in the kinetic experiments the activation of all mitomycin units in solution were monitored.

Cytotoxicities We tested whether mitomycin bis-disulfide **8** inhibited tumor growth.[40)] The *in vitro* antiproliferative activity tests were conducted at the Kyowa Hakko Pharmaceutical Company (Shizuoka, Japan) using the human lung adenocarcinoma cell line A549 (Table 4). This cell line was chosen because of its sensitivity to KW-2149 (**2**). Since compound **8** was designed to undergo activation under nucleophilic, acidic, and reductive conditions, we evaluated the antiproliferative activity of **8** under both aerobic and hypoxic conditions. We also list the activity ratio (IC_{50} (hypoxic)/IC_{50} (aerobic)) for these compounds under the two different test conditions. We recognized that the observed IC_{50} values do not serve as a measure of the extent and efficiency of DNA

Table 3. Extent of DNA ISC for **1**, **15** and **8** Using L-DTT and GSH[a)]

	Compound					
	1	**15**	**8**	**1**	**15**	**8**
Thiol	L-DTT			GSH		
DNA ISC	3%	10%	30%	3%	8%	7%
ss-DNA	97%	90%	70%	97%	92%	93%

a) DNA cross-linking experiments for **1** (0.1 mM), **15** (0.1 mM) and **8** (0.05 mM) using *Eco*RI-linearized pBR322 plasmid DNA and L-DTT and GSH (**1** and **15**: 10 eq; **8**: 20 eq). All reactions were incubated at room temperature (6 min). The percentage of DNA ISC was obtained by the quantitative analysis of DNA bands using a Storm™ 860 Phosphorimager and ImageQuant 5.0 software (Molecular Dynamics).

Table 4. Antiproliferative Activities for Mitomycins **8**, **1**, **2** and **15**[a)]

Compound	IC_{50} (μmol/l)		Ratio
	Aerobic	Hypoxic	[IC_{50} (hypoxic)/ IC_{50} (aerobic)]
8	9	>50	>5.7
15	97	>100	>1.0
1 (MMC)	2.4	20	8.2
2 (KW-2149)	0.12	0.41	3.4
7[b)]	41	>100	>2.4

a) A549 cell lines were used. The IC_{50} (μmol/l) value is the concentration that inhibits cell replication by 50% under the assay condition. *b*) Ref. 21.

ISC for the different mitomycins since we were not able to determine the relative levels of the mitomycins within the cells and the factors that contributed to inhibiting cell replication.

We found that **8** was less potent than **1** under aerobic and hypoxic conditions (Table 4). The activity ratio (hypoxic/aerobic) for **8** was >5.7 compared with 8.2 for **1**. Compound **8** was 10.8-fold more potent than its diol derivative, **15**, under aerobic conditions, and both compounds showed little cytotoxicity under hypoxic conditions (>50 μmol/l). It is important to note that bis-disulfide **8** was 4.6-fold more potent than dimer **7**,[21)] and this increased activity was retained when the data were normalized to account for the relative concentrations of mitomycin "units" per assay. In summary, **8** displayed weaker activity than either **1** or **2** and higher activity than **15** and **7**.

Conclusions

In this study, we report the evaluation of bis-disulfide **8**, a novel mitomycin that contained three structural elements expected to affect drug activation and DNA adduction in the presence of nucleophiles. First, a cyclic bis-disulfide linker permitted the release of two dimeric mitomycins. Second, a flexible, eight-carbon linker appended the two mitomycins in the released dimers. Third, conversion of **8** to **9** provided two dimeric molecules in which each mitomycin unit contained a substituted C(7) aminoethylene thiolate moiety ideally positioned for intramolecular activation of the mitomycin group. We found that under acidic conditions, **8** was activated in MeOH to afford mitosene **16**. Next, we compared the activation rate of **8** with the reference compound **15** in the absence and presence of nucleophiles. In the absence of an external nucleophile, neither **8** nor **15** underwent change (<10%) after 7 d (25 °C). Et_3P markedly increased the consumption rate of **8** but not of **15**. These findings are consistent with the notion that mitomycin activation of **8** proceeded by phosphine attack at the disulfide unit to generate thiolate species capable of activating the mitomycin units. Similar results were observed for **7** and Et_3P.[21)] Using Et_3P, the consumption rates for **8** were 3.1-times faster than those for **7**. We have attributed this difference, in part, to the difficulty in regenerating bis-disulfide **8** upon initial cleavage, compared with the corresponding reaction with **7**. Finally, we determined the efficiency of the DNA ISC processes of **8**. When the nucleophile was added (Et_3P, L-DTT), **8** generated higher levels of DNA ISC adducts than the reference compounds **1** and **15**. The increased levels of DNA ISC paralleled the increased levels of mitomycin consumption seen in the kinetic studies. This observation supports the proposed nucleophilic pathway for mitomycin activation (Chart 2). We observed that the levels of DNA ISC adducts for **8** were lower than that reported for **7**.[21)] This finding documented the importance of the length and the composition of the linker for efficient DNA ISC formation and is agreement with previous results.[22,23)]

The activation pathway for **8** differentiated this mitomycin from **1** and from other mitomycin analogs that rely on reductive and/or acid-catalyzed pathways for drug function. Our findings may pave the way for new analog development exhibiting different activity profiles, and where the mitomycin, the linker, and the activating group are finely tuned for maximal DNA adduction.

Experimental

General ^1H- (300 MHz) and ^{13}C- (75 MHz) NMR spectra were recorded on a Varian Gemini 2000 spectrometer. Mass spectral (MS) data were obtained by Dr. Mehdi Moini at the University of Texas at Austin. The low-resolution MS studies were run on a Finnegan TSQ-70 triple quadrupole mass spectrometer, and the high-resolution MS studies were conducted on a Micromass ZAB-E mass spectrometer. FT-IR spectra were run on a Mattson Galaxy Series FT-IR 5000 spectrometer. Melting points were determined in open capillary tubes using a Thomas–Hoover melting point apparatus and are uncorrected. pH Measurements were determined on a Radiometer pHM26 meter using a Radiometer pHC4000 glass electrode. LC-MS analyses were conducted with Agilent 1100 LC/MSD by Dr. Voyksner (LCMS Limited, Raleigh, NC, U.S.A.). The products were analyzed with a Zorbak C_{18} SB column (2.1×50 mm, 3.5 μm particles) using the following linear gradient condition: 80% A (0.025 M ammonium acetate in H_2O–CH_3CN (95 : 5), pH 6.5), 20% B (0.025 M ammonium acetate in H_2O–CH_3CN (5 : 95), pH 6.5) isocratic for 1 min, and then from 80% A, 20% B to 20% A, 80% B for 30 min. The flow rate was 0.3 ml/min, and the eluent was monitored at 365 and 313 nm. The mass spectral mode of operation was positive ion electrospray (+ESI) and the scan range was 300—1900 daltons with 45 psi of nebulization pressure.

Methanolysis of 8 to Give *cis*- and *trans*-C(1) Methoxymitosenes 16 Mitomycin **8** (3.5 mg, 0.0042 mmol) was dissolved in MeOH–$CHCl_3$ (1 : 1, 3 ml) and then the "pH" was adjusted to *ca.* 3.0 with a methanolic 2 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 (50% MeOH–$CHCl_3$) afforded the product as a red solid (3.0 mg, 86%); ^{1}H-NMR (CD_3OD–$CDCl_3$, 300 MHz) δ: 1.53—1.87 (16H, m, C(4″)H_2, C(5″)H_2), 1.97 (12H, br s, C(6)CH_3), 2.98 (4H, br s, C(3″)H), 3.51 (12H, br s, C(1)OCH_3), 3.62—3.92 (8H, m, C(7)NH–C$\underline{H}_2$), 4.10—4.24 (4H, m, C(3)$\underline{H}$H′), 4.26—4.49 (8H, m, C(3)H$\underline{H}$′, C(2)H), 4.98 (4H, br s, C(1)H), 5.15—5.33 (8H, m, C(10)H_2), the ^{1}H-NMR data were in agreement with the COSY spectrum. HPLC t_R 33.8—36.5 min (multiple peaks). *Rf*=0.09 (50% MeOH–$CHCl_3$). UV–vis (CH_3CN–H_2O) λ_{max}=223, 254, 315 nm. LC-MS *m/z*: 1703 $[M+Na]^+$, 863 $[0.5M+Na]^+$ (t_R 17.5—19.3 min).

General Procedure for the Mitomycin Activation Studies (Kinetic 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 (10—60 μl of 4 mM methanolic solution, final concentration 0.015—0.03 mM) was added a methanolic solution (5—50 μl) of the nucleophile of choice (stock solution: 4—20 mM, final nucleophile concentration 0.3—0.6 mM). The reaction was monitored by UV–vis spectroscopy (200—600 nm), and typically 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 $\pm$0.1 pH units of the original solution. The reaction products were analyzed by HPLC and the presence of unreacted staring materials and products (*e.g.*, **8**, **15**, **16**) were determined by coinjection of authentic samples in the HPLC and cospotting of authentic samples in the TLC. The λ_{max} of mitomycin (*ca.* 374 nm) was plotted *versus* time and found to decrease in a first-order decay (exponential decay) process. The nonlinear regression analysis to fit the observed exponential decay by SigmaPlot Program (SigmaPlot, 2001) yielded pseudo-first-order rate constants (k_{obs}) and half-lives ($t_{1/2}$). The reactions were done in duplicate and the results averaged.

General Procedure for Alkaline Agarose Gel Electrophoresis[38,39] 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 gel 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 *ca.* 85 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 Ar (15 min), the mitomycin (1—5 μl of 1—2 mM DMSO solution, final concentration 0.05—0.1 mM) and the nucleophile (1—5 μl of 1—20 mM DMSO solution, final concentration 1.0—2.0 mM) were added and the resulting solution (final volume 100 μl) was incubated at room temperature (6 min). 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 an aqueous 50 mM NH_4OAc and 10 mM β-mercaptoethanol solution (3 h). The gel was then analyzed by two methods. In one method, the gel was visualized by UV and photographed using Polaroid film 667. In the second method, the gel was analyzed with a Storm^{TM} 860 phosphorimager operating in the blue fluorescence mode and ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

General Procedure for Antiproliferative Activity Test[40] *In vitro* antiproliferative tests were conducted using human tumor cell line A549 (lung adenocarcinoma) by Dr. Hitoshi Arai (Kyowa Hakko Kogyo Co., Shizuoka, Japan). The cells (2×10^3 cells/well) were precultured at 37 °C (24 h) in 96-well microtiterplates containing the culture medium (RPMI-1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin) under either aerobic (5% of CO_2 and 95% of air) or hypoxic (5% of CO_2 and <2% of O_2) conditions. The cells were then treated with the drug candidates (1 h), washed twice with the medium and further incubated (71 h) in the drug-free medium. The antiproliferative activity of drugs against tumor cells was measured by MTT assay. Cell growth (%) was calculated by the equation, $\{[A-A_o]/[A_c-A_o]\}\times100$ (A: absorbance, A_o: blank absorbance, A_c: control absorbance), and the activity was expressed by IC_{50} values (concentration required for 50% inhibition).

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