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Reductive Activation of Mitomycin A by Thiols

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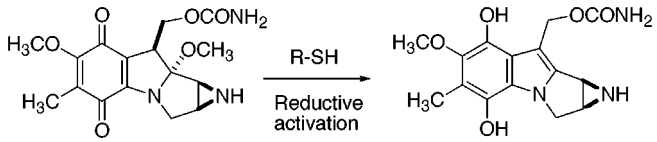
ABSTRACT



Mitomycin A

Mitomycin C is unchanged upon exposure to thiols under physiological conditions. Its more toxic variant, mitomycin A (MA), undergoes elimination of methanol to give a variety of mitosene derivatives, diagnostic of its activation to a reactive electrophile. Evidence is presented for a novel reductive mechanism, characterized by the transient addition of a thiol to the quinone of MA, followed by intramolecular electron transfer, leading to reduced quinone and oxidized thiol.

The mitomycins are a family of antitumor antibiotics that bind covalently to DNA. Mitomycin C (MC;[1] **1**), the best known member of this family, is used clinically for anticancer therapy. The mode of action of MC has been the subject of extensive research, and there is ample evidence that the in vivo activity of MC requires enzymatic reduction. The mechanism of the reductive activation of MC has been studied with both biological and chemical reducing agents, and it was concluded that the reduction of the quinone causes the elimination of methanol, exposing masked biselectrophilic indole hydroquinone **4**, which alkylates DNA to form mitosene[1] monoadducts and cross-links[2] (Scheme 1). A major byproduct of the reduction of MC is the mitosene **5a**, arising by isomerization of the hydroquinone **4** via a quinone methide intermediate.[3]

Another member of the mitomycin family is mitomycin A (MA; **2**), which differs from MC only in the substituent at the 7-position. MA presents the highest toxicity among the naturally occurring mitomycins, which precluded its clinical use. Its enhanced toxicity has been attributed to its relatively high reduction potential and, hence, to relatively more facile reductive activation, due to the 7-methoxy substituent.[4] The formation of DNA adducts of MA was studied in vitro, using the same reducing agents that activated MC (enzymatic, catalytic hydrogenation, sodium dithionite).[5] MA presented reactivity with DNA which was similar to that of MC except that the formation of DNA interstrand cross-links was not inhibited by aerobic conditions of the activation, in contrast to MC. This latter property of MA is likely related to its higher redox potential.[5] We report here a unique reductive activation mechanism of MA by showing that unlike its 7-amino substituted cousin MC, MA is reduced by thiols from quinone to hydroquinone.

The reaction of MA with thiols was monitored by a UV assay, based on the conversion of the 7-methoxymitosane chromophore (λ_{max} 317 nm) to the 7-methoxymitosene chromophore (λ_{max} 280 nm). Incubation of a 0.1 mM solution of **2** with 0.2 mM DTT at pH 6 resulted in quantitative

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(1) Abbreviations: MC, mitomycin C; MA, mitomycin A; MER, mercaptoethanol; *N*-Ac-Cys, *N*-acetylcysteine; GSH, glutathione; DTT, dithiothreitol. Mitosene: structure **5**, without substituents in the 2- and 7-positions; $[RSH]_0$, thiol concentration.

(2) Tomasz, M. *Chem. Biol.* **1995**, *2*, 575−579.

(3) Tomasz, M.; Lipman, R. *Biochemistry* **1981**, *20*, 5056−5061.

(4) Pan, S.; Gonzalez, H. *Mol. Pharmacol*. **1990**, *37*, 966−9.

(5) McGuinness, B. F.; Lipman, R.; Goldstein, J.; Nakanishi, K.; Tomasz, M. *Biochemistry* **1991**, *30*, 6445−6453.

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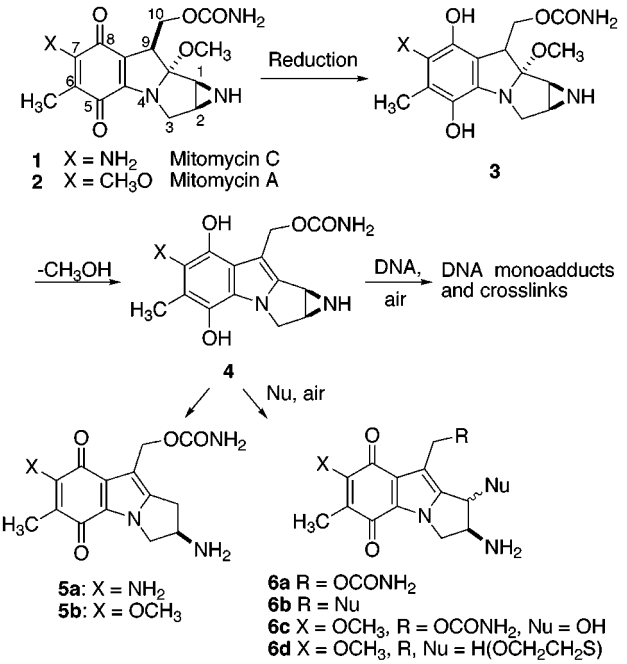
Scheme 1. Mechanism of Reductive Activation of Mitomycins C and A

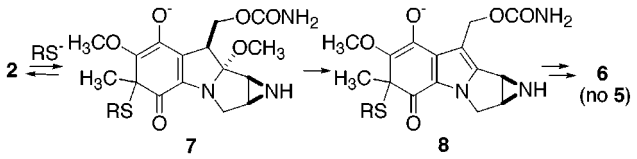


consumption of **2** in about 2 h (Figure 1). Using MER or *N*-Ac-Cys gave similar spectral changes, but the reaction occurred at a much slower rate, requiring a large excess of thiol (about 100- and 250-fold, respectively) and higher pH (7.5), to proceed at similar rates. MC was not consumed under any of these conditions.

We were interested in determining which reactions account for the consumption of MA. Irreversible addition of thiols to benzoquinone and mono-, di-, and trisubstituted quinones are well documented reactions, resulting in thioether-substituted hydroquinones.[6a] These reactions do not constitute reduction of quinones by thiols. In contrast, the tetrasubstituted quinone duroquinone was reported 60 years ago to be reduced directly by thioglycolic acid in alkali.[6c] More recent reports showed, however, that neither duroquinone[6b] nor 2,3-dimethoxynaphthoquinone[6d] reacted with the thiol GSH. Thus, the facile reaction of MA (also a tetrasubstituted quinone) with thiols, as described herein, does not appear to have a clear-cut precedent. Prior to our work, Kono et al. reported[7] that in the presence of thiols MA decomposed, and certain solvolytic mitosene products and disulfide derivatives could be identified in the complex reaction mixture.

The consumption of MA leading to the formation of mitosenes by the reaction of mitomycin A with thiols could in principle be attributed to a strictly nucleophilic activation (Scheme 2): Michael addition of thiol to the 6-position of

Scheme 2. Proposed Mechanism for Nonreductive, Nucleophilic Activation of Mitomycin A by Thiols



the quinone would disrupt the vinylogous amide, allowing for N-4 assisted elimination and subsequent nucleophilic substitutions at positions 1 and 10.[8a−c] One way to distinguish experimentally between a nucleophilic activation mechanism and a reductive mechanism is the analysis of the resulting MA metabolites. The nucleophilic activation (Scheme 2) should result in the formation of mitosenes substituted by nucleophiles at the 1 and 10 positions. However, if a reductive mechanism is involved, 1-unsubstituted mitosenes, e.g., **5b**, should also be observed (Scheme 1). We show below that thiol-induced activation of MA involves reduction, i.e., electron transfer from the thiol to the quinone ring, as evidenced by formation of **5b**, and other dihydromitosene derivatives, along with mono- and bifunctional nucleophilic substitution products **6**. We determined the mechanism of the reduction by a kinetic study, also described below.

HPLC analysis of the reaction between MA and DTT at pH 6 showed the predominant formation of a single mitosene metabolite (Figure 2A), which was characterized as 2-amino-7-methoxymitosene **5b** by ESIMS, ^{1}H NMR,[8b] and conversion to 2,7-diaminomitosene **5a**[3] by ammonia treatment. The yield of **5b** was pH-dependent, decreasing at higher pH values in favor of 1,10-dihydroxymitosenes.

The finding that **5b** is a major product indicates that the major path of the activation cascade involves an intermediate

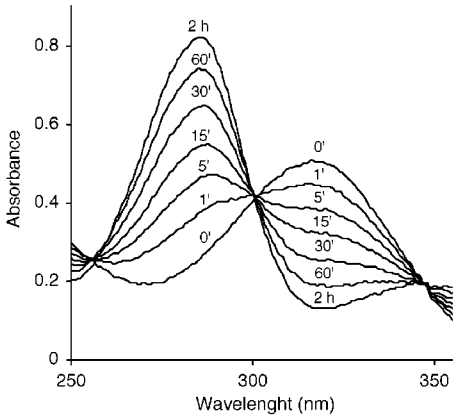


Figure 1. UV assay of the reduction of mitomycin A by DTT under aerobic conditions. Reaction times are indicated on top of each spectra.

(6) (a) Wardman, P. *Free Radical Res. Commun.* **1990**, *8*, 219−299. (b) Butler, J.; Hoey, B. M. *Free Radical Bio. Med.* **1992**, *12*, 337−345. (c) Snell, J. M.; Weissberger, A. *J. Am. Chem. Soc.* **1939**, *61*, 450−453. (d) Gant, T. W.; Rao, D. N. R.; Mason, R. P.; Cohen, G. M. *Chem. Biol. Interact.* **1988**, *65*, 157−173.

(7) Kono, M.; Saitoh, Y.; Kasai, M.; Shirahata, K.; Morimoto, M.; Ashizawa, T. *J Antibiot.* **1993**, *46*, 1428−1438.

(8) (a) Wang, S.; Kohn, H. *J Med. Chem.* **1999**, *42*, 788−790. (b) Subramaniam, S.; Kohn, H. *J. Am. Chem. Soc.* **1993**, *115*, 10519−10526. (c) Kohn, H.; Wang, S. *Tetr. Lett.* **1996**, *37*, 2337−2340.

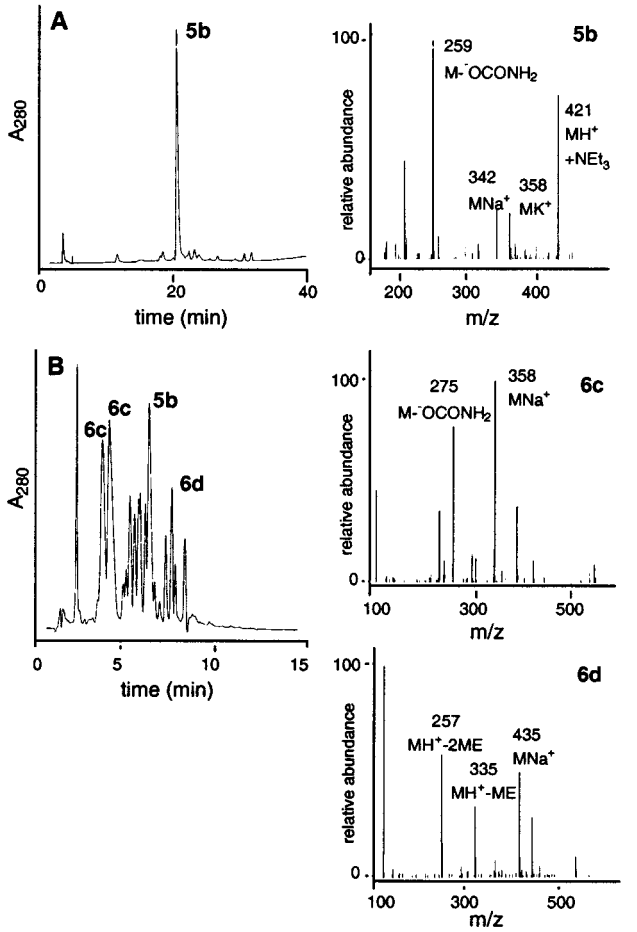


Figure 2. (A) HPLC analysis of the reaction of mitomycin A and DTT and ESIMS of the major peak, idenfified as **5b**. (B) HPLC analysis of the reaction of 1 mM mitomycin A and 10 mM mercaptoethanol after 2 h, and ESIMS of three selected peaks, idenfified as **6c** (two isomers) and **6d**.

hydroquinone **3** (X = OCH_3), and therefore the activation of MA with DTT occurs predominantly through a reductive mechanism and not a nucleophilic mechanism. The same applies to the activation of MA by MER: HPLC analysis of the reaction at pH 7.5 showed multiple peaks (Figure 2B), one of which again corresponds to 2-amino-7-methoxymitosene **5b**. The peaks were characterized by ESIMS and UV as mitosene derivatives of MA, with various substitution patterns in positions 1 and 10, including additional dihydromitosene as reduction products. Figure 2 shows three selected ESIMS, which correspond to **5b**, **6c** (1-α and 1-β isomers), and **6d**. The **6c** isomers are known,[9] and their indentity was further confirmed by direct comparison by HPLC with the authentic compound.

To gain insight into the mechanism of the reaction of MA and thiols, we decided to perform kinetic measurements by a UV assay of the decrease of the absorbance from the MA chromophore (λ_{max} 317 nm). Experiments were performed with different thiols (MER, *N*-Ac-Cys or DTT), each at several different thiol concentrations and different pH values. In all cases, the rate of disappearance of MA could be fit satisfactorily to a first-order rate law (see Supporting Information). For each thiol, the value of the observed rate constant k_{obs} was dependent on the thiol concentration and the pH, indicating an involvement of a thiolate anion directly in (or preceding) the rate-determining step. A summary of the observed rate constants (k_{obs}) is shown in Table 1.

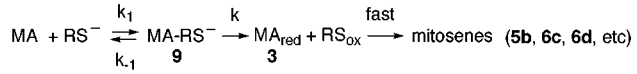
Table 1. Rate Constants for the Reaction of MA with Thiols[a]

pH	[RSH]	R^2	k_{obs} (min^{-1})	k' [b] (min^{-1})
7.8	25 mM N-Ac-Cys	0.994	0.067	144
8.0	10 mM N-Ac-Cys	0.992	0.041	140
8.0	25 mM N-Ac-Cys	0.994	0.131	178
8.0	50 mM N-Ac-Cys	0.994	0.234	160
8.2	25 mM N-Ac-Cys	0.994	0.171	142
7.8	25 mM MER	0.997	0.178	600
8.0	10 mM MER	0.998	0.080	429
8.0	25 mM MER	0.995	0.296	633
8.0	50 mM MER	0.998	0.764	817
8.2	25 mM MER	0.997	0.569	776
6.1	0.2 mM DTT	0.999	0.280	1.76×10^6
6.3	0.2 mM DTT	0.998	0.454	1.80×10^6
6.5	0.2 mM DTT	0.997	0.649	1.62×10^6
6.8	0.2 mM DTT	0.998	1.249	1.56×10^6
7.0	0.2 mM DTT	0.993	2.076	1.65×10^6
6.1	0.05 mM DTT	0.999	0.080	2.02×10^6
6.1	0.10 mM DTT	0.999	0.143	1.80×10^6
6.1	0.20 mM DTT	0.980	0.311	1.96×10^6
6.1	0.50 mM DTT	0.997	0.751	1.89×10^6
6.1	1.00 mM DTT	0.972	1.538	1.93×10^6

[a] See Supporting Information for experimental details. [b] Calculated as $k' = k_{obs}([H^+] + K_a)/K_a[RSH]_0$. See Supporting Information for derivation.

We argue that these observations support the mechanism shown in Scheme 3. According to this mechanism, an initial

Scheme 3. Proposed Mechanism for the Reductive Activation of Mitomycin A by Thiols

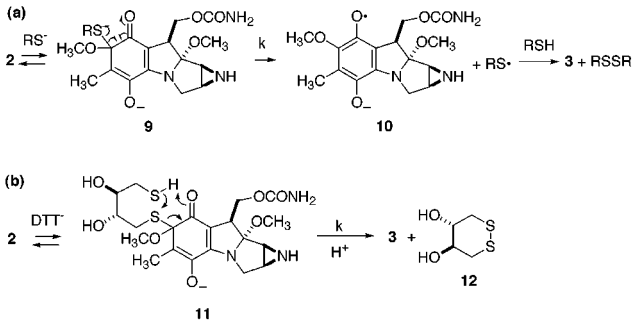


Michael addition of thiolate anion to the quinone generates an intermediate thiol−mitomycin adduct MA-RS^- **9** or **11** (Scheme 4) which then undergoes a rate-determining intramolecular redox reaction to give a reduced derivative of MA (**3**) capable of forming the observed 1-dihydro mitosene **5b** as an end product, as well as nucleophilic-substituted mitosene products **6** (cf. Scheme 1).

The rate of disappearance of MA will be given by k[MA-RS^-], and the rate constant can be expressed as $k = k_{obs}$-$([H^+] + K_a)/K_aK_{eq}[RSH]_0$, where K_a is the acidity constant of the thiol and K_{eq} is the equilibrium constant of the formation of the MA-RS^- adduct **9**.[10] Although the values of K_{eq} are not known, we can assume that they will be similar

(9) Taylor, W. G.; Remers, W. A. *J. Med. Chem.* **1975**, *18*, 307−311

Scheme 4. Proposed Mechanism for the Activation of Mitomycin A by Monothiols (a) and DTT (b)



for the three thiols. This assumption allows us to calculate values of k', which is related to k by the same constant K_{eq} ($k' = K_{eq}k$) for the three thiols. Table 1 shows the values of k' calculated from the equation $k' = k_{obs}([H^+] + K_a)/K_a[RSH]_0$. The values of k' (min^{-1}) obtained for the reactions with *N*-Ac-Cys, MER, and DTT at various concentrations of the thiol and various pH's are in excellent agreement within the same thiol: $(1.5 \pm 0.1) \times 10^2$ (*N*-Ac-Cys); $(6.6 \pm 1.2) \times 10^2$ (MER); $(1.80 \pm 0.12) \times 10^6$ (DTT). The excellent fit of the experimental data supports the proposed mechanism, applicable to all three thiols.[11]

We then investigated the source of the large difference between the rates (k') for monothiols and the bisthiol DTT, about 4 orders of magnitude. If the Michael addition was the rate-limiting step of the reaction, the values of k' specify k_1 (rate of addition of thiolate to the quinone of MA). The values obtained for k' rule out this scenario: DTT, like MER and *N*-Ac-Cys, is an aliphatic thiol, and it is implausible that it adds 10^4 times faster than *N*-Ac-Cys as a nucleophile with quinones. The rate-limiting step must be the internal redox reaction k as shown above. The large observed differences of k' can only be explained by proposing a difference in the mechanism of this step between DTT and the monothiols. We propose that the mechanism for the reaction with MER and *N*-Ac-Cys is analogous to the mechanism we previously proposed for disulfide-substituted mitomycin C derivatives:[12] the rate-limiting electron-transfer step is a homolytic dissociation of the quinone−sulfur bond, resulting in the formation of the semiquinone of MA (**10**) and the thiyl radical of MER or *N*-Ac-Cys (Scheme 4). The semiquinone **10** then reacts rapidly with another thiol molecule to form the hydroquinone **3**. For the 10 000-fold faster DTT activation, we propose that the mechanism of the rate-limiting step is also a C−S bond dissociation, but it proceeds via an intramolecular ene-like reduction, which produces directly the hydroquinone of MA (**3**) and oxidized DTT (Scheme 4). Since the products of the reduction with DTT by this mechanism (**3** and **12**) are much more stable than the intermediates of the reaction with the monothiols (**10** and RS•), according to the Hammond postulate the activation energy of the transition state of the reduction of MA by DTT is much lower as well; this explains the observed rate differences.[13]

The masked alkylating functions of the pro-drug MA are shown to be activated by thiols. This process may have significance for the observed high toxicity of MA relative to that of MC. GSH, present in up to 5 mM concentration in mammalian cells, could generate activated MA which would exceed the concentration of enzymatically activated MC formed in the cells under equivalent drug dose conditions. The higher level of activated MA may lead to additional toxic lesions. Another interesting prediction stemming from the present work is that in the cell MA may act as a selective oxidant of important bifunctional thiols, e.g., thioredoxin or lipoate enzymes, inhibiting their function. The feasibility of this prediction will be tested in in vitro systems.

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Supporting Information Available: Experimental procedures for the determination of the kinetics of the reaction of MA with thiols. Calculations of the rate costants k_{obs} and k'. Procedures for HPLC and ESIMS. Preparation of **5a**, **5b**, and **6c** isomers. UV, ESIMS, and HPLC proofs of structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Adduct **9** (C-7 thiol adduct) is the most likely regioisomer of six possible quinone adducts, all of which could react by the same mechanism.

(11) An alternative, direct electron transfer from RS^- to quinone is not ruled out rigorously by our data. It is unlikely, however, since such reactions were estimated to be 10^{11} times slower, if they occurred at all, than Michael addition of RS^- to quinones.[6b] Furthermore, it would not explain the large rate increase observed with DTT.

(12) He, Q.-Y.; Maruenda, H.; Tomasz, M. *J. Am. Chem. Soc.* **1994**, *116*, 9349−9350.

(13) A simpler difference, such as intermolecular vs intramolecular reaction of a second R−SH with the MA-RS^- adduct, involving otherwise the same mechanism for the monothiols and DTT would not account for the large (10^4-fold) magnitude of the rate difference. Furthermore, the observed dependence of k_{obs} on thiol concentration is essentially identical in the three thiols (Table 1).