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7-*N*,7′-*N*′-(1″,2″-Dithianyl-3″,6″-dimethylenyl)bismitomycin C: synthesis and nucleophilic activation of a dimeric mitomycin†

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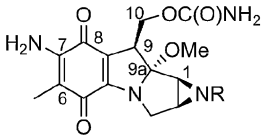
Dimeric alkylating agents that modify complementary DNA strands have engendered significant interest. We have prepared the novel dimeric mitomycin, 7-*N*,7′-*N*′-(1″,2″-dithianyl-3″,6″-dimethylenyl)bismitomycin C (**9**), in which the mitomycins are bridged by a dithiane unit. Dimer **9**, like the clinically tested acyclic disulfides KW-2149 (**3**) and BMS-181174 (**4**), was designed to activate under nucleophilic and reductive conditions. Successive nucleophile-mediated disulfide cleavage transformations of **9** are expected to generate thiol species ideally positioned to render the two mitomycin systems vulnerable to nucleophilic attack and permit DNA interstrand cross-link formation. The dithiane linker, strategically positioned between the two mitomycins, distinguished **9** from **3** and **4**. Nucleophilic activation of this cyclic disulfide permitted both activated mitomycins to remain tethered to one another. We report the synthesis of **9**, and show that the nucleophile Et_3P markedly enhances the activation and consumption of **9**, compared with the reference compound 7-*N*, 7′-*N*′-(cyclohexanyl-*trans*-1″,4″-dimethylenyl)bismitomycin C (**27**). We further demonstrated that **9** provides higher levels of DNA interstrand cross-links than either the dimeric reference compounds, **27** and 7-*N*,7-*N*′-(2″,5″-dihydroxy-1″,6″-hexanediyl)bismitomycin C (**28**), or the monomeric mitomycins, **1** and **3**, when Et_3P is added to solutions containing *Eco*RI-linearized pBR322 DNA.

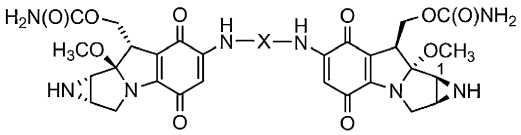
Introduction

Mitomycin C (**1**) is a clinical anticancer agent of significant importance.[1] Bioreduction of **1** leads to the sequential activation of the C(1) and C(10) sites necessary for the generation of mono- and bis-(cross-linked) alkylated DNA adducts.[2] While both DNA lesions contribute to the antitumor activity of **1**, it is the DNA interstrand cross-link adducts (DNA ISC) that have attracted the most interest. The cross-linking of complementary DNA strands by **1** (**1**–DNA) is expected to inhibit DNA replication and subsequent cell proliferation.[2] Consistent with this notion **1**–DNA ISC have been shown to be *ca.* 60 times more lethal than the corresponding **1**–DNA monoadducts.[3]



1 R = H
8 R = Me

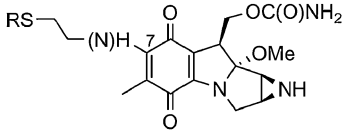
There has been an increased interest in the design and evaluation of novel dimeric agents that target DNA.[4,5] Among these compounds are intercalating and irreversible alkylating agents that cross-link complementary strands of DNA. We have prepared a series of dimeric mitomycins (**2**) designed to take advantage of the enhanced reactivity of the C(1) site in **1**, compared with the C(10) position.[6] Modification of DNA by



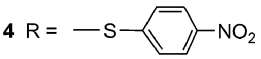
2 X=$(CH_2)_n$ and $(CH_2)_n$ Y$(CH_2)_n$ where Y=O, NH

these dimeric mitomycins at the distal C(1) positions would lead to DNA ISC. These dimeric mitomycins were tethered at the mitomycin C(7) amino moieties with a hydrocarbon, alkyl ether, or a dialkylamine unit linker. DNA cross-linking experiments using a denaturing-gel electrophoresis-based assay showed that the extent of DNA ISC for select dimeric mitomycins exceeded those of **1** and that the reaction proceeded, in part, at the two distal C(1) sites in the dimer. Recently, information was reported concerning the site(s) of DNA ISC bonding for this class of dimeric mitomycins.[7] Moreover, evidence was presented that these dimeric mitomycins were activated by the same reductases that catalyze **1** utilisation and that DNA adduction proceeded with the apparent 5′-CpG sequence specificity previously documented for **1**.[8,9]

The clinical successes and limitations of **1** has led to the syntheses and evaluation of more than 1000 analogues.[10] In the 1980s, two mitomycins were disclosed, **3** (KW-2149)[11] and **4** (BMS-181174),[12] where each contained a C(7) aminoethylene disulfide moiety in place of the C(7) amino unit in **1**. Significantly, **3** was active in **1**-resistant P388 and non-hypoxic



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



5 R = H

cells.[13] Both **3** and **4** were entered into clinical trials, and **3** progressed to phase II testing. Mechanistic studies using **3**, **4**, and structurally related compounds indicated that these agents likely underwent nucleophile-assisted cleavage [*e.g.* glutathione (GSH)] of the C(7) aminodisulfide unit to provide **5**, and that **5** initiated drug–DNA adduction.[14–16]

Compounds **3** and **4** are members of an emerging class of anticancer agents that contain a multi-sulfide linkage. Cytotoxicity in these agents is associated, in part, with a nucleophile-assisted,

† Dedicated to Laurence H. Hurley on the occasion of his 60th birthday.

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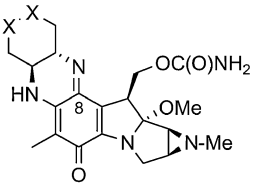
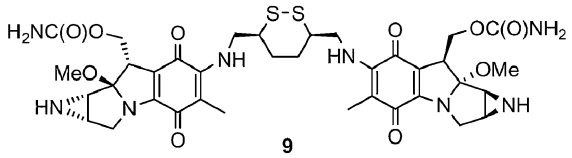
sulfur–sulfur cleavage transformation.[11,12,17–22] These reports led us to prepare C(8) iminoporfiromycin **6** that contained a cyclic disulfide unit and its hydrocarbon counterpart **7**.[23] Porfiromycin



6 X = S
7 X = CH_2

(**8**) is the N(1a) methyl derivative of **1**.[2a] We showed that phosphine nucleophiles accelerated **6** activation and solvolysis, compared with **7**, and that it also provided increased levels of DNA ISC, compared with **7**.[23]

In this study, we report the synthesis of the dimeric mitomycin **9**, which contains a cyclic disulfide unit. Dimer **9** was designed to undergo facile DNA ISC by a nucleophile-assisted disulfide cleavage process permitting successive modification of the two mitomycin distal C(1) sites that remain tethered together. We demonstrated that phosphines dramatically accelerate the activation and utilisation of **9** compared with **1** and provide enhanced levels of DNA ISC adducts.



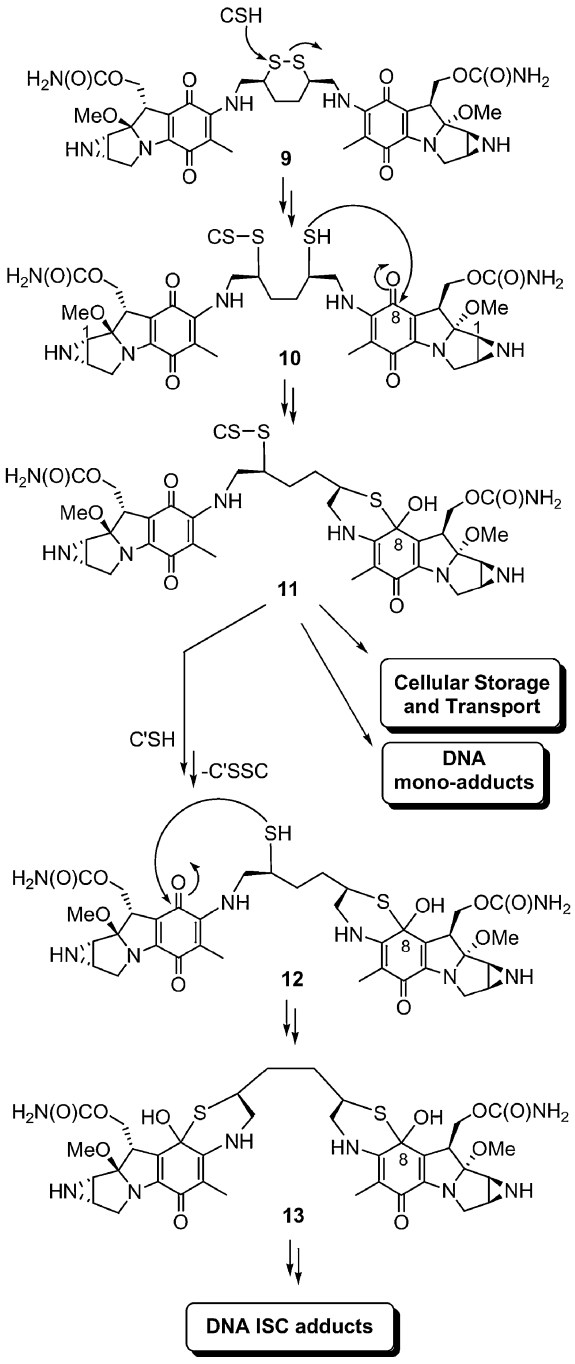
9

Results and discussion

Selection of dimeric mitomycin 9

Compound **9** retained the key structural features found for **1** and for many semi-synthetic mitomycins. Thus, we expected that this dimeric mitomycin would likely be activated by both reductases and acids, pathways previously documented for **1**.[2] Four structural elements are, however, unique to **9** and were incorporated into our design of this mitomycin to promote nucleophile-assisted DNA ISC. First, **9** had two mitomycin units to permit DNA adduction at the reactive C(1) site.[6] Second, the disulfide unit was strategically placed three atoms away from the C(7) position in both mitomycins. This arrangement has been important for intramolecular thiol-mediated mitomycin activation processes.[16] Third, the dithiane linker in **9** ensured that the two mitomycin units would remain together after activation and DNA adduction. This feature differentiated **9** from **3** and **4** where disulfide cleavage leads to a monomeric species. Fourth, the dithiane linker is of sufficient size to permit modification of the two distal mitomycin C(1) sites in **9** by guanine residues on complementary DNA strands. We estimate with computational programs (Sybyl 6.0, HyperChem 7.1) that the distance for **9** and its expected intermediates to be between 7–25 Å depending upon their conformation. This distance can cross-link guanines on different DNA strands separated by as many as five base pairs.

The envisaged nucleophile-mediated route unique to **9** is shown in Scheme 1. Activation of **9** begins with intracellular thiol- or serum albumin (CSH)-mediated disulfide cleavage[14–16,24–26] to give **10**, which is then converted to **11** through intramolecular cyclization. In Scheme 1, we show that intramolecular cyclization occurs at the C(8); however, we recognize that alternative cyclization sites [C(7), C(6)][14,16,23] may initiate mitomycin activation. A second round of disulfide cleavage beginning with **11** provides **12**, which is then converted to **13** by intramolecular cyclization. Formation of **11** and **13** disrupts the N(4)–C(4a)–C(8a)–C(8)–O conjugated system and is expected to lead to the rapid loss of methanol from C(9) and



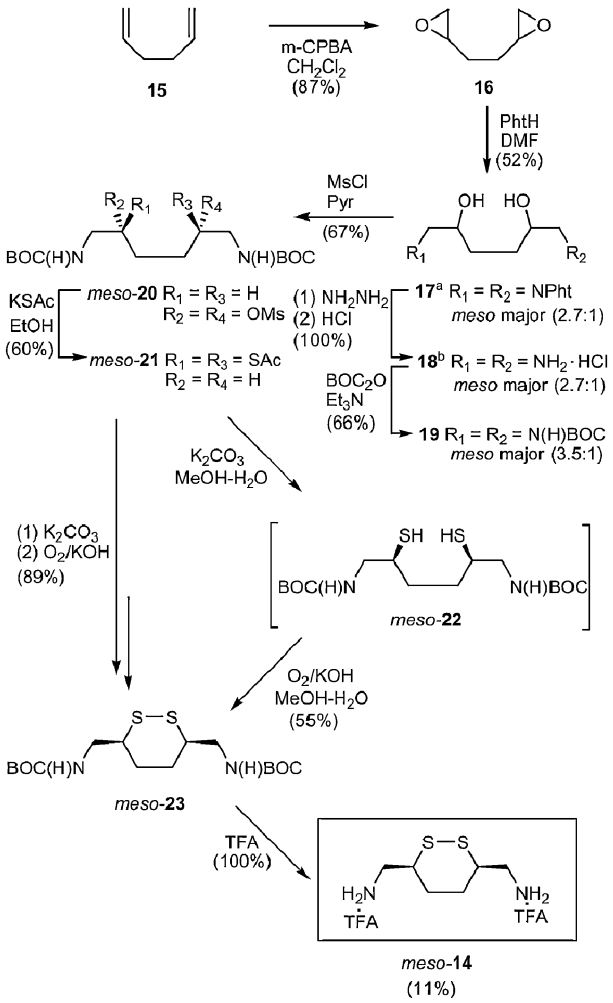
Scheme 1 Proposed nucleophile-mediated activation pathway for **9**.

C(9a) and mitosene formation, permitting the successive DNA adduction at the two distal C(1) sites in the dimer. If correct, **9** nucleophile-mediated activation in the presence of DNA will lead to DNA ISC.

Thus, three objectives were established for this study: the synthesis and characterization of **9**, the documentation of the nucleophile-mediated activation pathway for **9**, and the assessment of the **9** DNA ISC efficiencies under nucleophile-mediated conditions using linearized pBR322 DNA. We also determined the *in vitro* cellular cytotoxicities of **9** against human lung adenocarcinoma cell line A549. While we found it interesting, the cytotoxicity information for **9** was not used in our assessment of the structural requirements for nucleophile activation of this mitomycin since neither the effective concentration of **9** within the A549 cells nor the factors that contributed to the mitomycin inhibition of cell replication were determined.

Synthesis of the cyclic disulfide bridge: (3*R*,6*S*)-*meso*-3,6-bis(aminomethyl)-1,2-dithiane di-trifluoroacetate salt (14)

Synthesis of **9** required the preparation of (3*R*,6*S*)-*meso*-3,6-bis(aminomethyl)-1,2-dithiane di-trifluoroacetate salt (**14**) (Scheme 2). We began by converting 1,5-hexadiene (**15**) to diepoxide **16** using *m*-chloroperbenzoic acid.[27,28] Epoxide cleavage with phthalimide in DMF gave **17**.[28] Three stereoisomers of **17** exist: *meso*-(*R*,*S*) and the enantiomeric pair, *threo*-(*R*,*R*), and *threo*-(*S*,*S*). We found that the isolated product existed as a mixture of diastereomers (2.7 : 1, ^{13}C NMR analysis).[28] Recrystallization (twice) of **17** in DMF (80 °C) provided a sample enriched in one isomer (9.4 : 1, ^{13}C NMR analysis) in 64% yield.[28] X-Ray crystallographic analysis identified this adduct as *meso*-**17**,[28] indicating that the major product in the initially isolated **17** mixture (2.7 : 1 diastereomeric mixture) was the *meso*-adduct. Hydrazine deprotection of the phthalimide units in **17** (*meso* : *dl* = 2.7 : 1) gave **18**[29] (*meso* : *dl* = 2.7 : 1, ^{13}C NMR analysis) in near quantitative yields after acid work-up. Subsequent protection of the amino groups in **18** with BOC_2O and Et_3N afforded **19** in moderate yield (66%) as a mixture of diastereomers (*meso* : *dl* = 3.5 : 1, ^{13}C NMR analysis). Recrystallization of **19** from ethyl acetate provided a single isomer (≥10 : 1, ^{13}C NMR analysis) (75% yield). The product yields and the ratio of diastereomers obtained for the conversion of **17** to **19** indicated that the major **19** diastereomer in the 3.5 : 1 mixture was the *meso*-isomer. Several methods were explored to replace the hydroxyl groups in **19** by thioacetate units. Mitsunobu reaction[30] of **19** with thioacetic acid produced no reaction (data not shown). Next, we treated **19** (*meso* : *dl* = 3.5 : 1) with MsCl in pyridine to give **20** in a moderate yield (67%). ^{13}C NMR analysis showed that **20** was isolated as a single diastereomer. Once again, the observed yield for this transformation indicated that this compound was the *meso*-isomer. We suspect that conditions employed in the isolation work-up of this reaction (addition of H_2O) led to the selective precipitation of the *meso*-**20**. Displacing mesylate units in **20** with KSAc in EtOH gave **21** in a 60% yield. ^{13}C NMR analysis showed only a single isomer, thus providing evidence that substitution proceeded with stereochemical control by an S_N2 pathway. We have, therefore, assigned **21** as the *meso*-stereoisomer. Hydrolysis of the thioacetate groups in **21** with aqueous methanolic K_2CO_3 gave the dithio derivative **22**, which was directly oxidized in basic methanol with O_2 to yield cyclic **23** (55% yield). Cyclic disulfide **23** was also obtained in 89% yield from **21** by sequential hydrolysis and oxidation without isolation of **22**. The BOC groups in **23** were removed in near quantitative yield by trifluoroacetic acid (TFA) to give cyclic disulfide **14**. The overall yield for **14** from **15** was 11% (9 steps) and our reaction sequence provided **14** as a single diastereomer (*meso*).

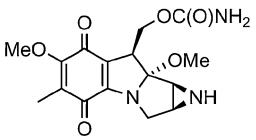


[a]Reference 28. [b]Reference 29.

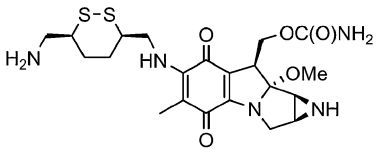
Scheme 2 Synthesis of cyclic disulfide *meso*-**14**.

Synthesis of mitomycin dimers 9, 27, and 28

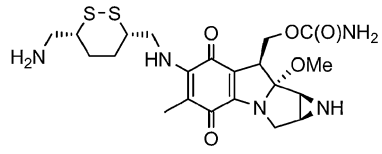
Treatment of mitomycin A[31] (MMA, **24**) with **14** in the presence of Et_3N gave **9** (53% yield) along with the mono-substituted products **25a** and **25b** (21% yield). Several reaction conditions



24
(MMA)



25a

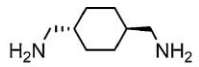


25b

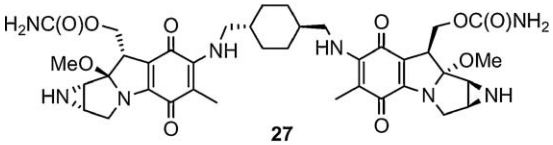
and solvents (*i.e.* dichloromethane, ethanol, methanol, chloroform) were examined for this transformation, and we found that methanol (room temperature, 1 d) gave the best results. In the synthesis of **9**, the solution color changed from violet to dark blue as the reaction proceeded (1 d), with monomers **25a** and **25b** being formed initially and then converted to dimer **9** (TLC and HPLC analyses). On TLC, **25a** and **25b** exhibited lower R_f values (0.15) than dimer **9** (0.47). After 1 day the product ratio of dimer **9** to monomers **25** was *ca.* 2.5. Using HPLC, we observed two peaks of equal intensity for the monomers (t_R 21.8, 22.3 min) that were consistent with the formation of two diastereomers (**25a**, **25b**); only one HPLC peak (t_R 29.8 min) was observed for **9**.

Using similar reaction conditions, we prepared the two dimeric mitomycins, **27** (70% yield) and **28** (59% yield), upon treatment of **24** with 1,4-bis(aminomethyl)cyclohexane (**26**) and **18**, respectively. Compound **27** was chosen as the reference compound for the chemical studies. In **27**, we replaced the dithiane moiety in **9** with a cyclohexyl moiety to determine the importance of the disulfide cleavage processes for

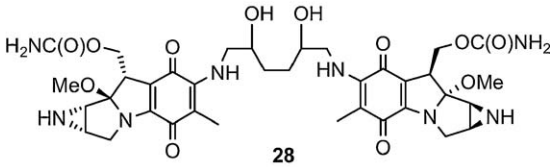
nucleophile-mediated mitomycin activation. We recognized that **27** existed as the *trans*-isomer while **9** was the *cis*-isomer. Our decision to prepare **27** was based on the commercial availability of *trans*-**26**. While this structural difference should not affect



26



27



28

(1.6:1 diastereomeric mixture)

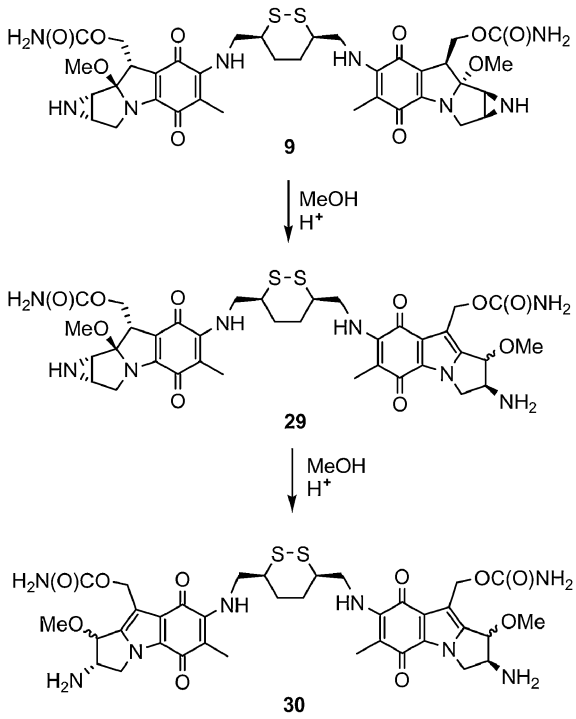
the compounds' reactivities with nucleophiles, it can impact their DNA adduction and cytotoxicity properties. Thus, we used **28** along with **27** as reference compounds for **9** for the biochemical studies. Significantly, the hydroxyl groups in diol **28** are spatially positioned at the same sites as the thiol units in projected intermediates **10** and **12** (Scheme 1). We prepared diastereomeric **28** (1.6 : 1 mixture) by treating **18** (*meso* : *dl* = 2.7 : 1) with **24**. On the basis of the yield obtained for **28** (59%) and the starting composition of **18** we have termed the major isomer in the 1.6 : 1 diastereomeric mixture (^{13}C NMR analysis) as (2″*R*,5″*S*)-**28**.

Chemical reactivity of dimeric mitomycins

We determined the rate of methanolysis of **9** and **27** in the absence of nucleophiles and in the presence of a phosphine and thiols to learn if this mitomycin is activated by nucleophiles (see later in Tables 1 and 2). The kinetic measurements were monitored by UV-vis spectroscopy (200–600 nm) or HPLC using UV-vis detection for greater than two half-lives, when possible, and then the absorbance of the starting mitomycin (374 nm) was plotted against time. We used non-linear regression analysis to fit the observed exponential decay of **9** using the SigmaPlot Program 2001 to provide pseudo-first-order rate constants. The reactions were conducted in duplicate, and the results averaged. The products were verified, when possible, by HPLC and TLC analyses with authentic samples.

The methanolysis of **9** was first monitored by HPLC using a MeOH–$CHCl_3$ (1 : 1) solution (effective 'pH' 3, room temperature, 3 d). The HPLC chromatograms for **9**, **29** and **30** are shown in Fig. 1(a)–(c), respectively, and are distinguished by their distinctive UV-vis profiles, which show either the mitomycin (374 nm, *e.g.* **9**) or mitosene (316 nm, *e.g.* **30**) chromophore[32] or both (*e.g.* **29**). As the reaction proceeded, we observed that **9** disappeared (t_R 29.8 min) and four peaks that corresponded to the four diastereomers for **29** [t_R 29.6, 30.0, 30.3 and 30.8 min (1 : 1.1 : 0.9 : 1)] appeared with the subsequent emergence of three peaks for **30** (t_R 30.6, 31.3 and 32.1 min) in an approximate 1 : 2 : 1 ratio. We have assumed that two of the four **30** diastereomeric adducts overlapped in the HPLC.

Purification of the product mixture by preparative thin layer chromatography (PTLC) afforded an authentic sample of the C(1) methoxymitosenes **30** as a mixture of diastereomers.



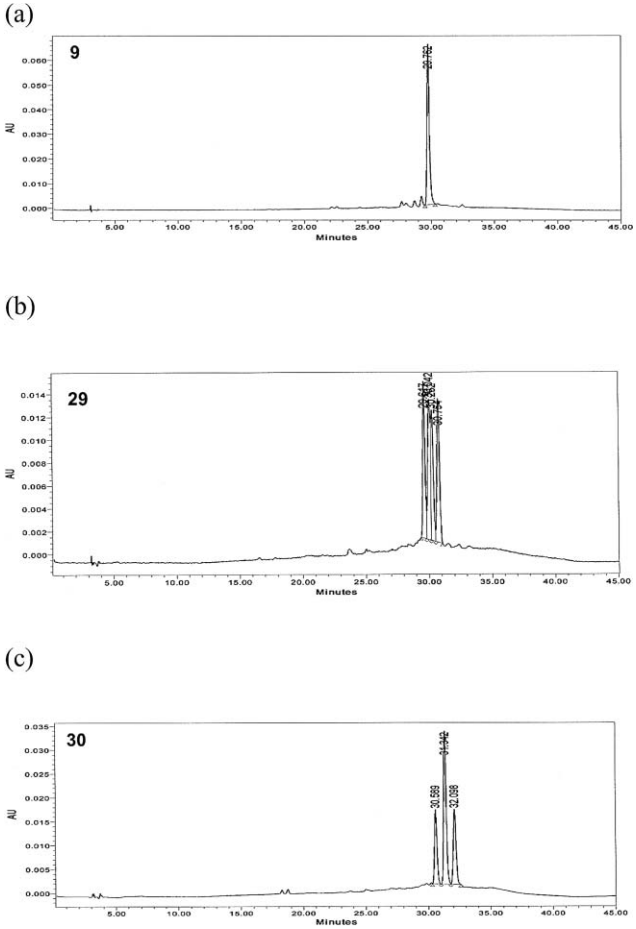


Fig. 1 HPLC profiles of **9** (a), **29** (b) and **30** (c). The reactions were monitored at 374 nm for **9** (t_R 29.8 min) and 316 nm for **29** (t_R 29.6, 30.0, 30.3 and 30.8 min) and **30** (t_R 30.6, 31.3 and 32.1 min).

Solvolytic products **30** were identified by their HPLC, UV-vis, ^{1}H NMR and mass spectrometric properties. In the ^{1}H NMR spectra for **30** we observed the expected resonance (δ 3.51) for the C(1) methoxy units and the downfield resonances (δ 5.73 and 5.78) for the C(10) methylene unit.[33] The expected molecular ion

Table 1 Methanolysis rates for **9** at effective 'pH' 7.4, 5.5, and 4.0[a]

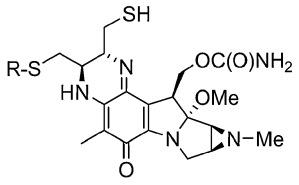
'pH'	k_{obs}/d^{-1}	$t_{1/2}$/d
7.4	0.0347	20[b]
5.5	0.0533	13
4.0	1.65	0.42

[a] Reactions were run in buffered methanolic solution (0.1 M Tris·HCl, pH 7.4; 0.1 M bis-Tris·HCl, pH 5.5; 0.1 M bis-Tris·HCl, pH 4.0) at 25 °C. All reactions were run in duplicate and the values averaged. The concentration of **9** was 0.03 mM and the data were obtained using a Cary 3Bio Varian UV-visible spectrophotometer and the reactions monitored at 374 ± 2 nm unless otherwise indicated. [b] The data were obtained using HPLC.

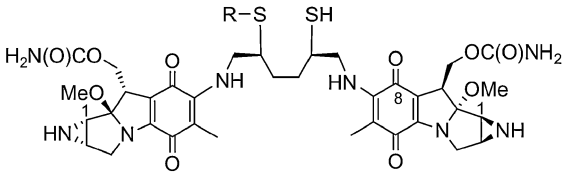
peak (*m*/*z* 813 $[M + 1]^+$) for the C(1) methoxymitosenes **30** was observed in the low-resolution mass spectrum.

We next determined the rate of methanolysis for mitomycin dimer **9** in buffered methanolic solutions at effective 'pH' 7.4, 5.5 and 4.0 in the absence of nucleophiles at 25 °C (Table 1). We observed that reduction of pH led to enhanced solvolysis rates. The observed rate increases were not proportional to the pH. We observed only a modest increase in the conversion of **9** to **29** and to **30** as we reduced the pH from 7.4 to 5.5. The observed half-life ($t_{1/2}$) for **9** at pH 5.5 was comparable with that reported for **1** ($t_{1/2}$ = 13.7 d).[34] These results suggested that **9** underwent acid-catalyzed activation along a pathway similar to that of **1**.[32,35] A more substantial rate increase was observed as the pH was lowered from 5.5 to 4.0. We observed a clear isosbestic point at 337 nm (data not shown). Analysis of the rate data was consistent with our assumption that the methanolysis followed pseudo-first-order kinetics and that the two mitomycin units underwent change independent of one another. Using HPLC we observed the steady depletion of **9**, the appearance of **29** followed by appearance of **30**, and the concomitant disappearance of **29** over a 75 h period (data not shown). When we examined the solvolysis of **27** at pH 7.4 by UV-vis spectroscopy we observed little solvolysis after 10 d.

Since **9** and **27** underwent little change at pH 7.4, we determined if the solvolysis rates of these dimers were increased in the presence of thiols [L-dithiothreitol (L-DTT), and glutathione (GSH)] at pH 7.4 (Table 2). Adding L-DTT (10, 20 equiv.) gave no significant increases (≤5%) in the consumption of either **9** or **27** after 6–10 d, compared with solutions without L-DTT. Likewise, when GSH was used as the nucleophile (20 equiv.) the activation rates of **9** and **27** were not affected (<5%) after 10 d. The similarity of the **9** and **27** rate data and the lack of detectable rate enhancements as the thiol and the thiol concentration were varied led us to conclude that L-DTT and GSH did not contribute to the methanolysis of mitomycins **9** and **27** at pH 7.4. This finding mirrored the low activation level observed for C(8) iminoporfiromycin **6** when L-DTT was added.[23] For **6**, we suggested that the thiol-cleaved C(8) iminoporfiromycin product **31**, if formed, can revert back to **6** due to the ease of formation of the 6-membered ring.[23] A similar pathway may have occurred with the L-DTT–**9** cleaved product **32**.



31 R = L-DTT



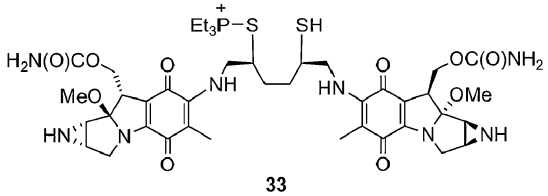
32 R = L-DTT

We next asked if phosphines affected the activation of **9** and **27** (Table 2). Previously, Gates and co-workers showed that Ph_3P triggered the activation of leinamycin.[22] We subsequently showed that Et_3P increased the activation of C(8) iminoporfiromycin **6**.[23] We found that Et_3P (10–50 equiv.) markedly enhanced **9** activation. Rate increases for **9** were only observed upon addition of ≥10 equiv. of Et_3P; a similar finding was observed for **6**.[23] We suspect that this represented the threshold level of Et_3P needed for mitomycin nucleophilic activation under our conditions, and that sub-threshold levels of Et_3P are consumed by the trace amounts of O_2 not purged by Ar. For 10, 20 and 50 equiv. of Et_3P we observed a 77-, 154- and 345-fold increase, respectively, in the rate of mitomycin consumption and the formation of mitosene products (Table 2), documenting that the activation rate was linear with Et_3P concentration. In contrast to **9**, no appreciable rate enhancements (<5%) were observed for **27** upon addition of Et_3P (10–50 equiv.) (Table 2). Our findings that Et_3P affected only the consumption of **9** and not of **27** led us to conclude that the disulfide group in **9** underwent cleavage to thiol **33** thereby activating the appended mitomycin units.

Table 2 Methanolysis rates for **9** and **27** in the presence of nucleophiles at effective 'pH' 7.4[a]

Reagents		**9**		**27**	
Nu^-	Equiv.	k_{obs}/d^{-1}	$t_{1/2}$/d	k_{obs}/d^{-1}	$t_{1/2}$/d
None	—	0.0347[b]	20	[c]	[c]
L-DTT	10	[d]	[d]	[c]	[c]
	20	[d]	[d]	[c]	[c]
GSH	20	[c]	[c]	[c]	[c]
	2	[c]	[c]	—	—
	5	[d]	[d]	—	—
Et_3P	10	2.67	0.26	[cb]	[c]
	20	5.33[e]	0.13	[cb]	[c]
	50	12.0	0.058	[cb]	[c]

[a] Reactions were run in buffered methanolic solution (0.1 M Tris·HCl, pH 7.4) at 25 °C. The reactions were run in duplicate and the values averaged. The data were obtained using a Cary 3Bio Varian UV-visible spectrophotometer and the reactions monitored at 374 ± 3 nm unless otherwise indicated. The concentration of the mitomycin was 0.03 mM. [b] The data were obtained using HPLC. [c] No appreciable change in 10 d (less than 5% of original amount). The unreacted mitomycin was identified by HPLC and TLC. [d] No appreciable change in 6 d (less than 5% of original amount). The unreacted mitomycin was identified by HPLC and TLC. [e] The data were obtained by UV spectroscopy and are in agreement with the HPLC data ($t_{1/2}$ = 0.13 d).



33

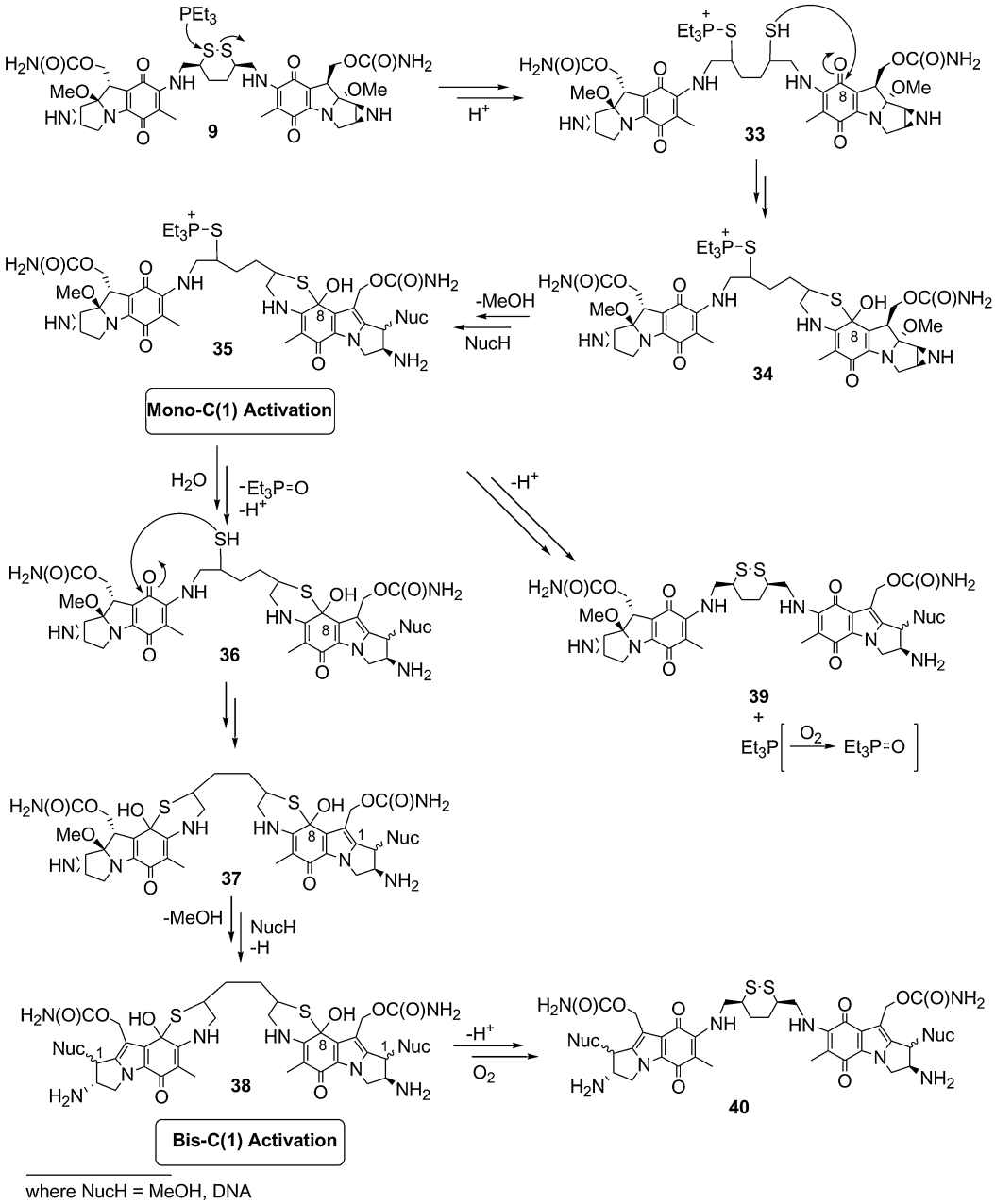
When the reactions of **9** with excess Et_3P were monitored by UV-vis spectroscopy we observed the progressive increase in the 314 nm absorption with time, which was consistent with the formation of mitosene products. Careful inspection of the HPLC chromatograms for the **9** plus Et_3P (10 equiv.) reaction revealed minor amounts of the C(1) substituted products **29** and **30** during the initial stages (0–45 min) of the reaction. The products disappeared with time with the concomitant appearance of a new, unidentified peak (t_R 24.0 min) that showed UV-vis

absorption maxima at 233 and 314 nm (data not shown). When **9** was treated with 20 equiv. of Et_3P we observed only **29** in the HPLC at the initial stages of the reaction followed by the appearance of the unidentified 24 min peak. Several attempts to identify this new peak by LC–MS and after chemical treatment (*e.g.* hydrolysis, oxidation) gave inconclusive results (data not shown).

Our finding that **9** consumption was nearly linear with Et_3P concentration and that Et_3P mediated the conversion of **9** to mitosenes **29** and then to **30** led us to conclude that phosphine cleavage of the disulfide unit in **9** provided thiol(s) (*i.e.* **33**) capable of activating the quinone ring in **33**. The kinetic data further suggest that the disulfide cleavage is the rate-limiting step. In Scheme 3, we propose a phosphine-mediated activation pathway for **9** that is consistent with these findings. This mechanism is similar to that suggested for C(8) iminoporfiromycin **6**.[23] The generation of thiol **33** is key to this pathway. We expect that **33** undergoes intramolecular cyclization to give hemi-thioketal **34** leading to the disruption of the N(4)–C(4a)–C(8a)–C(8)–O conjugated system. Mitosene formation and aziridine ring-opening then occur to give the C(1)-activated product **35**. A second round of activation is initiated after the decomposition of the thiophosphonium species **35** by H_2O (or MeOH)[36,37] to provide thiol **36**. Similar to the conversion of **33** to **35**, intramolecular cyclization of thiol **36** leads to **37** and then **38**, in which both C(1) sites in the two distal mitomycin units have been modified. The reaction is completed when **38** converts to **40**. Our pathway derives support from recent studies showing that Et_3P rapidly promotes L-DTT disulfide cleavage,[37] and comparable activation results obtained for C(8) iminoporfiromycin **6** with phosphines.[23]

DNA bonding profiles for mitomycin dimers 9, 27, and 28

The ability of dimeric mitomycins **9**, **27** and **28** to cross-link complementary *Eco*RI-linearized pBR322 DNA was determined using denaturing alkaline agarose gel electrophoresis as reported by Cech[38a] and Tepe and Williams.[38b] The size of the DNA product(s) was estimated using λ DNA digested with *Hin*dIII as a molecular weight marker. The extent of DNA ISC formation for **9**, **27** and **28** was determined in the absence and the presence of Et_3P and L-DTT. We also included **1** and **3** in this study.



Scheme 3 Proposed phosphine-mediated activation pathway for **9**.

We first examined the extent of **9** DNA ISC induced by Et_3P for varying concentrations of **9** (0.1–0.01 mM) and Et_3P (5, 10 equiv.). The experiments were run at room temperature (2 h) in aqueous buffered solutions (pH 7.4). We found that treatment of 0.1 mM concentrations of **9** with Et_3P provided appreciable levels of DNA ISC adducts, and we used this mitomycin concentration in our subsequent studies. Next, we added Et_3P (5 equiv.) and compared the extent of DNA ISC for **9** with those obtained for **1**, **27** and **28** (Fig. 2). We observed that **9** efficiently generated DNA ISC (83%) while **27** and **28** generated 14 and 21%, respectively. Under these conditions, **1** gave 5% of DNA ISC. These findings differentiate dimeric mitomycins **9**, **27**, and **28** from their monomeric counterpart **1**, and distinguish dimeric mitomycins that contain a disulfide-containing linker (**9**) from those that do not (**27**, **28**). Dimers **9**, **27**, and **28** all showed higher levels (14–83%) of DNA ISC than **1** (5%). Since **1** can only generate DNA ISC by sequential adduction at C(1) and C(10) but **9**, **27**, and **28** can cross-link complementary strands by DNA adduction processes that occur at the two mitomycins, we have tentatively attributed the increased levels of DNA ISC for the dimers, compared with **1**, in part, to C(1) modification processes. This finding is in agreement with our earlier studies that **2** gave enhanced levels of DNA ISC compared with **1** under reductive ($Na_2S_2O_4$) conditions[6] and was supported by the recent study of Tomasz and co-workers.[7] Significantly, when we compared the extent of DNA ISC for **9**, **27**, and **28** we observed a 4.0–5.9-fold increase in DNA ISC upon inclusion of a disulfide unit in the linker. We have attributed the enhanced level of DNA ISC for **9**, in part, to the functional role provided by the disulfide unit in the Et_3P-mediated mitomycin activation process (Scheme 3). Significantly, the extent of DNA ISC for **9** (0.1 mM) under Et_3P-mediated conditions exceeded those reported for dimeric mitomycins **2** (0.2 mM) under reductive conditions ($Na_2S_2O_4$).[6]

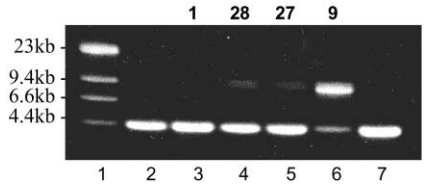


Fig. 2 Denaturing 1.2% alkaline agarose gel for **1**, **9**, **27** and **28** (0.1 mM) using Et_3P (5 equiv.). DNA cross-linking experiments using 0.1 mM concentrations and *Eco*RI-linearized pBR322 plasmid DNA with Et_3P (5 equiv.). All reactions were incubated at room temperature (2 h). Lane 1: λ *Hin*dIII DNA molecular weight marker. Lane 2: control (only linearized pBR322). Lane 3: **1** + Et_3P (5 equiv.). Lane 4: **28** + Et_3P (5 equiv.). Lane 5: **27** + Et_3P (5 equiv.). Lane 6: **9** + Et_3P (5 equiv.). Lane 7: only Et_3P (5 equiv.).

We also examined the effect of L-DTT on mitomycin **9**–DNA ISC and compared these findings with **1**, **27** and **28**. Using the conditions established for Et_3P, we treated aqueous buffered solutions (pH 7.4) containing **1**, **9**, **27** and **28** (0.1 mM) and DNA with L-DTT (5 equiv.) at room temperature (2 h) (Fig. 3). We found that L-DTT (5 equiv.) led to detectably higher levels of DNA ISC for **9** (77%) than **27** (24%) or **28** (31%). Significantly, we observed noticeable amounts of DNA ISC for dimers **27** (24%) and **28** (31%) using L-DTT, and these values exceeded those seen with Et_3P (Fig. 2). Dimers **27** and **28** cannot undergo disulfide activation yet DNA ISC adducts were observed. We are uncertain if the observed differences in the DNA ISC levels for **9** and **27** (**28**) are due to L-DTT-mediated cleavage of the disulfide unit in **9**. The kinetic experiments showed that the consumption of **9** and **27** were not affected by L-DTT (10 equiv.) (Table 2). Thus, we have attributed the **27** and **1** DNA ISC products, in part, to trace levels of L-DTT activation pathways other than the one outlined for **9**, which proceeded through disulfide cleavage (Scheme 3). Among these, the most likely routes are quinone reductive and base-mediated C(9) proton abstraction pathways. Our finding that L-DTT treatment of **9** led to substantial levels of DNA ISC (Fig. 3) yet provided only trace levels of mitosene products (Table 2) documented the sensitivity of the denaturing agarose gel electrophoresis assay. Generation of only a single DNA ISC within the 4361 bp *Eco*RI pBR322 DNA leads to a marked change in the electrophoretic mobility of the DNA adduct.

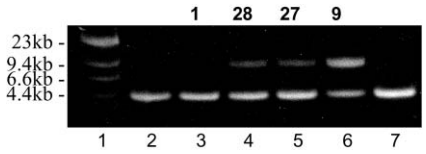


Fig. 3 Denaturing 1.2% alkaline agarose gel for **1**, **9**, **27** and **28** (0.1 mM) using L-DTT (5 equiv.). DNA cross-linking experiments using 0.1 mM mitomycins and *Eco*RI-linearized pBR322 plasmid DNA with L-DTT (5 equiv.). All reactions were incubated at room temperature (2 h). Lane 1: λ *Hin*dIII DNA molecular weight marker. Lane 2: control (only linearized pBR322). Lane 3: **1** + L-DTT (5 equiv.). Lane 4: **28** + L-DTT (5 equiv.). Lane 5: **27** + L-DTT (5 equiv.). Lane 6: **9** + L-DTT (5 equiv.). Lane 7: only L-DTT (5 equiv.).

Similar DNA-bonding profiles were obtained when the reaction time was reduced from 2 h to 6 min and the amount of nucleophile was increased from 5 to 10 equiv. For Et_3P, we observed that **9** gave moderate levels of DNA ISC (58%), and the extent of DNA ISC for **27**, **28**, and **1** was 8, 12%, and 3%, respectively (data not shown). For L-DTT (10 equiv.), we found that **9** gave moderate levels of DNA ISC (30%), and **27**, **28**, and **1** gave reduced levels, 13, 16, and 3%, respectively (data not shown).

Mitomycin **9** was more efficient than **1** in generating DNA ISC upon treatment with Et_3P (Fig. 2). But **9** is a dimeric mitomycin with an appended disulfide unit and **1** is a monomeric mitomycin without an attached disulfide moiety. Thus, we compared **1** with **3** to determine the importance of the C(7) aminoethylene disulfide unit for DNA ISC. Significantly, McAdam *et al.* showed that **3** upon treatment with thiols provided DNA ISC.[25b] Treatment (6 min) of 0.1 mM solutions of **1** and **3** with Et_3P (10 equiv.) gave levels of DNA ISC corresponding to 5 and 14%, respectively (data not shown). Since the effective monomeric mitomycin unit concentration of a 0.1 mM solution of dimeric **9** is twice that of a 0.1 mM solution of **3**, the observed 14% DNA ISC for **3** will likely increase with increasing **3** concentration. Nonetheless, the 58% DNA ISC found for **9** indicates that the two mitomycin units and the cyclic disulfide bridge contributed to the DNA ISC efficiency of this agent.

The effect of Et_3P on **9** and **27** DNA ISC transformations paralleled the observed consumption rates for these mitomycins (Table 2). However, the effect of Et_3P on the kinetic data appeared greater than its effect on the extent of DNA ISC formation. The DNA ISC experiments did not permit us to measure the number of ISC adducts within *Eco*RI DNA (4631 bp) for **9**, **27** or **28**. Thus, the true effect of Et_3P in these experiments remains unknown. Moreover, we have not determined the site(s) of mitomycin covalent adduction [*i.e.* C(1), C(10)]. Dimeric mitomycin activation may have given tri- and tetra-functionalized adducts,[7] and DNA products where linkages exist between duplexes. These questions await further study. Nonetheless, we concluded that the enhanced levels of DNA ISC for **9** compared with **27**, **28**, and **3** stemmed, in part, from nucleophile-mediated mitomycin activation processes leading to DNA adduction (Scheme 3).

Cytotoxicities

Using an antiproliferative activity assay we tested whether mitomycin dimer **9** inhibited tumor cell line replication.[39] As discussed earlier, we did not use the IC_{50} values as a measure of the extent and efficiency of DNA ISC for the mitomycins in this test group since we did not determine the relative

Table 3 Antiproliferative activities for mitomycin dimers **9**, **27**, and **28** [a]

Compound	IC_{50}/μmol L^{-1}		
	Aerobic	Hypoxic	IC_{50} (hypoxic)/IC_{50} (aerobic)
9	41	>100	>2.4
27	51	>100	>2.0
28	63	>100	>1.6
1	2.4	20	8.2
3	0.12	0.41	3.4

[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.

levels of the mitomycins within the cells and the factors that contributed to the inhibition of cell replication. The *in vitro* antiproliferative activity tests were conducted at the Kyowa Hakko Pharmaceutical Company (Shizuoka, Japan) using the human tumor cell line A549 (lung adenocarcinoma). This cell line was chosen because of its sensitivity to KW-2149 (**3**). Since compound **9** was designed to undergo non-reductive, acidic, and nucleophile-mediated activation, we were also interested in evaluating the antiproliferative activities of this compound under both aerobic and hypoxic conditions and determining the activity ratio [IC_{50} (hypoxic)/IC_{50} (aerobic)] of **9** compared with **1**, **3**, **27** and **28**.

The results for *in vitro* antiproliferative activity tests for mitomycin dimer **9** and reference compounds **1**, **3**, **27** and **28** are listed in Table 3. We found that under aerobic and hypoxic conditions, **9** was 17-fold and >5-fold, respectively, less potent than its monomer prototype, mitomycin C (**1**). Similar differences were previously observed for dimeric mitomycins **2** tethered by alkyl linkers with a central heteroatom [N(H), O] against the A549 tumor cell line.[6] The activity ratio (hypoxic/aerobic) for **9** was >2.4 while for **1** and **3** it was 8.2 and 3.4, respectively. When **9** was compared with **27** and **28**, we found small differences under aerobic and hypoxic conditions. The activity ratios (hypoxic/aerobic) for **9**, **27** and **28** appeared to be similar (>1.6 to >2.4) and these values did not permit us to differentiate the compounds. Consequently, we have concluded that for the A549 cell line incorporating the disulfide unit within the dimeric assembly did not measurably contribute to cell death and that the advantages of this unit observed for nucleophile-mediated mitomycin activation and DNA adduction processes did not extend to cell cytotoxicity.

Conclusions

In this study, the synthesis and evaluation of the novel mitomycin dimer **9** is reported. This mitomycin contained four key structural elements that were expected to improve drug activation and DNA adduction upon nucleophilic activation. First, there was the inclusion of two mitomycin units in **9**. This feature permits DNA adduction processes to occur at the two reactive mitomycin C(1) sites. Second, there was the disulfide moiety. Cleavage of this unit in **3** and **4** is likely to be responsible for the activities of these mitomycins in **1**-resistant and non-hypoxic cells.[14–16] Similarly, nucleophilic cleavage of the disulfide unit in **9** generates a thiol species ideally positioned to render the ring system vulnerable to nucleophilic attack (Scheme 1, **10**→**11**, **12**→**13**). Third, the disulfide unit was incorporated within a dithiane permitting the two mitomcyin moieties to remain tethered upon disulfide cleavage. This feature differentiated **9** from **3** and **4**, in which disulfide cleavage leads to monomeric mitomycins. Fourth, the dithiane linker allowed for guanines on complementary DNA strands to modify the two distal mitomycin C(1) sites to give DNA ISC adducts.

We compared the rate of methanolysis of **9** with the cyclohexyl-linked dimeric mitomycin **27** in the presence and absence of nucleophiles. Et_3P markedly increased the rate of **9** consumption and mitosene product formation but not for **27**. The kinetic studies indicated that Et_3P-mediated **9** activation proceeded by phosphine attack at the disulfide unit to give **33** (Scheme 3) and was supported by a previous investigation using DTT and Et_3P.[37]

The efficiency of mitomycin nucleophile-mediated activation processes for DNA ISC was determined using pBR322 DNA and a denaturing alkaline agarose gel electrophoresis assay.[38] We found that **9** generated higher levels of DNA ISC adducts upon Et_3P addition than did the reference compounds **1**, **3**, **27**, and **28**. The extent of DNA ISC for **9** with Et_3P exceeded that of dimeric mitomycins **2** under reductive conditions.[6] The higher levels of DNA ISC adducts for **9** compared with dimeric mitomycin **27** paralleled the rates of mitomycin consumption observed in the kinetic studies. Taken together, these findings indicated that the proposed nucleophile-mediated mechanism for disulfide cleavage (Scheme 3) contributed to the increased levels of DNA ISC. This pathway complements the reductive and acid-catalyzed DNA ISC pathways previously established for the mitomycins.[2,40] The DNA ISC efficiency of this non-reductive activation route for **9** warrants the further investigation of this transformation where the sequence specificity, site of mitomycin adduction [*i.e.* C(1), C(10)], and the extent of inter-duplex cross-linking products are determined.

Experimental

General

Mitomycins A (**24**) and C (**1**) used in this study were generously provided by Kyowa Hakko Co., Ltd. (Shizuoka, Japan). ^1H and ^{13}C NMR spectra were recorded on a General Electric QE-300 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 of aqueous solutions were determined on a Radiometer pHM26 meter using a Radiometer pHC4000 glass electrode. The effective 'pH' of the buffered methanolic solutions was similarly determined and the meter and electrode was standardized against aqueous buffer solutions.

LC–MS analyses were conducted with Agilent 1100 LC/MSD by Dr Voyksner (LCMS Limited, Raleigh, NC). 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^{-1}, and the eluent was monitored at 365 and 313 nm. The mass spectral mode of operation was positive ion electrospray (+ESI) and scan range was 300–1900 Da with 45 psi of nebulization pressure.

1,6-Diamino-2,5-hexanediol dihydrochloride (18) (ref. 29). A mixture of 1,6-bis(phthalimido)-2,5-hexanediol (**17**, *meso* : *dl* = 2.7 : 1, 867 mg, 2.1 mmol), EtOH (19 mL) and $NH_2NH_2{\cdot}H_2O$ (0.26 mL, 5.3 mmol) was heated under reflux (3 h). After cooling at room temperature, the solvent was removed *in vacuo*. H_2O (22 mL) and concentrated HCl (11 mL) was added to the residue and the mixture was heated under reflux (1 h). After cooling to 0 °C, the precipitate was removed by filtration. The filtrate was then concentrated under reduced pressure and the wet residue was dissolved in H_2O (30 mL). A small amount of insoluble matter was removed by filtration and the clear filtrate was concentrated under reduced pressure to afford **18**

(yellow solid) as a mixture of diastereomers (*meso* : *dl* = 2.7 : 1, ^{13}C NMR analysis): yield, 469 mg (*ca.* 100%); mp 227–240 °C [lit.,[29] bp (free amine) 130 °C (2 mm Hg)]; ν_{max} (KBr)/cm^{-1} 3381, 3069, 2952, 1560, 1260, 1042 and 977; δ_H (300 MHz; CD_3OD–D_2O) 1.43–1.74 (m, 4 H, CH_2CH_2), 2.77–2.88 (m, 2 H, C*H*H′N), 3.07 (app d, *J* = 12.6 Hz, 2 H, CH*H*′N), and 3.81 (br s, 2 H, C*H*OH), the ^{1}H NMR data were in agreement with the COSY spectrum; δ_C (75 MHz; CD_3OD–D_2O) for the major diastereomer, 31.4 (*C*H_2CH), 45.7 (CH_2N), and 68.7 (CHOH); δ_C (75 MHz; CD_3OD–D_2O) for the minor diastereomer, 31.1 (*C*H_2CH), and 68.3 (CHOH), one signal was not detected and is believed to overlap with one of the observed peaks; *m*/*z* (+CI) 149 ([M + 1]$^+$, 100%).

1,6-Bis(*tert*-butyloxycarbonylamino)-2,5-hexanediol (19). To a stirred solution of 1,6-diamino-2,5-hexanediol dihydrochloride (**18**, 222 mg, 1.0 mmol, *meso* : *dl* = 2.7 : 1) and Et_3N (0.84 mL, 6.0 mmol) in H_2O–DMF (1 : 1, 6 mL) was added a solution of di-*tert*-butyl dicarbonate (BOC_2O) (448 mg, 2.2 mmol) in DMF (2 mL). After warming to 50 °C, stirring was continued (5 h) and then the solvent was removed *in vacuo*. H_2O (40 mL) was added to the residue and the mixture was extracted with EtOAc (2 × 40 mL). The combined organic layers were successively washed with aqueous 0.1 N HCl (40 mL), saturated aqueous $NaHCO_3$ (40 mL) and H_2O (40 mL). The organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. Purification by PTLC (2 : 1 EtOAc/hexanes) afforded **19** (white solid) as a mixture of diastereomers (*meso* : *dl* = 3.5 : 1, ^{13}C NMR analysis): yield, 230 mg (66%); mp 115–120 °C; R_f 0.15 (2 : 1 EtOAc/hexanes); ν_{max} (KBr)/cm^{-1} 3373, 2964, 2927, 1694, 1525, 1276, 1173, 1003, and 642; δ_H (300 MHz; $CDCl_3$) 1.34–1.69 [m, 22 H, CH_2CH_2, $OC(CH_3)_3$], 2.95–3.16 (m, 2 H, C*H*H′N), 3.24 (app d, *J* = 13.5 Hz, 2 H, CH*H*′N), 3.55 (br s, 2 H, CHO*H*), 3.71 (br s, 2 H, C*H*OH), and 5.10 (br s, 2 H, NHCO), the ^{1}H NMR data were in agreement with the COSY spectrum; δ_C (75 MHz; $CDCl_3$–CD_3OD) for the major diastereomer, 28.4 [OC(*C*H_3)$_3$], 30.4 (*C*H_2CH), 46.5 (CH_2N), 71.2 (CHOH), 79.7 [O*C*(CH_3)$_3$], and 157.0 (NHCO); δ_C (75 MHz; $CDCl_3$–CD_3OD) for the minor diastereomer, 31.3 (*C*H_2CH), 46.7 (CH_2N), and 71.5 (CHOH), the other signals were not detected and are believed to overlap with the observed peaks; *m*/*z* (+CI) *m*/*z* 349 ([M + 1]$^+$, 100%); HRMS (+CI) $C_{16}H_{32}N_2O_6$ [M + 1]$^+$ requires 349.23386; found 349.23347.

Resolution of 1,6-bis(*tert*-butyloxycarbonylamino)-2,5-hexanediol (19) by fractional recrystallization. A mixture of the purified product [**19**, 220 mg, a mixture of diastereomers (*meso* : *dl* = 3.5 : 1, ^{13}C NMR analysis), mp 115–120 °C] and EtOAc (15 mL) was heated to 45 °C and then filtered to remove the insoluble impurities. The solution was allowed to stand overnight (room temperature) leading to the precipitation of a white solid. The precipitate was filtered and washed with hexanes to give resolved **19** as an apparent single diastereomer (*meso*, ^{13}C NMR analysis): yield, 141 mg (64%); mp 124–126 °C; δ_H (300 MHz; $CDCl_3$) 1.32–1.68 [m, 22 H, CH_2CH_2, $OC(CH_3)_3$], 2.94–3.14 (m, 2 H, C*H*H′N), 3.24 (app d, *J* = 13.5 Hz, 2 H, CH*H*′N), 3.54 (br s, 2 H, CHO*H*), 3.70 (br s, 2 H, C*H*OH), and 5.12 (br s, 2 H, NHCO); δ_C (75 MHz; $CDCl_3$–CD_3OD) 28.1 [OC(*C*H_3)$_3$], 30.0 (*C*H_2CH), 45.8 (CH_2N), 70.5 (CHOH), 79.4 [O*C*(CH_3)$_3$], and 156.9 (NHCO).

(2*R*,5*S*)-*meso*-1,6-Bis(*tert*-butyloxycarbonylamino)-2,5-hexanediol dimethanesulfonate (*meso*-20). To a cooled (0 °C) solution of 1,6-bis(*tert*-butyloxycarbonyl)amino-2,5-hexanediol [**19**, 139 mg, 0.4 mmol, a mixture of diastereomers (*meso* : *dl* = 3.5 : 1)] in pyridine (1.5 mL) was slowly added methanesulfonyl chloride (105 μL, 1.4 mmol) for 20 min. After warming to room temperature, stirring was continued (3 h) and then the mixture was poured into a cooled aqueous 2 M HCl solution (15 mL) leading to the precipitation of a white solid. After stirring at 0 °C (1 h), the reaction mixture was filtered and successively washed with H_2O (10 mL) and EtOH (2 mL) to afford **20** (white solid) as an apparent single diastereomer (*meso*, ^{13}C NMR analysis): yield, 135 mg (67%); mp 235–243 °C; R_f 0.51 (2 : 1 EtOAc/hexanes); ν_{max}(KBr)/cm^{-1} 3345, 2983, 2937, 1685, 1533, 1347, 1173, and 911; δ_H (300 MHz; $CDCl_3$) 1.42 [s, 18 H, $OC(CH_3)_3$], 1.82 (br s, 4 H, CH_2CH_2), 3.06 (s, 6 H, OMs), 3.25–3.48 (m, 4 H, CH_2N), 4.72 (br s, 2 H, C*H*OMs), and 5.04 (br s, 2 H, NHCO); δ_C (75 MHz; $CDCl_3$) 27.3 (*C*H_2CH), 28.3 [OC(*C*H_3)$_3$], 38.4 (OMs), 43.5 (CH_2N), 80.0 [O*C*(CH_3)$_3$], 80.9 (*C*HOMs), and 156.0 (NHCO); *m*/*z* (+CI) 505 ([M + 1]$^+$, 100%); HRMS (+CI) $C_{18}H_{37}N_2O_{10}S_2$ [M + 1]$^+$ requires 505.18896; found 505.18777.

(2*S*,5*R*)-*meso*-1,6-Bis(*tert*-butyloxycarbonylamino)-2,5-bis(acetylthio)hexane (*meso*-21). To a stirred solution of (2*R*,5*S*)-*meso*-1,6-bis(*tert*-butyloxycarbonylamino)-2,5-hexanediol dimethanesulfonate (*meso*-**20**, 107 mg, 0.21 mmol) in DMF (2.5 mL) was added KSAc (54 mg, 0.48 mmol). After warming to 60 °C, stirring was continued (3 h) and then the solvent was removed *in vacuo*. H_2O (40 mL) was added to the residue and the mixture was extracted with EtOAc (2 × 40 mL). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. Purification by PTLC (1 : 2 EtOAc/hexanes) afforded **21** (white solid) as an apparent single diastereomer (*meso*, ^{13}C NMR analysis): yield, 59 mg (60%); mp 122–125 °C; R_f 0.50 (1 : 2 EtOAc/hexanes); ν_{max} (KBr)/cm^{-1} 3378, 2979, 2928, 1693, 1518, 1260, and 1169; δ_H (300 MHz; $CDCl_3$) 1.41 [s, 18 H, $OC(CH_3)_3$], 1.50–1.62 (m, 2 H, C*H*H′CH), 1.70–1.87 (m, 2 H, CH*H*′CH), 2.30 (s, 6 H, SAc), 3.15–3.39 (m, 4 H, CH_2N), 3.43–3.58 (m, 2 H, C*H*SAc), and 4.89 (br s, 2 H, NHCO), the ^{1}H NMR data were in agreement with the COSY spectrum; δ_C (75 MHz; $CDCl_3$) 28.3 [OC(*C*H_3)$_3$], 28.8 (*C*H_2CH), 30.7 (CO*C*H_3), 43.8 (CH_2N), 44.9 (*C*HSAc), 79.4 [O*C*(CH_3)$_3$], 155.9 (NHCO), and 195.2 (*C*OCH_3); *m*/*z* (+CI) 465 ([M + 1]$^+$, 100%); HRMS (+CI) $C_{20}H_{37}N_2O_6S_2$ [M + 1]$^+$ requires 465.20931, found 465.20927.

(3*R*,6*S*)-*meso*-3,6-Bis(*tert*-butyloxycarbonylaminomethyl)-1,2-dithiane (*meso*-23). To a stirred solution of (2*S*,5*R*)-*meso*-1,6-bis(*tert*-butyloxycarbonylamino)-2,5-bis(acetylthio)hexane (*meso*-**21**, 40 mg, 0.086 mmol) in MeOH–H_2O (5 : 1, 4.4 mL) was added K_2CO_3 (70 mg, 0.51 mmol). After stirring at room temperature (30 min), KOH (10 mg, 0.18 mmol) was added and O_2 was bubbled through the solution (5 h). The solvent was removed *in vacuo* and H_2O (15 mL) was added to the residue. The mixture was extracted with EtOAc (2 × 15 mL) and the combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. Purification by PTLC (1 : 2 EtOAc/hexanes) afforded **23** (white solid) as an apparent single diastereomer (*cis*, ^{13}C NMR analysis): yield, 29 mg (89%); mp 118–120 °C; R_f 0.55 (1 : 2 EtOAc/hexanes); ν_{max} (KBr)/cm^{-1} 3376, 2976, 2926, 1691, 1524, 1252, and 1169; δ_H (300 MHz; $CDCl_3$) 1.45 [s, 18 H, $OC(CH_3)_3$], 1.73–2.11 (m, 4 H, CH_2CH_2), 2.87–3.02 (m, 2 H, CHS), 3.31–3.50 (m, 4 H, CH_2N), and 4.88 (br s, 2 H, NHCO), the ^{1}H NMR data were in agreement with the COSY spectrum; δ_C (75 MHz; $CDCl_3$) 28.3 [OC(*C*H_3)$_3$], 28.5 (*C*H_2CH), 42.5 (CH_2N), 44.2 (CHS), 79.6 [O*C*(CH_3)$_3$], and 155.8 (NHCO); *m*/*z* (+CI) 379 ([M + 1]$^+$, 100%) HRMS (+CI) $C_{16}H_{31}N_2O_4S_2$ [M + 1]$^+$ requires 379.17253, found 379.17198.

(3*R*,6*S*)-*meso*-3,6-Bis(aminomethyl)-1,2-dithiane di-trifluoroacetate salt (*meso*-14). (3*R*,6*S*)-*meso*-3,6-Bis(*tert*-butyloxycarbonylamino)-1,2-dithiane (*meso*-**23**, 14 mg, 0.04 mmol) was dissolved in trifluoroacetic acid (1.5 mL) and stirring was continued at room temperature (30 min). The reaction was concentrated *in vacuo* to afford **14** as a viscous oil: yield, 15 mg (*ca.* 100%); ν_{max} (neat)/cm^{-1} 2924, 1677, 1194, 1136, 838, and 722; δ_H (300 MHz; D_2O) 1.80–2.42 (m, 4 H, CH_2CH_2), and 3.15–3.70 (m, 6 H, NCH_2CH); δ_C (75 MHz; D_2O) 29.5 (*C*H_2CH), 42.1 (CH_2N), and 42.6 (CHS); *m*/*z* (+CI) 179 ([M − 2TFA + 1]$^+$,

100%); HRMS (+CI) $C_6H_{15}N_2S_2$ [M − 2TFA + 1]$^+$ requires 179.06767, found 179.06685.

Procedure for mitomycins 9 and 25. To an anhydrous methanolic solution (1 mL) of *meso*-14 (2.0 mg, 0.005 mmol) and triethylamine (4 μL, 0.03 mmol) was added **24** (3.4 mg, 0.01 mmol). The reaction solution was stirred at room temperature (1 d) and then the solvent was removed *in vacuo*. Purification of the reaction mixture by PTLC (20% MeOH–$CHCl_3$) afforded the desired products.

*7-N,7′-N′-(1″,2″-Dithianyl-3″,6″-dimethylenyl)bismitomycin C (**9**).* Yield, 2.2 mg (53%); HPLC t_R 29.8 min; R_f 0.47 (20% MeOH–$CHCl_3$); λ_{max} ($CHCN–H_2O$)/nm 222, and 374; δ_H (300 MHz; pyridine-d_5) 1.83–2.04 [m, 4 H, C(3′)H_2], 2.14 [s, 6 H, C(6)CH_3], 2.75 [d, J = 3.9 Hz, 2 H, C(2)H], 3.15 [d, J = 3.9 Hz, 2 H, C(1)H], 3.22 [s, 6 H, C(9a)OCH_3], 3.62 [br d, J = 12.6 Hz, 2 H, C(3)HH′], 3.83–3.95 [m, 4 H, C(1′)H_2], 4.02 [dd, J = 10.8, 3.9 Hz, 2 H, C(9)H], 4.56 [d, J = 12.6 Hz, 2 H, C(3)HH′], 5.06 [dd, J = 10.8, 10.2 Hz, 2 H, C(10)HH′], and 5.41 [dd, J = 10.2, 3.9 Hz, 2 H, C(10)HH′], the signals for the C(2′)H, C(7)NH, C(10)OC(O)NH_2 and N(1a)H protons were not detected and are believed to overlap with the observed peaks, the ^{1}H NMR data were in agreement with the COSY spectrum; δ_C (75 MHz; pyridine-d_5) 11.7 [C(6)CH$_3$], 30.2 [C(3′)], 34.4 [C(2)], 38.4 [C(1)], 46.0 [C(9)], 46.7 [C(2′)], 48.1 [C(1′)], 51.3 [C(9a)OCH$_3$], 52.3 [C(3)], 64.1 [C(10)], 106.3 [C(6)], 108.6 [C(9a)], 112.6 [C(8a)], 148.7 [C(7)], 157.4 [C(4a)], 159.8 [C(10a)], 178.5 [C(8)], and 180.9 [C(5)]; m/z (+FAB) 813 ([M + 1]$^+$, 100%); HRMS (+FAB) $C_{36}H_{45}N_8O_{10}S_2$ [M + 1]$^+$ requires 813.27001, found 813.26906.

*7-N-(6′-Aminomethyl-1′,2′-dithianyl-3′-methylenyl)mitomycin C (**25a** and **25b**).* Yield, 0.5 mg (21%); HPLC t_R 21.8, 22.3 min (two peaks, 1 : 1); R_f 0.15 (20% MeOH–$CHCl_3$); λ_{max} ($CH_3CN–H_2O$)/nm 222 and 374; δ_H (300 MHz; pyridine-d_5) 1.75–2.06 [m, 4 H, C(3′)H_2, C(4′)H_2], 2.13 [s, 3 H, C(6)CH_3], 3.14 [d, J = 4.8 Hz, 1 H, C(1)H], 3.21 [s, 3 H, C(9a)OCH_3], 3.22–3.38 [m, 3 H, C(2′)H, C(6′)H_2], 3.62 [br d, J = 12.9 Hz, 2 H, C(3)HH′], 3.78–3.95 [m, 2 H, C(1′)H_2], 3.99 [dd, J = 10.8, 3.9 Hz, 1 H, C(9)H], 4.56 [d, J = 12.9 Hz, 1 H, C(3)HH′], 5.06 [dd, J = 10.8, 10.2 Hz, 1 H, C(10)HH′], and 5.41 [dd, J = 10.2, 3.9 Hz, 1 H, C(10)HH′], the signals for the C(5′)H, C(7)NH, C(10)OC(O)NH_2 and N(1a)H protons were not detected and are believed to overlap with the observed peaks, the signal for the C(2)H proton overlapped with the $(CH_3CH_2)_3N$ peak; m/z (+FAB) 496 ([M + 1]$^+$, 100%); HRMS (+FAB) $C_{21}H_{30}N_5O_5S_2$ [M + 1]$^+$ requires 496.16884, found 496.17002.

7-*N*,7′-*N*′-(Cyclohexanyl-*trans*-1″,4″-dimethylenyl)bismitomycin C (27). Using the procedure employed for **9** and MeOH (2 mL), **26** (a mixture of diastereomers: *cis* : *trans* = 1 : 10, ^{13}C NMR analysis, 3.1 mg, 0.02 mmol), and **24** (15 mg, 0.043 mmol) gave **27** (12 mg, 70%) after PTLC purification (10% MeOH–$CHCl_3$); HPLC t_R 30.1 min; R_f 0.14 (10% MeOH–$CHCl_3$); λ_{max} ($CH_3CN–H_2O$)/nm 223 and 374; δ_H (300 MHz; $CDCl_3$) 0.98–1.09 [m, 4 H, C(3′)HH′], 1.58–1.78 [m, 2 H, C(2′)H], 1.86 [d, J = 7.5 Hz, 4 H, C(3′)HH′], 2.00 [s, 6 H, C(6)CH_3], 2.82 [d, J = 3.9 Hz, 2 H, C(2)H], 2.90 [d, J = 3.9 Hz, 2 H, C(1)H], 3.21 [s, 6 H, C(9a)OCH_3], 3.38 [t, J = 5.8 Hz, 4 H, C(1′)H_2], 3.52 [br d, J = 13.0 Hz, 2 H, C(3)HH′], 3.60 [dd, J = 10.8, 4.3 Hz, 2 H, C(9)H], 4.30 [d, J = 13.0 Hz, 2 H, C(3)HH′], 4.49 [dd, J = 10.8, 10.2 Hz, 2 H, C(10)HH′], 4.70 [dd, J = 10.2, 4.3 Hz, 2 H, C(10)HH′], and 6.44 [t, J = 5.8 Hz, 2 H, C(7)NH], the signals for the C(10)OC(O)NH_2 and N(1a)H protons were not detected and are believed to overlap with the observed peaks; δ_C (75 MHz; $CDCl_3$) 9.9 [C(6)CH$_3$], 30.0 [C(3′)], 32.6 [C(2)], 36.5 [C(1)], 39.2 [C(2′)], 42.8 [C(9)], 49.8 [C(1′), C(9a)OCH$_3$], 51.1 [C(3)], 62.6 [C(10)], 103.6 [C(6)], 106.2 [C(9a)], 109.7 [C(8a)], 147.2 [C(7)], 155.9 [C(4a)], 156.4 [C(10a)], 176.0 [C(8)], and 178.7 [C(5)]; m/z (+CI) 777 ([M + 1]$^+$, 100%); HRMS (+CI) $C_{38}H_{49}N_8O_{10}$ [M + 1]$^+$ requires 777.35717, found 777.35877.

7-*N*,7′-*N*′-(2″,5″-Dihydroxy-1″,6″-hexanediyl)bismitomycin C (28). Using the procedure for **9** and MeOH (1.5 mL), **18** (a mixture of diastereomers: 2.7 : 1, ^{13}C NMR analysis, 4.8 mg, 0.022 mmol), triethylamine (18 μL, 0.13 mmol), and **24** (15 mg, 0.043 mmol) gave **28** as a mixture of diastereomers (1.6 : 1, ^{13}C NMR analysis) in 59% yield (10 mg) after PTLC purification (30% MeOH–$CHCl_3$); HPLC t_R 22.8 min; R_f 0.40 (30% MeOH–$CHCl_3$); λ_{max} ($CH_3CN–H_2O$)/nm 222 and 371; δ_H (300 MHz; pyridine-d_5) 1.83–2.03 [m, 4 H, C(3′)H_2], 2.17 [s, 6 H, C(6)CH_3], 2.73 [d, J = 3.9 Hz, 2 H, C(2)H], 3.12 [d, J = 3.9 Hz, 2 H, C(1)H], 3.20 [s, 6 H, C(9a)OCH_3], 3.60 [br d, J = 12.7 Hz, 2 H, C(3)HH′], 3.67–3.78 [m, 2 H, C(1′)HH′], 3.79–3.93 [m, 2 H, C(1′)HH′], 3.97 [dd, J = 10.5, 3.8 Hz, 2 H, C(9)H], 4.03–4.15 [m, 2 H, C(2′)H], 4.55 [d, J = 12.7 Hz, 2 H, C(3)HH′], 5.03 [dd, J = 10.5, 10.2 Hz, 2 H, C(10)HH′], 5.37 [dd, J = 10.2, 3.8 Hz, 2 H, C(10)HH′], and 7.48 [br s, 2 H, C(7)NH], the signals for the C(10)OC(O)NH_2 and N(1a)H protons were not detected and are believed to overlap with the observed peaks, the ^{1}H NMR data were in agreement with the COSY spectrum; δ_C (75 MHz; pyridine-d_5) for the major diastereomer, 10.2 [C(6)CH$_3$], 32.3 [C(3′)], 32.8 [C(2)], 36.7 [C(1)], 44.4 [C(9)], 49.6 [C(9a)OCH$_3$], 50.6 [C(3)], 51.3 [C(1′)], 62.5 [C(10)], 70.6 [C(2′)], 103.6 [C(6)], 107.0 [C(9a)], 110.6 [C(8a)], 147.8 [C(7)], 156.3 [C(4a)], 158.1 [C(10a)], 176.8 [C(8)], and 179.0 [C(5)]; δ_C (75 MHz; pyridine-d_5) for the minor diastereomer, 32.0 [C(3′)], 32.7 [C(2)], 44.5 [C(9)], 70.3 [C(2′)], 147.9 [C(7)], and 156.2 [C(5a)], the other signals were not detected and are believed to overlap with the observed peaks; m/z (+FAB) 783 ([M + 1]$^+$, 100%); HRMS (+FAB) $C_{36}H_{47}N_8O_{12}$ [M + 1]$^+$ requires 783.33135, found 783.33250.

Methanolysis of 9 to give *cis*- and *trans*-C(1) methoxymitosenes 30. Mitomycin **9** (3.5 mg, 0.0043 mmol) was dissolved in MeOH–$CHCl_3$ (1 : 1, 3 mL) and then the effective pH was adjusted to *ca.* 3.0 with a methanolic 2 M HCl solution. The reaction solution was stirred at room temperature (3 d) and then the solvent was removed under reduced pressure. Purification of the reaction mixture by PTLC (20% MeOH–$CHCl_3$) afforded **30** as a red solid: yield, 1.5 mg (40%); HPLC t_R 30.6, 31.3, 32.1 min (three peaks, 1 : 2 : 1); R_f 0.33 (20% MeOH–$CHCl_3$); λ_{max} ($CH_3CN–H_2O$)/nm 214, 255, and 316; δ_H (300 MHz; pyridine-d_5) 1.84–2.09 [m, 4 H, C(3′)H_2], 2.24 [br s, 6 H, C(6)CH_3], 3.24 [br s, 2 H, C(2′)H], 3.51 [br s, 6 H, C(1)OCH_3], 3.84–4.05 [m, 6 H, C(2)H, C(1′)H_2], 4.14–4.32 [m, 2 H, C(3)HH′], 4.41–4.80 [m, 4 H, C(3)HH′, C(1)H], 5.73 [1/2ABq, J = 13.0 Hz, 2 H, C(10)HH′], 5.78 [1/2ABq, J = 13.0 Hz, 2 H, C(10)HH′], and 6.77–6.89 [m, 2 H, C(7)NH], the signals for the C(10)OC(O)NH_2 and C(2)NH_2 protons were not detected and are believed to overlap with the observed peaks, the ^{1}H NMR data were in agreement with the COSY spectrum; m/z (+ESI) 813 ([M + 1]$^+$, 100%); (LC–MS) 835 [M + Na]$^+$ (t_R 15.4–15.6 min), 835 [M + Na]$^+$ (t_R 15.6–16.2 min), 835 [M + Na]$^+$ (t_R 16.2–16.3 min).

General procedure for the solvolysis of mitomycins (kinetic studies)

To a buffered methanolic solution (0.1 M Tris·HCl, pH 7.4; 0.1 M bis-Tris·HCl, pH 5.5 and 4.0) (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 0.03–0.6 mM). The reaction was monitored by UV-visible spectroscopy (200–600 nm), and typically followed for greater than two half-lives. The effective 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 products were identified by coinjection of authentic samples where possible in the HPLC and cospot of authentic samples where possible in the TLC. The absorbance for 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 non-linear 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[23,38]

The agarose gels were prepared by adding 1.2 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 *Eco*RI-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 0.01–0.1 mM) and a nucleophile of choice (1–5 μL of 1–20 mM DMSO solution, final concentration 0.05–1.0 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 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 in 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™ 860 phosphorimager operating in the blue fluorescence mode and ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

General procedure for antiproliferative activity test[39]

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 per 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^{-1} of penicillin, and 100 μg mL^{-1} 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, $\{[A - A_o]/[A_c - A_o]\} \times 100$ (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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