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Design, synthesis, and mode of action studies of a mitomycin tetramer inducing double activations with a single probe

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ABSTRACT

We report design, synthesis, and mechanistic studies of a new mitomycin tetramer **9** along with a new mitomycin dimer **10**. Mitomycin **9** is a tetramer connected by the disulfide linker **11**, and easily undergoes disulfide cleavage to provide two dimeric structures **9r** that each contains a single thiol probe for activations. So, tetramer **9** as a precursor of **9r** was specifically targeted to undergo *double activations with a single probe*. A tetramer **9** was synthesized using **1** and key intermediate **11**, and a dimer **10** was synthesized from **1** and diamine **12**. Activation studies revealed that **9** underwent effective double activations with a single probe by nucleophiles while the reference **10** did not. Evaluations of DNA ISC formations showed that **9** generated substantial levels of DNA ISC by nucleophilic activation while the references **10** and **2** did not. The effectiveness of **9** in activation and formation of DNA ISC per probe was verified by comparing with dimers **5**–**8** of double activations with two probes. These findings highlighted the role of a single thiol in **9r** and demonstrated the intended *double activations with a single probe*, which marks the first case in mitomycin studies.

1. Introduction

Mitomycins constitute a class of antitumor agents that display antiproliferative activity by DNA alkylation,[1,2] and recently, studies on synthesis and mode of actions of mitomycinoid alkaloids were broadly reviewed.[2b,c] Despite the reports of mitomycin A (MMA, **1**) and its numerous derivatives, only mitomycin C (MMC, **2**) has been a useful agent of clinical importance[1,2a] (Fig. 1). Continuing efforts to overcome the drug resistance and side effects of **2**[2a] led to the findings of disulfide mitomycins **3** (KW-2149)[3] and **4** (BMS-181174).[4] In general, mitomycins have to undergo proper activation processes by which they are converted to good electrophiles for chemical reactivity and biological activities. For **3** and **4**, it was believed that a different activation mechanism through the key function of the disulfide group[5–7] in **3** and **4** led to their improved pharmacological properties and DNA adduction[6–8] compared to **2**. The thiol generated by disulfide cleavage of **3** and **4** would trigger the activation of the mitomycin ring by intramolecular cyclization to a quinone ring, which leads to the generation of highly electrophilic C(1) site (activation) that proceeds to react with DNA (DNA adduction). Hence, the thiol group could serve as a good probe to activate the mitomycin ring by nucleophilic activation mechanisms that differ from traditional and/or reductive activation mechanisms.[2a,d]

Among several types of DNA adducts of mitomycin, the DNA interstrand cross-link (DNA ISC) adducts by bis-alkylation of DNA by mitomycin have drawn special interest because they are ~60 times more lethal than the corresponding monoadducts of **2**.[9] Considering that the reactivity of C(1) site toward DNA was 10–100 times higher than that of C(10) site,[10] we focused on the dimerization of mitomycin units that would provide two C(1) sites of higher reactivity, which could eventually provide higher levels of DNA ISC by bis-alkylation of DNA by both C(1) sites. So, aiming to enhance the formation of DNA ISC, we previously reported the design, synthesis, and activation studies of strategically-designed disulfide mitomycin dimers, **5**,[11] **6**,[12] **7**,[13,14] and **8**[15] (Scheme 1). Interestingly, dimers **5**–**8** all contained a medium-sized cyclic disulfide group as a linker to connect two mitomycin units. Cleavage of disulfide group in dimers **5**–**8** would provide **5r**–**8r** (reduced form) containing two thiols that could serve as good probes to initiate the mitomycin activation, leading to double activations with two thiol probes. As a result, dimers **5**–**8** underwent fast activations (k_{obs} (d^{-1}): 2.7–16) and produced high levels of DNA ISC (83–98%) adducts, which represented the important function of thiol probes. Based on the results of double activations with two thiol probes, we became interested in the strategy of *double activations with a single thiol probe*.

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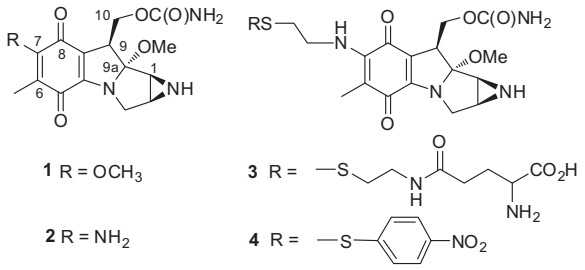


Figure 1. Structures of mitomycins **1**–**4**.

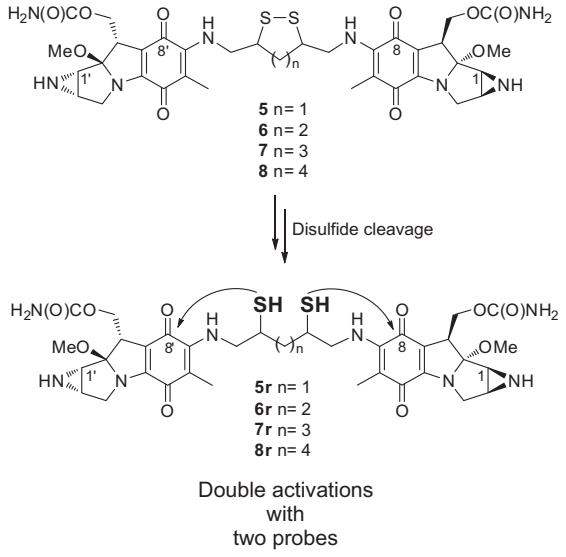
2. Results and discussion

2.1. Design of 9 and study objectives

In continuing efforts to identify the function of a thiol probe in mitomycin activations, we wished to see if the intended double activations with a single probe indeed occurred, and if so, to assess the effectiveness of the activations and DNA adductions. In addition, we were interested in whether the distance and flexibility of the linker would have any effect on those activations. As part of our studies for this purpose, we report herein new mitomycins **9** and **10** in the context of the strategy of double activations with a single probe (Scheme 2), and their comparisons with the precedent examples (e.g., **5**–**8**). Compound **9** contains several structural features associated with mechanistic aspects. First, although compound **9** is a tetramer, it could be seen as a 'dimeric dimer' since it can easily undergo disulfide cleavage (or reduction) to provide two dimeric species **9r** with a single thiol. The thiol–disulfide exchange and, in particular, the disulfide cleavage by a proper nucleophile were observed in our previous studies[11–15] and thus, expected to occur in compound **9**. As mentioned above, our primary interest lay in double activations with a single probe and we therefore aimed to create an appropriate mitomycin dimer with a single thiol probe (e.g., **9r**). However, mitomycin **9r** was believed to be very unstable due to the facile and reversible attack of the thiol to quinone ring and it was very difficult to isolate, which was consistent with the previous observation that the thiol species generated by disulfide cleavage of **3** or **4** was also very unstable.[7] Accordingly, instant generation from a disulfide precursor could be one of the best ways to create the specifically-designed dimer (e.g., **9r**) for our purpose. As a result, we envisioned dimerization of the same dimeric unit **9r** to afford dimeric dimer **9**. Second, compound **9** (and **9r**) contained a 2-mercaptoethylamino unit at C(7), which is an important structural requirement for mitomycin activation. Only in this structural condition could the thiol easily attack the quinone ring by forming a six-membered cyclic structure. Taken together, compound **9** was aimed to undergo efficient nucleophilic double activations with a single probe and corresponding facile DNA adduction. On the other hand, hydroxy mitomycin **10** contains hydroxy linker instead of disulfide linker with the same carbon skeleton as in **9r** and could therefore serve as a good reference to identify the net effect of thiol probe (or disulfide) in **9r** (or **9**). This is the first case in mitomycin studies where double activations with a single thiol probe were tested.

We also attempted to compare target mitomycin **9** with **5**–**8** in terms of the function of thiol probes and the distances and flexibilities of the linker. Although compounds **5r**–**8r** with two thiol probes seemed to be more advantageous for double activations of mitomycin rings, the two thiols in **5r**–**8r** could easily revert back to disulfide to form **5**–**8**, which might retard the activations because activations could be induced by thiol itself. In this regard, it was simply presumed that compound **9r** with a single probe could be sufficient for double activations as there was no possibility of intramolecular disulfide formation. In addition, the flexibility and distance between the two C(1) sites in **9r** would be lower and shorter, respectively, than those in **5r**–**8r**, which might affect the mitomycin activations and subsequent DNA adductions.

So, the objectives of this work were studies on design, synthesis, activation mechanism, and DNA ISC formations for **9** compared to **10**, and elucidation of the strategy of double activations with a single probe in comparison with **5**–**8**. Notably, considering **9** and **10** still contained units of mitomycin C (**2**), it was believed that these mitomycins would undergo activations under reductive[16] and acidic conditions,[17] and thus exhibit basic levels of cytotoxicity. Furthermore, since our main interest lay in the activation mechanism of these peculiar mitomycins, the cytotoxicity data of **9** and **10** were not included in the scope of our present studies. Despite comprehensive investigations of mitomycin activations, much still remains unknown about the precise activation processes and the structures of intermediates and activated products.



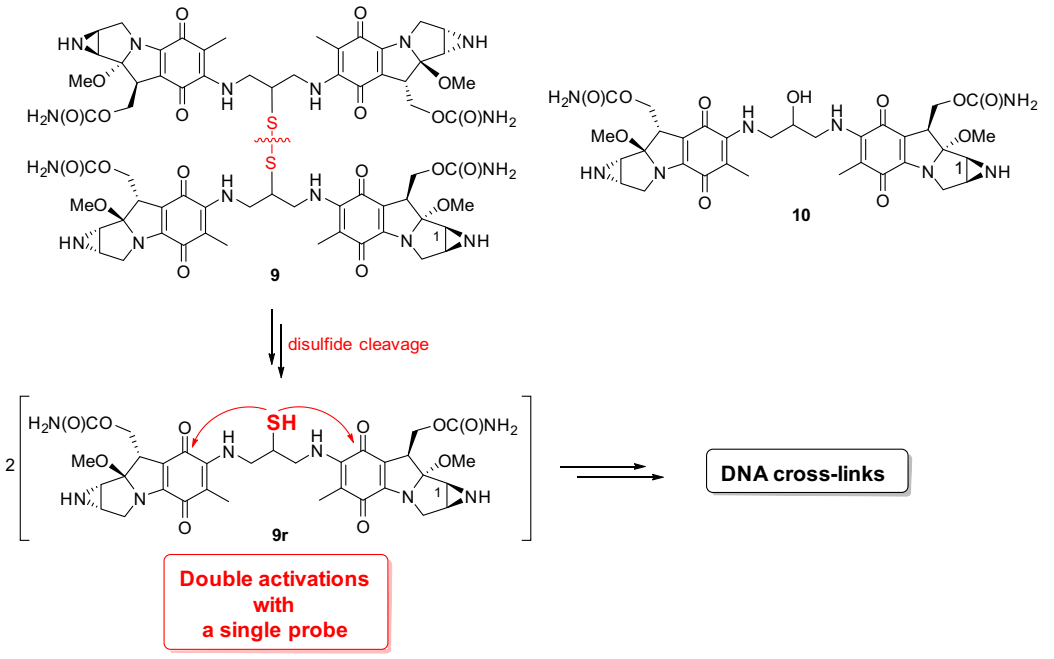
Scheme 1. Disulfide cleavage of mitomycin dimers **5**–**8** to give **5r**–**8r**.

2.2. Chemistry

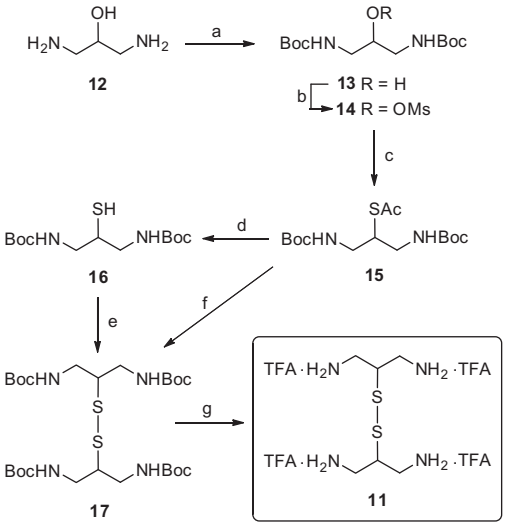
2.2.1. Synthesis of disulfide intermediate 11

Prior to synthesis of the target mitomycins **9** and **10**, we established the synthesis of the required key intermediate, disulfide **11**[18] as shown in Scheme 3.

At first, we treated amine **12** with di-*t*-butyl dicarbonate (Boc_2O) to afford Boc-protected derivative **13**[19] in 99% yield. Next, we reacted **13** with methanesulfonyl chloride (MsCl) to obtain mesyl derivative **14**[19] in 92% yield. Subsequent replacement of the mesyl group with acetylthio group using potassium thioacetate (KSAc) in DMF gave acetylthio derivative **15**[20] along with substantial formation of a side product with higher R_f value (0.80, 1:2 EtOAc/hexanes) than that (0.70) of product **15**. The optimized yield of this step was 61% and further efforts to improve the yield and identify the side product were not successful. It was believed that the adjacent Boc-amino group might interfere with the replacement process, which was also observed in the previous example of 1,5-bis (*tert*-butyloxycarbonylamino)-2,4-pentanediol dimethanesulfonate.[11] Hydrolysis of **15** by potassium carbonate (K_2CO_3) was then conducted to afford thiol derivative **16**.[20] Although the reaction seemed to proceed smoothly, the yield was generally low (less than 50%) probably due to the instability of thiol species, which is consistent with our previous results that thiol could undergo easy



Scheme 2. Disulfide cleavage of mitomycin tetramer **9** to give **9r**.

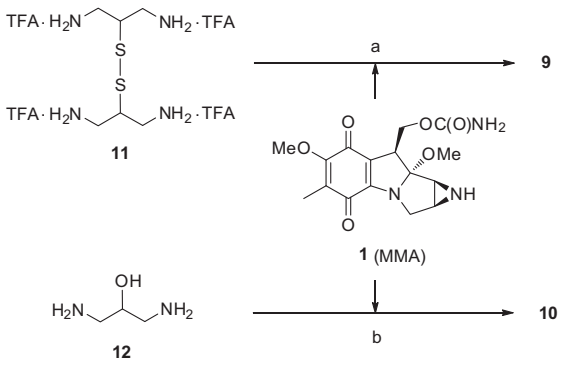


Scheme 3. Synthesis of key intermediate, disulfide **11**. Reagents and conditions: (a) Boc_2O, Et_3N, DMF–H_2O (1:1), room temperature, 3 h, 99%; (b) MsCl, Et_3N, CH_2Cl_2, 0 °C, 5 h, 92%; (c) KSAc, Et_3N, DMF, 60 °C, 1 d, 61%; (d) K_2CO_3, MeOH–H_2O (5:1), room temperature, 1 h, 57%; (e) I_2, Et_3N, $CHCl_3$, room temperature, 3 h, 67%; (f) K_2CO_3, MeOH–H_2O (5:1), room temperature, 1 h, then I_2, Et_3N, MeOH–H_2O–$CHCl_3$ (5:1:2), room temperature, 6 h, 77% (for two steps); (g) TFA, room temperature, 2 h, ~100%.

oxidation and polymerization.[11,13] Hence, we conducted careful hydrolysis and work-up procedure under a purge of nitrogen gas to afford the thiol **16** in 57% yield. We then performed the disulfide formation (oxidation) using I_2/Et_3N condition, leading to the generation of disulfide **17** in 67% yield. In order to improve these two reactions by avoiding the complexity related to the thiol species, we performed the two reactions, hydrolysis and disulfide formation, in situ without isolation of thiol **16**, finally affording disulfide **17** in good yield (77%). Deprotection of Boc group in **17** was achieved by employing trifluoroacetic acid (TFA) to afford amine·TFA salt **11** in near quantitative yield. Consequently, synthesis of the key intermediate, disulfide **11**, was achieved through a six-step synthetic sequence from **12** in a good overall yield (43%).

2.2.2. Synthesis of mitomycins 9 and 10

New target mitomycins **9** and **10** were synthesized using mitomycin A (MMA, **1**, Kyowa Hakko Kirin Co.) and the corresponding intermediates **11** and **12**, respectively, as shown in Scheme 4. Treatment of **1** with intermediate **11** in MeOH provided the target mitomycin **9** in 32% yield. Further efforts to improve the results of this reaction were inconclusive, probably due to the complexity of the reaction. In reality, this step is composed of four consecutive reactions of a tetraamine **11** with four molecules of **1**, affording tetramer **9** that is very polar and long-tailing on the TLC. During the reaction, the reaction media could turn into a kind of buffer solution, due to the presence of excess amount of amine and TFA, and therefore, the reaction rate seemed to gradually decrease. In addition, disulfide could undergo further reactions including disulfide cleavage and/or polymerization. These phenomena hampered us to improve the results. The synthesis of tetramer **9** seemed to be more difficult than those of dimers **5**–**8**. In addition, treatment of **1** with amine **12** in MeOH afforded hydroxy mitomycin **10** in 86% yield, which was employed as a reference for the studies of **9**.



Scheme 4. Synthesis of mitomycins **9** and **10**. Reagents and conditions: (a) Et_3N, MeOH, room temperature, 4 d, 32%; (b) Et_3N, MeOH, room temperature, 2 d, 86%.

2.3. Mode of action studies of mitomycins 9 and 10

2.3.1. Activation of mitomycin 9 using acidic methanolysis

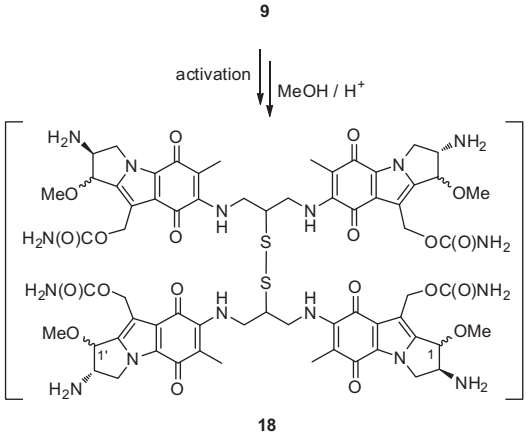
Prior to the nucleophilic activation of **9**, the activation of **9** under acidic conditions was evaluated (Scheme 5). In order to imitate the activation and subsequent DNA adduction for **9** in real biological system, we applied methanolysis reaction as a chemical model system where **9** was induced to undergo activation and react with methanol. So, we conducted the methanolysis of **9** in acidic media (1:1 MeOH–$CHCl_3$ solution at nonaqueous effective pH ('pH') 2.5–3.0) for ~3 d, expecting the formation of tetra-activated mitosene product, C(1)-methoxymitosene **18**. We monitored the reaction using TLC and UV–vis spectroscopy, and in particular, the UV–vis spectra were diagnostic in identifying the activation process. The established UV–vis absorption pattern of mitomycin activations[17,21] implied that activated mitosene products such as **18** display absorption maximum at ~313 nm and the starting mitomycin such as **9** at ~370 nm. We observed the gradual disappearance of starting mitomycin **9** along with gradual and stepwise generation of multi-activated products on TLC and UV–vis spectroscopy, which enabled us to confirm the progress of the activation of **9**. However, the activated products were highly polar and displayed long tailing with low R_f value on TLC, which made them difficult to purify. HPLC chromatograms also showed multiple peaks between ~32 and ~35 min with absorption maxima at 313 nm. We believe that the multi-activated products including **18** are highly polar due to the presence of multiple primary amines generated at each C(2) site and also exist as a mixture of many diastereomers due to the uncontrolled stereochemistry at each C(1) site, which could all cause the complexity of the reaction. Nevertheless, we attempted to isolate the products by PTLC with partial success of low yields (~20%).

2.3.2. Kinetic studies of nucleophilic activations of 9 and 10

Using the methanolysis reactions, we performed kinetic studies of nucleophilic activations induced by appropriate nucleophiles to see if the target mitomycin **9** efficiently undergoes nucleophilic activation compared to reference hydroxy mitomycin **10**, and more significantly, if the intended double activations with a single probe for **9** (or **9r**) indeed occurred by the thiol group in **9r**. In general, the rates of activation of mitomycins under the methanolysis reaction as a chemical model system were believed to directly reflect the real efficiency of activation and adduction of the mitomycins.[11] So, we conducted kinetic studies of nucleophilic activations by measuring the rate of methanolysis of mitomycins, employing Et_3P, L-dithiothreitol (L-DTT), and glutathione (GSH) as proper nucleophiles to induce disulfide cleavage and sequential activations. The activation reactions were conducted in buffered methanolic solutions (0.1 M Tris·HCl, 'pH' 7.4) and monitored by UV–vis spectroscopy (200–500 nm) for more than two half-lives. The absorption was periodically measured at ~370 nm for starting mitomycins and at ~313 nm for activated mitosene products. Generally, the reactions followed pseudo first-order kinetics, and the reaction constant (k_{obs}) and half-lives ($t_{1/2}$) were calculated using the absorption values at 370 nm. The reactions were run in duplicate, and the results averaged, which were summarized in Table 1.

First, we measured the activation rates of **9** and **10** without nucleophile and observed no distinct decrease (less than 10% of original amount) of starting mitomycins in 5 d, which means no noticeable activation without nucleophile. Next, we checked the effect of thiol nucleophiles, L-DTT and GSH, on the activation of **9** and **10**. We observed modest activations of mitomycin **9** with L-DTT (k_{obs}: 0.69 d^{-1} with 10 equiv; 1.3 d^{-1} with 20 equiv). Similarly, we found comparable activation of **9** with GSH (k_{obs}: 1.0 d^{-1} with 20 equiv). However, we observed no appreciable activation of reference mitomycin dimer **10** with these thiol nucleophiles. It was surprising to see the activation of **9** with thiol nucleophiles since we did not observe noticeable activation of disulfide mitomycin dimers **5**–**8** with these thiols in our previous studies.[11,12] Mitomycin **9** (and its reduced form **9r**) differs from mitomycin dimers **5**–**8** (and their reduced forms **5r**–**8r**), and in particular, the longer lifetime of thiol in **9r** compared to **5r**–**8r** was believed to induce weak activations of **9**. Taken together, the target mitomycin **9** underwent modest activation by thiol nucleophiles, which was not previously demonstrated. Thus, we concluded that the weak double activations with a single probe for **9** were induced by thiol nucleophiles.

We then checked the effects of Et_3P as a phosphine nucleophile on activations of tetramer **9** compared to reference **10**. We observed a significant decrease of **9** in the range of 5–50 equiv of Et_3P, which represented the rapid nucleophilic activation of **9** by Et_3P. The k_{obs} values of activation reactions ranged from 0.72 d^{-1} (5 equiv) to 6.9 d^{-1} (50 equiv) and, in general, the activation rates were proportional to the amount of Et_3P employed. However, the reference hydroxy mitomycin **10** showed no appreciable decrease in 5 d even with 50 equiv of Et_3P, which indicated that **10** did not undergo appreciable activation by Et_3P. Considering that the only structural difference of **9** (or **9r**) compared to **10** is the presence of a disulfide (or thiol) group, the nucleophilic activation by Et_3P for only **9** but not **10** must be directly attributed to the function of the disulfide (thiol) unit. Consequently, we confirmed the intended double activations with a single probe for **9** in the



Scheme 5. Activation of **9** to give mitosene product **18**.

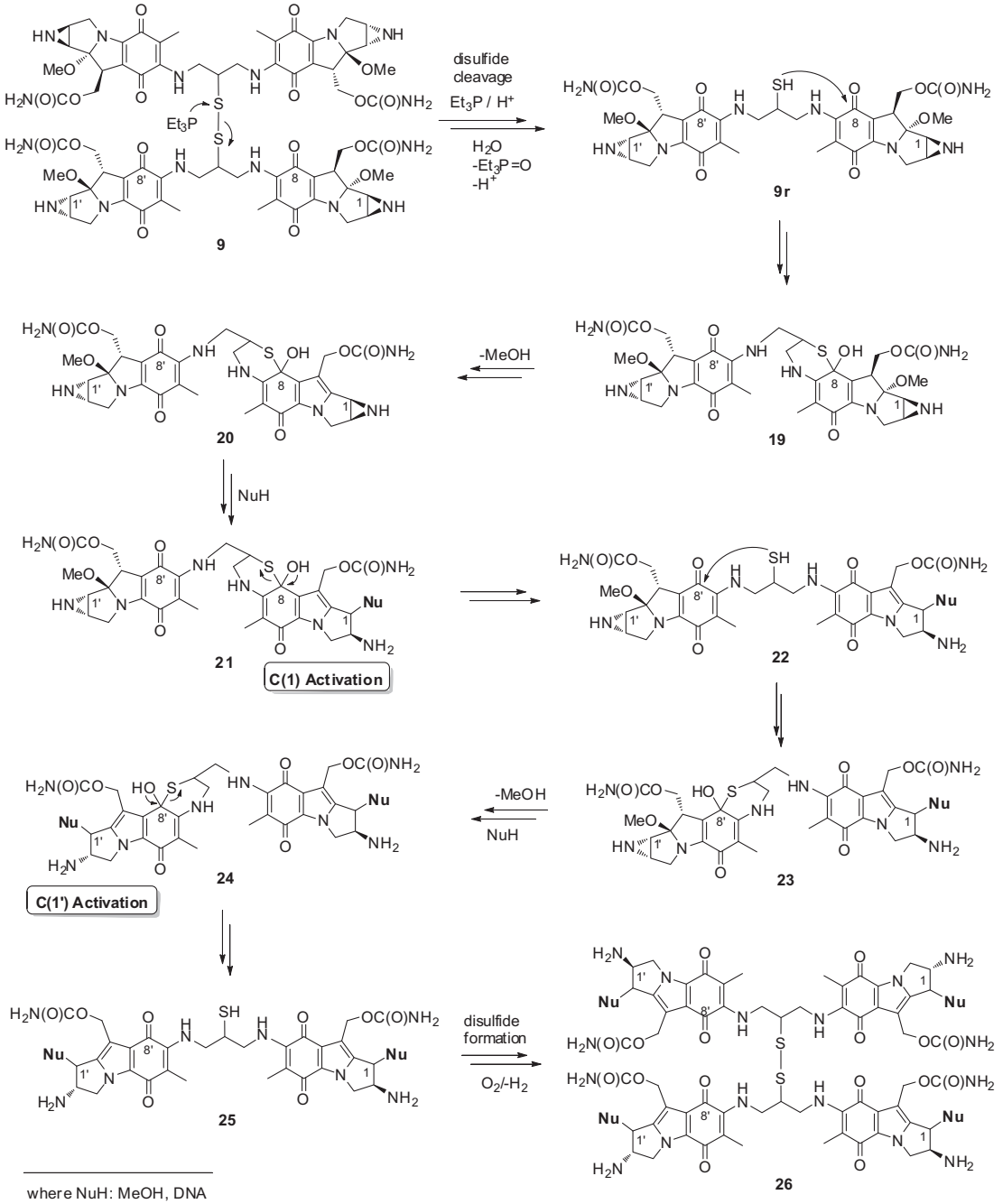
Table 1
Activation rates for **9** and **10** at 'pH' 7.4[a]

Reagents		9		10	
Nu	equiv	k_{obs} (d^{-1})	$t_{1/2}$ (d)	k_{obs} (d^{-1})	$t_{1/2}$ (d)
No Nu		[b]	[b]	[b]	[b]
L-DTT	10	0.69	1.0	[b]	[b]
	20	1.3	0.53	[b]	[b]
GSH	20	1.0	0.69	[b]	[b]
Et_3P	2	[b]	[b]	—	—
	5	0.72	0.96	—	—
	10	1.3	0.53	[b]	[b]
	20	2.9	0.24	[b]	[b]
	50	6.9	0.10	[b]	[b]

[a] Reactions were run in buffered methanolic solution (0.1 M Tris·HCl, 'pH' 7.4) at 25 °C. The reactions were run in duplicate and the values averaged. The results were obtained using a Shimadzu UV-1800 spectrophotometer and the reactions monitored at ~370 nm unless otherwise indicated. The concentrations of the mitomycins were 0.015 mM for tetramer **9** and 0.030 mM for dimer **10**.
[b] No appreciable change in 5 d (less than 10% of the original amount).

presence of Et_3P. When the activation reactions by Et_3P were monitored, we observed similar phenomena as in acidic activations: a gradual disappearance of starting mitomycin **9** at 370 nm and a continual appearance of products at 313 nm, and the formation of multiple peaks (t_R 32–35 min, λ_{max} ~313 nm) on HPLC, which again confirmed the formation of activated mitosene products. However, intensive efforts to identify these multiple peaks were inconclusive, probably due to the formation of many polar diastereomers even at low concentrations (~0.015 mM).

Based on the information obtained by methanolysis using nucleophiles, we propose a mechanism for nucleophilic activation of tetramer **9** by Et_3P as shown in Scheme 6. Mitomycin **9** undergoes disulfide cleavage by Et_3P and subsequent decomposition of thiophosphonium ion by H_2O (or MeOH)[22] to give a thiol in **9r** that could serve as an activation probe. We believed that the involvement of a thiolate instead of a thiol in the activation process is also a possibility.[23] The thiol is expected to attack C(8) carbon of quinone ring by intramolecular cyclization to provide hemi-thioketal **19**, thereby destabilizing the nonbonding electron pair at N(4). Here we showed that intramolecular cyclization occurs at the C(8); however, we recognized that the C(6) or C(7) may be another alternative site.[23b] This destabilization would induce the elimination of the adjacent methoxide at C(9a) to give an iminium ion at N(4)–C(9a) and then mitosene structure **20**. In structure **20**, formation of a highly conjugate allylic-type carbon at C(1) along with electron push from N(4) would facilitate opening of the aziridine ring of high ring-strain. Accordingly, the C(1) site becomes electrophilic enough to react with a



Scheme 6. Proposed mechanism of nucleophilic activation of **9** by Et_3P.

nucleophile (e.g., MeOH or DNA) to afford **21**, which constitutes C(1) activation and adduction. Then, reverting the hemi-thioketal in **21** back to quinone would regenerate the thiol in **22** by which a second round of activation would be initiated. Similar sequential transformations (**22** → **23** → **24**) would afford **24**, which constitutes another C(1′) activation and adduction. Again, reverting the hemi-thioketal back to quinone would give thiol species **25** that could undergo proper oxidation or disulfide formation reaction, finally affording **26**. Taken together, mitomycin **9** successfully induced double activations at C(1) and C(1′) sites with a single thiol probe. This proposed pathway seems to be consistent with our previous studies on the activations of **5**–**8**.[11–15] To the best of our knowledge, this marks the first study of *double activations with a single probe* in a series of mitomycin compounds.

2.4. Studies on the formation of DNA ISC for 9 and 10

We wished to evaluate the ability of the target mitomycin **9** to produce DNA ISC compared to reference **10** highlighting the function of disulfide in **9**, and significantly, to demonstrate the strategy of double activations with a single probe. So, we applied the methods by Cech,[24] and Tepe and Williams[25] with minor modifications for our studies. Linearized pBR322 DNA obtained by linearization of pBR322 plasmid DNA with *Eco*RI enzyme was treated with mitomycins in nucleophilic activation conditions. Then, the purified DNA mixture was applied to denaturing alkaline agarose gel electrophoresis to identify the generated DNA ISC along with λ DNA digested with *Hin*dIII as a molecular weight marker.

We first needed to determine the proper range of concentrations of mitomycin at which the ability of target compound to generate DNA ISC would be noticeably differentiated. Our previous studies[11] indicated that the proper concentrations for dimeric compounds **5**–**8** were 0.1 mM whereas that for monomeric compound **2** was 0.2 mM, which keeps the concentrations of mitomycin unit the same, regardless of the number of units in a molecule. Considering this, we applied a range of concentrations of 0.025–0.20 mM at room temperature for 2 h in the presence of Et_3P (5 equiv), and found that the levels of DNA ISC ranged from 17% to 68%, as shown in Table 2. Thus, we chose 0.05 mM (41% DNA ISC) for the target mitomycin **9**, in order to sufficiently differentiate the levels of DNA ISC and to compare the results in parallel with those of compounds **5**–**8** while keeping the concentration of mitomycin units the same.

First, we measured the formation of DNA ISC in the presence of Et_3P (5 equiv), and the results were shown in Figure 2. The target mitomycin **9** produced modest levels of DNA ISC (~41%) under these conditions compared to references **10** (5%) and **2** (2%). Accordingly, we concluded that mitomycin **9** underwent more efficient activations and DNA ISC formation by the action of Et_3P on the disulfide unit, compared to references **10** and **2** that do not contain the disulfide unit. In addition, when comparing the DNA ISC data with the rate data of kinetic studies (Table 1), we found reasonable correlations based on the observation that only mitomycin **9** underwent facile activations by Et_3P (5 equiv: k_{obs} = 0.72 d^{-1}) while references **10** and **2** did not. Thus, **9** was confirmed to produce DNA ISC

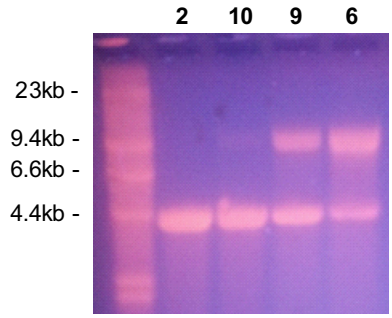


Figure 2. Denaturing 1.2% alkaline agarose gel for **2**, **10**, **9** and **6** using Et_3P. DNA cross-linking experiments using 0.2 mM (monomer **2**), 0.1 mM (dimers **10** and **6**), and 0.05 mM concentrations of mitomycins (tetramer **9**), and *Eco*RI-linearized pBR322 plasmid DNA with 5 equiv of Et_3P. All reactions were incubated at room temperature (2 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: **2** + Et_3P. Lane 3: **10** + Et_3P. Lane 4: **9** + Et_3P. Lane 5: **6** + Et_3P.

by the proposed process of double activations with a single probe. Furthermore, in this experiment we also used dimer **6** of double activations with two probes for comparison with **9**. The levels of DNA ISC for dimer **6** were 81%, which was similar to the values (83%) reported in our previous studies.[12] Although the levels of DNA ISC for tetramer **9** were nearly half of those for **6**, the levels of DNA ISC *per probe* were nearly the same (~41%), which supported the strategy of double activations with a single probe.

2.5. Reflections on the strategy of double activations with a single probe

As discussed above, one of our main interests in this study lay in the strategy of double activations with a single probe. We wished to see if the intended double activations with a single probe indeed occurred and, if so, to assess the effectiveness of the activations and DNA adductions. To assess this strategy, we compared **9** (or **9r**) with an appropriate reference. Among mitomycins **5**–**8** that represent double activations with two probes, we chose **6** (or **6r**) as a reference considering the ring size of the cyclic disulfide linker and the distances between the two mitomycin rings. Thus, we compared **9** with **6** in terms of activation rates and the levels of DNA ISC as shown in Table 3. In the activation rate studies, the target mitomycin **9** definitely underwent activations through double activations with a single probe, and moreover, the activation rate (e.g., k_{obs} = 1.3 d^{-1}) per probe of **9** (or **9r**) was nearly the same as that (e.g., k_{obs} = 1.35 d^{-1}) per probe of mitomycin **6** (or **6r**). As discussed above, the levels of DNA ISC per probe for both **9** and **6** were also nearly identical (41% for **9**; 40.5% for **6**), which seemed reasonable and consistent based on the function of each thiol.

We considered several factors such as the number and lifetime of thiol, the distance between C(1) and C(1′) sites, and the flexibility of two mitomycin rings. Our previous studies[11–15] using mitomycins **6**–**8** indicated that as the ring size of the cyclic disulfide linker increased from six-membered ring (**6**) to eight-membered ring (**8**), the lifetime of thiol(s) increased due to slower cyclization to cyclic disulfide and the activation rates increased. Thus, in the

Table 2
The levels of DNA ISC depending on concentrations of **9**[a]

Drug concentration (mM)	0.025	0.050	0.10	0.20
DNA ISC (%)	17	41	51	68

[a] DNA cross-linking experiments for **9** using *Eco*RI-linearized pBR322 plasmid DNA with Et_3P (5 equiv). All reactions were incubated at room temperature (2 h). The percentage of DNA ISC was obtained by Bio-Rad Smartspec™ 3000 and/or UV Trans Illuminator with a digital camera.

Table 3
Comparison of **9** with **6** in activation rates and DNA ISC formations using Et_3P

Compounds	Activation rates[a] k_{obs} (d^{-1})	DNA ISC[b] (%)
9	1.3	41
6[c]	2.7	81

[a] Activation rates with 10 equiv of Et_3P.
[b] DNA ISC formations with 5 equiv of Et_3P.
[c] Ref. 12.

case of **9**, the lifetime of the generated thiol was believed to be longer than those for **6**–**8** since there is no possibility for intramolecular cyclic disulfide formation, which might facilitate the activations. However, the number of thiols should be considered at the same time, and the single thiol in **9r** has to activate two mitomycin rings while each thiol in **6r** has to activate one mitomycin ring, which might influence the whole activation rates. Thus, it seems reasonable that the activation rates and DNA ISC per probe of **9** would be similar to those of **6**. Regarding the distance and flexibility, the distances between C(1) and C(1′) in **9** ranged from 7 to 20 Å according to their conformations (Sybyl 6.0, HyperChem 7.1) and were a little shorter than those (7–25 Å) in dimer **6**.[12] Therefore, the distances between the two reaction sites, in a limited range, did not significantly affect the formation of DNA ISC. Flexibility did not significantly influence DNA ISC based on the observations using **9** and **6**. However, much still remains unknown and many other factors must be associated with the activation and DNA adduction, and thus, advanced elucidation of a working mechanism must be further pursued. Taken together, despite the complexities discussed above, double activations with a single probe for **9** and the effectiveness of this strategy were successfully demonstrated, which constitutes a new approach in mitomycin activation studies.

3. Conclusion

We present the design, synthesis, and mode of action of novel mitomycins **9** and **10**. Mitomycin **9** features a unique structural motif of a tetramer tethered through the disulfide linker **11** and could therefore be viewed as a dimeric dimer; this motif specifically induces *double activations with a single probe*. Compound **9** easily undergoes disulfide cleavage (or reduction) to provide dimer **9r** containing a single probe in a dimeric structure. So, tetramer **9** was aimed to undergo efficient nucleophilic activations and corresponding facile DNA adduction.

Synthesis of the key intermediate, disulfide **11**, was achieved through an efficient six-step sequence from **12** in an excellent overall yield (43%). Treatment of the intermediate **11** with **1** in MeOH provided new target mitomycin **9** in 32% yield. Similarly, use of the amine **12** and **1** in MeOH afforded new reference mitomycin **10** in 86% yield. Then, through mechanistic studies of the nucleophilic activation of **9** compared to reference **10**, we found that mitomycin **9** underwent effective activation by nucleophiles (e.g., Et_3P), while reference **10** did not, implying that the generated thiol in **9** activated both mitomycin rings. Thus, we demonstrated the intended *double activations with a single probe* for **9** in the presence of Et_3P. Based on these observations, we also proposed a nucleophilic activation pathway for **9**. Through the evaluation of the ability of mitomycin **9** to produce DNA ISC, we found that **9** produced substantial levels of DNA ISC (~41%) in the presence of Et_3P while the references **10** and **2** yielded trace levels (5% and 2%, respectively), which again confirmed the double activations with a single probe and highlighted the key role of the thiol group. The effectiveness in activations and formation of DNA ISC per probe for **9** were verified by comparing with dimers **5**–**8**. This strategy using specifically-designed **9** marks the first case among the mitomycin activations and constitutes a new approach for the activations of mitomycins and other compounds that require prior activations.

4. Experimental

4.1. General

Melting points were measured by Buchi B-545 melting point apparatus using open capillary tubes and are uncorrected. FT-IR spectra were recorded on a Perkin–Elmer Spectrum GX spectrometer. ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were obtained by a Bruker DRX 300 spectrometer. Mass spectra were obtained by CI, EI or FAB ionization method. UV–vis spectra were recorded on a Shimadzu UV-1800 Spectrophotometer. The measurements of pH in aqueous solutions were conducted using a IQ Scientific Instruments IQ-240 meter, and the measurements of nonaqueous effective pH ('pH') in buffered methanolic solutions were similarly conducted with long equilibration time. Thin layer chromatography was conducted on silica gel plates (20 × 20 cm; Aldrich No. Z12272-6). HPLC analyses were executed using the following Waters Associate Units: 515 A pump, 515 B pump, dual λ absorbance 2487 detector, 717 plus autosampler, and Hypersil ODS column (4.6 × 300 mm). The products were analyzed by linear gradient condition: 90% A (aqueous 0.025 M triethylammonium acetate, pH 6.5), 10% B (acetonitrile) isocratic for 5 min, then from 90% A, 10% B to 45% A, 55% B in 30 min. The flow rate was 1 mL/min, and the eluent was monitored from 200 to 400 nm. The HPLC solvents were filtered (aqueous solution with Millipore HVLP, 0.45 mm; acetonitrile with Millipore HV, 0.45 mm) and degassed immediately before use.

4.2. Procedures for synthesis of intermediates and mitomycins

4.2.1. 1,3-Bis(*t*-butyloxycarbonylamino)-2-propanol (13)[19]

To a stirred solution of 1,3-diamino-2-propanol (**12**, 500 mg, 5.6 mmol) and Et_3N (4.6 mL, 33 mmol) in DMF–H_2O (1:1, 32 mL) was added a solution of di-*tert*-butyl dicarbonate (Boc_2O) (3.0 g, 14 mmol) in DMF. After stirring at rt (3 h), H_2O (15 mL) was added and the mixture was extracted with EtOAc (2 × 15 mL). The combined organic layers were washed with aqueous 0.1 N HCl (25 mL), saturated aqueous $NaHCO_3$ (25 mL) and H_2O (25 mL), and dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:2 EtOAc/hexanes) afforded the product as a white solid (1.6 g, 99%). Mp 88–91 °C; R_f 0.45 (1:2 EtOAc/hexanes); IR (KBr) 3358, 2977, 1545, 1515, 1366, 1251, 1170, 989, 860, 782, 615 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 18H, OC(CH_3)$_3$), 3.17 (1/2ABMXqt, J = 14.3, 5.7 Hz, 2H, C*H*HCH), 3.28 (ABMXqt, J = 14.3, 5.7 Hz, 2H, CH*H*CH), 3.71 (br s, 2H, OH), 3.75 (quin, J = 4.9 Hz, 1H, CH_2CH), 5.16 (br s, 2H, NHCO); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.6 (OC(*C*H_3)$_3$), 43.7 (*C*H_2CH), 71.1 (CH_2*C*H), 80.0 (O*C*(CH_3)$_3$), 157.4 (NHCO); MS m/z 291 $[M+H]^+$; HRMS (+FAB) calcd for $C_{13}H_{27}N_2O_5$ $[M+H]^+$: 291.1920, found: 291.1924.

4.2.2. 1,3-Bis(*t*-butyloxycarbonylamino)-2-propanol methanesulfonate (14)[19]

To a cooled (0 °C) solution of 1,3-bis(*t*-butyloxycarbonylamino)-2-propanol (**13**, 500 mg, 1.7 mmol) in CH_2Cl_2 (5 mL) was added Et_3N (0.50 mL, 3.4 mmol) and methanesulfonylchloride (0.2 mL, 2.1 mmol). After stirring at the same temperature (5 h) the solvent was removed in vacuo. H_2O (80 mL) was added to the residue and then the mixture was extracted with EtOAc (2 × 80 mL). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (80 mL) and H_2O (80 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. Crystallization of crude product in EtOAc/hexanes mixture afforded the product as a white solid (580 mg, 92%). Mp 138–141 °C; R_f 0.60 (1:2 EtOAc/hexanes); IR (KBr) 3446, 2976, 1704, 1511, 1366, 1265, 1175, 916, 738, 528 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 18H, OC(CH_3)$_3$), 3.09 (s, 3H, OS(O)$_2$$CH_3$), 3.31 (1/2ABMXqt, J = 14.7, 5.8 Hz, 2H, C*H*HCH), 3.50 (1/2ABMXqdd, J = 14.7, 7.2, 4.5 Hz, 2H, CH*H*CH), 4.66 (quin, J = 5.0 Hz, 1H, CH_2CH), 5.17 (br s, 2H, NHCO); ^{13}C NMR (75 MHz, DMSO-d_6) δ 28.5 (OC(*C*H_3)$_3$), 38.1 (OS(O)$_2$$CH_3$), 41.7 (*C*$H_2$CH), 78.5 (O*C*($CH_3$)$_3$), 79.9 ($CH_2$*C*H), 156.1 (NHCO); MS m/z 369 $[M+H]^+$; HRMS (+FAB) calcd for $C_{14}H_{29}N_2O_7S$ $[M+H]^+$: 369.1695, found: 369.1704.

4.2.3. 1,3-Bis(*t*-butyloxycarbonylamino)-2-acetylthiopropane (15)[20]

To a stirred solution of 1,3-bis(*t*-butyloxycarbonylamino)-2-propanol methanesulfonate (**14**, 300 mg, 0.81 mmol) in DMF (5 mL) was added KSAc (112 mg, 0.97 mmol) and Et_3N (82 mg, 0.81 mmol). After warming to 60 °C, stirring was continued (1 d) and then the solvent was removed in vacuo. H_2O (2 × 80 mL) was added to the residue and the resulting mixture was extracted with EtOAc (2 × 80 mL). The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:2 → 1:1 EtOAc/hexanes) afforded the product as a white solid (175 mg, 61%). Mp 92–93 °C; R_f 0.70 (1:2 EtOAc/hexanes); IR (KBr) 3366, 2977, 1720, 1511, 1366, 1251, 1167, 956, 864, 737, 632 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 18H, OC(CH_3)$_3$), 2.34 (s, 3H, SC(O)CH_3), 3.21–3.34 (m, 2H, C*H*HCH), 3.35–3.45 (m, 2H, CH*H*CH), 3.62 (quin, J = 5.7 Hz, 1H, CH_2CH), 5.18 (br s, 2H, NHCO); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.4 (OC(CH_3)$_3$), 30.9 (SC(O)*C*H$_3$), 40.8 (*C*H$_2$CH), 45.4 (CH_2*C*H), 79.7 (O*C*(CH_3)$_3$), 156.3 (NHCO), 194.8 (S*C*(O)CH$_3$); MS m/z 349 $[M+H]^+$; HRMS (+FAB) calcd for $C_{15}H_{29}N_2O_5S$ $[M+H]^+$: 349.1797, found: 349.1794.

4.2.4. 1,3-Bis(*t*-butyloxycarbonylamino)-2-mercaptopropane (16)[20]

To a stirred solution of 1,3-bis(*t*-butyloxycarbonylamino)-2-acetylthiopropane (**15**, 60 mg, 0.17 mmol) in MeOH–H_2O (5:1, 6 mL) was added K_2CO_3 (140 mg, 1.0 mmol). After stirring at room temperature (1 h), H_2O (50 mL) was added to the residue. The mixture was extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with H_2O (50 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:4 → 1:3 EtOAc/hexanes) afforded the product as a white solid (30 mg, 57%). Mp 91–93 °C; R_f 0.72 (1:2 EtOAc/hexanes); IR (KBr) 2925, 1692, 1505, 1390, 1252, 1168, 780 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.22–1.30 (m, 1H, SH), 1.45 (s, 18H, OC(CH_3)$_3$), 2.92–3.20 (m, 3H, C*H*HC*H*), 3.40–3.62 (m, 2H, CH*H*CH), 5.27 (br s, 2H, NHCO); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.6 (OC(*C*H$_3$)$_3$), 40.6 (CH_2*C*H), 44.2 (*C*H$_2$CH), 79.9 (O*C*(CH_3)$_3$), 156.0 (NHCO); MS m/z 307 $[M+H]^+$; HRMS (+FAB) calcd for $C_{13}H_{27}N_2O_4S$ $[M+H]^+$: 307.1692, found: 307.1686.

4.2.5. Bis(1,3-bis(*t*-butyloxycarbonylamino)-2-propyl)disulfide (17)

4.2.5.1. Method A. To a stirred solution of 1,3-bis(*t*-butyloxycarbonylamino)-2-mercaptopropane (**16**, 30 mg, 0.098 mmol) and Et_3N (34 μL, 0.25 mmol) in $CHCl_3$ (1.5 mL) was dropwise added a saturated $CHCl_3$ solution of iodine (~1.0 mL) at room temperature until a slight excess of iodine was evidenced by its color. After stirring at room temperature (3 h) the solution was treated with saturated aqueous $Na_2S_2O_3$ (50 mL) and the mixture was extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with aqueous 0.1 N HCl (50 mL), saturated aqueous $NaHCO_3$ (50 mL) and H_2O (50 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:5 EtOAc/hexanes) afforded the product as a white solid (20 mg, 67%). Mp 125–127 °C; R_f 0.55 (1:2 EtOAc/hexanes); IR (KBr) 3053, 2984, 2305, 1705, 1505, 1422, 1265, 1167, 895, 739 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 36H, OC(CH_3)$_3$), 3.01 (quin, J = 5.5 Hz, 2H, CH_2*CH*), 3.07–3.20 (m, 4H, C*H*HCH), 3.48–3.65 (m, 4H, CH*H*CH), 5.52 (br s, 4H, NHCO); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.4 (OC(*C*H$_3$)$_3$), 40.6 (*C*H$_2$CH), 52.2 (CH_2*C*H), 79.7 (O*C*(CH_3)$_3$), 156.5 (NHCO); MS m/z 611 $[M+H]^+$; HRMS (+FAB) calcd for $C_{26}H_{51}N_4O_8S_2$ $[M+H]^+$: 611.3148, found: 611.3143.

4.2.5.2. Method B. To a stirred solution of 1,3-bis(*t*-butyloxycarbonylamino)-2-acetylthiopropane (**15**, 60 mg, 0.17 mmol) in MeOH–H_2O (5:1, 6 mL) was added K_2CO_3 (140 mg, 1.0). After stirring at room temperature (1 h), Et_3N (59 μL, 0.43 mmol) and a saturated $CHCl_3$ solution of iodine (~2.0 mL) was then added drop wise at room temperature until a slight excess of iodine was evidenced by its color. After stirring at room temperature (6 h) the solution was treated with saturated aqueous $Na_2S_2O_3$ (100 mL) and the mixture was extracted with EtOAc (2 × 70 mL). The combined organic layers were washed with aqueous 0.1 N HCl (70 mL), saturated aqueous $NaHCO_3$ (70 mL) and H_2O (70 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. Purification by column chromatography (1:5 EtOAc/hexanes) afforded the product as a white solid (40 mg, 77%), which was identified as the same compound obtained in method A.

4.2.6. Bis(1,3-diamino-2-propyl)disulfide·4TFA (11)[18]

Bis(1,3-bis(*t*-butyloxycarbonylamino)-2-propyl)disulfide (**17**, 13 mg, 0.021 mmol) was dissolved in trifluoroacetic acid (0.5 mL) and stirring was continued at room temperature (2 h). The reaction mixture was concentrated in vacuo to give the product as a brown solid (13 mg, ~100%). Mp 185–186 °C; R_f 0.10 (1:2 MeOH/CH_2Cl_2); IR (KBr) 3433, 1673, 1522, 1431, 1392, 1206, 837, 721, 598 cm^{-1}; ^1H NMR (300 MHz, MeOD-d_4) δ 3.11–3.23 (m, 4H, C(1)*H*H, partly overlapped with H_2O peak), 3.31 (dd, J = 13.9, 5.4 Hz, 4H, C(1)H*H*), 3.50–3.62 (m, 2H, C(2)H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 46.9 (CH_2CH), 54.8 (CH_2*C*H); MS m/z 211 $[M-4TFA+H]^+$; HRMS (+FAB) calcd for $C_6H_{19}N_4S_2$ $[M-4TFA+H]^+$: 211.1051, found: 211.1055.

4.2.7. (7-*N*,7′-*N*′-Bis(1″,3″-propanediyl)-2″-disulfide) tetrakismitomycin C (9)

To a stirred solution of bis(1,3-diamino-2-propyl)disulfide·4TFA (**11**, 1.7 mg, 2.8 μmol) and Et_3N (4.0 μL, 29.0 μmol) in MeOH (0.4 mL) was added MMA (**1**, 5.0 mg, 14 μmol). The reaction solution was stirred at room temperature (4 d) and then the solvent was removed in vacuo. Purification of the reaction mixture by PTLC (1:3 MeOH/$CHCl_3$) afforded the product as a dark blue solid (1.3 mg, 32%). R_f 0.68 (3:7 MeOH/$CHCl_3$); HPLC t_R 38.6 min (96%); UV–vis (MeOH) λ_{max}: 370 nm; ^1H NMR (300 MHz, pyridine-d_5) δ 2.19 (s, 12H, C(6)CH_3), 2.72 (br s, 4H, C(2)H), 3.13 (br s, 4H, C(1)H), 3.25 and 3.26 (2s, 12H, C(9a)OCH_3 and C(9a′)OCH_3), 3.56 (d, J = 11.8 Hz, 4H, C(3)*H*H), 3.73 (app t, J = 6.4 Hz, 2H, C(2″)H), 3.90–4.09 (m, 4H, C(9)H), 4.11–4.38 (m, 8H, C(1″)H_2), 4.50 (app d, J = 12.6 Hz, 4H, C(3)H*H*), 5.08–5.20 (m, 4H, C(10)*H*H, partly overlapped with H_2O peak), 5.32–5.45 (m, 4H, C(10)H*H*), 7.25–7.43 (m, 4H, C(7)NH), the signals for the N(1a)H and C(10)OC(O)NH_2) protons were not detected and are believed to overlap with the observed peaks; MS m/z 1501 $[M+Na]^+$; HRMS (+ESI) calcd for $C_{66}H_{78}N_{16}NaO_{20}S_2$ $[M+Na]^+$: 1501.4917, found: 1501.4913.

4.2.8. 7-*N*,7′-*N*′-(2″-Hydroxy-1″,3″-propanediyl)bismitomycin C (10)

To a stirred solution of 1,3-diamino-2-propanol (**12**, 0.50 mg, 5.5 μmol) and Et_3N (4.8 μL, 35 μmol) in MeOH (0.2 mL) was added MMA (**1**, 4.0 mg, 11 μmol). The reaction solution was stirred at room temperature (2 d) and then the solvent was removed in vacuo. Purification of the reaction mixture by PTLC (1:3 MeOH/$CHCl_3$) afforded the product as a dark blue solid (3.4 mg, 86%). R_f 0.44 (3:7 MeOH/$CHCl_3$); HPLC t_R 23.3 min (97%); UV–vis (MeOH) λ_{max}: 370 nm; ^1H NMR (300 MHz, pyridine-d_5) δ 2.18 and 2.19 (2s, 6H, C(6)CH_3 and C(6′)CH_3), 2.74 (d, J = 3.6 Hz, 2H, C(2)H), 3.13 (app d, J = 3.6 Hz, 2H, C(1)H), 3.23 and 3.24 (2s, 6H, C(9a)OCH_3 and C(9a′)OCH_3), 3.54–3.65 (m, 2H, C(3)*H*H), 3.76–3.98 (m, 4H, C(1″)H_2), 3.99 (dd, J = 11.2, 3.9 Hz, 2H, (C(9)H), 4.27–4.37 (m, 1H, C(2″)H), 4.53 (d, J = 12.6 Hz, 2H, C(3)H*H*), 4.96–5.12 (m, 2H, C(10)*H*H, partly overlapped with H_2O peak), 5.37 (dd, J = 10.5, 3.9 Hz, 2H, C(10)H*H*), 7.36–7.71 (m, 6H, C(7)NH, C(10)OC(O)NH_2), the signal for the N(1a)H proton was not detected and is believed

to overlap with the observed peaks; MS m/z 725 $[M+H]^+$; HRMS (+FAB) calcd for $C_{33}H_{41}N_8O_{11}$ $[M+H]^+$: 725.2895, found: 725.2902.

4.2.9. C(1) methoxymitosenes (18)

Mitomycin **9** (2.0 mg, 1.4 μmol) was dissolved in a solution of MeOH–$CHCl_3$ (1:1, 2 mL) and then the 'pH' was carefully adjusted to 2.5–3.0 with a dilute methanolic HCl solution (0.02 M). The reaction solution was stirred at room temperature while the pH of the solution was maintained at near 3.0 by periodically adding the methanolic HCl solution. After stirring for 3 d, the reaction solution was neutralized (pH ~7) with a dilute methanolic solution of Et_3N (0.02 M). Purification of the reaction mixture by PTLC (1:1 MeOH/$CHCl_3$) afforded the product **18** as a red solid (0.40 mg, 20%). HPLC t_R 32.0–35.0 min (multiple peaks); UV–vis (MeOH) λ_{max}: 313 nm; MS m/z 1479 $[M+H]^+$.

4.3. General procedure for the mitomycin activation studies

To a buffered methanolic solution (0.1 M Tris·HCl 'pH' 7.4) (final volume 1.5 mL) containing the mitomycins (for tetramer **9**: 23 μL of 1.0 mM methanolic solution, final concentration 0.015 mM; for dimer **10**: 45 μL of 1.0 mM methanolic solution, final concentration 0.03 mM) was added a methanolic solution (18–113 μL) of the nucleophile of choice (stock solution: 5–20 mM, final nucleophile concentration 0.06–1.5 mM). The reaction was monitored by UV–vis spectroscopy (200–500 nm), and generally followed for greater than two half-lives. The 'pH' of the solution was determined at the conclusion of the reaction and found to be within ±0.1 pH units of the original solution. The reaction solutions were analyzed by HPLC and unreacted staring materials and products (e.g., **9**, **10**, and **18**) were determined by coinjection of authentic samples in the HPLC and cospotting of authentic samples in the TLC. The λ_{max} of mitomycin (~370 nm) was plotted versus time and found to decrease in a first-order decay (exponential decay) process. The nonlinear regression analysis to fit the observed exponential decay by SigmaPlot Program (SigmaPlot, 2001) yielded pseudo-first-order rate constants (k_{obs}) and half-lives ($t_{1/2}$). The reactions were done in duplicate and the results averaged.

4.4. General procedure for alkaline agarose gel electrophoresis[24,25]

Agarose gels were prepared by adding 1.20 g of agarose to 100 mL of an aqueous 100 mM NaCl and 2 mM EDTA solution (pH 8.0). The suspension was heated in a microwave oven until all of the agarose was dissolved (1 min). The hot solution was poured and then allowed to cool and solidify at room temperature (1 h). The gel was soaked in an aqueous alkaline running buffer solution (50 mL) containing 40 mM NaOH and 1 mM EDTA (1 h) and then the comb was removed. The buffer solution was refreshed prior to electrophoresis.

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

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

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References and notes

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