The Reactions of Mitomycin C with Dithiols

III. Mysterious Reactions

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Abstract

We report two unexpected findings ocurring after treatment of the antitumor drug mitomycin C with dithiols. The first finding relates to the reaction of mitomycin C with exactly one molar equivalent of dithiol to form a mitosene-like material with unusual physico-chemical properties. The second finding was the formation of unexpected dimeric mitosenes when the reaction of MC with dithiols was quenched at short reaction times. We hypothesize that both events are originated by an unprecedetended deaziridination reaction.

Introduction

The outcome of the reaction of the clinically used antittumor drug mitomycin C[1] with dithiols was studied. The outcome of the reaction is extremely dependent on the dithiol/MC ratio and the reaction time. With sub-stoichiometric amounts of dithiols 1-hydroxymitosenes **5a** are observed as major products[2] and with excess dithiol and long reaction times the major products were dithiol cross-links **8**.[3] When one mol of MC was treated with one mol of dithiol an unsoluble mitosene (mitosene **X**) was obtained, and when the reaction of MC with excess dithiol was performed a unexpected dimeric mitosene was observed (mitosene **Y**). While we were unable to isolate mitosenes **X** and **Y**, some clues on its identity were attained, and they are presented here.

Results and discussion

The distribution and relative amounts of products formed in the reaction of MC with different concentrations of DTT or 1,3-propanedithiol was studied and the results of these experiments are shown in Figure 1.
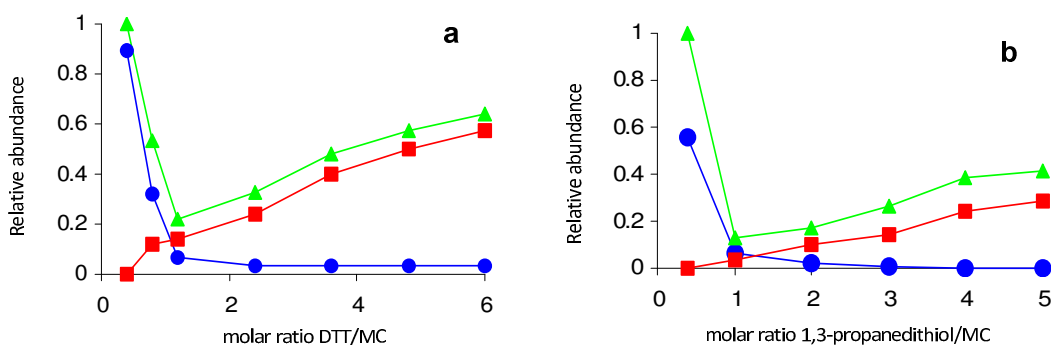

Figure 1. Dependence of the reaction of MC and dithiols on the concentration of dithiol: (a) DTT; (b) 1,3-propanedithiol. Reaction mixtures containing MC with increasing concentrations of dithiol were analyzed by HPLC (λ = 310 nm). Lines are: hydroxymitosenes **5a** (blue circles); cross-links **8** (red squares); sum of HPLC-detectable mitosenes (green triangles). The relative abundance of each compound was determined from the area of the observed peaks. The relative abundance of each compound was normalized (1 for the sum of all mitosenes observed with the lowest ratio dithiol/MC).

The reactions with substoichiometric dithiols resulted in the formation of 1-hydroxymitosenes **5a** as major products, as previously reported.[2] The use of molar ratios dithiol/MC higher than two suppressed the formation of **5a**, and resulted in the formation of dithiol cross-links **8** as major products.[3] When equimolar amounts of dithiol and MC were used, minimal amounts of HPLC-detectable mitosenes were observed and an insoluble red-brown precipitate was observed for both dithiols assayed. This material, thereafter termed mitosenes **X**, was collected by centrifugation and was washed with several solvents, as the solid was insoluble in common organic solvents, and could only dissolved in DMSO and DMF to give deep-red solutions. Mass recoveries for mitosenes **X** averaged 80%

of the original MC mass. UV spectra for mitosene X showed absorbance maximums at wavelengths characteristic of mitosene structures. Several MS and NMR experiments did not provide decipharable information.[2] The derivatization of mitosenes **X** with Ac_2O or Boc_2O resulted, in same cases, in the formation of mitosene materials that were soluble in organic solvents, but all attempts to ascertain its identity failed. The elemental analysis of samples of mitosene **X** from DTT indicated that on average it contained two mitosene molecules per molecule of DTT, while the material obtained from 1,3-propanedithiol the ratio mitosene/dithiol was 1:1.[2] As the only reacting species are MC, water and dithiol, the most plausible interpretation is that the autocataytic activation of MC generates **4**, that then reacts with 1,3-propanedithiol to give monoadduct **10** as the initial product. Afterwards, compound **10** evolves by an unknown pathway to form the unsoluble material, perhaps a polymer formed as a result of the electrophilic /nucleophilic nature of **10** and other mitosenes. A hypothesis for the evolution of **10** by a deaziridination reaction (Scheme 1) is discussed below.
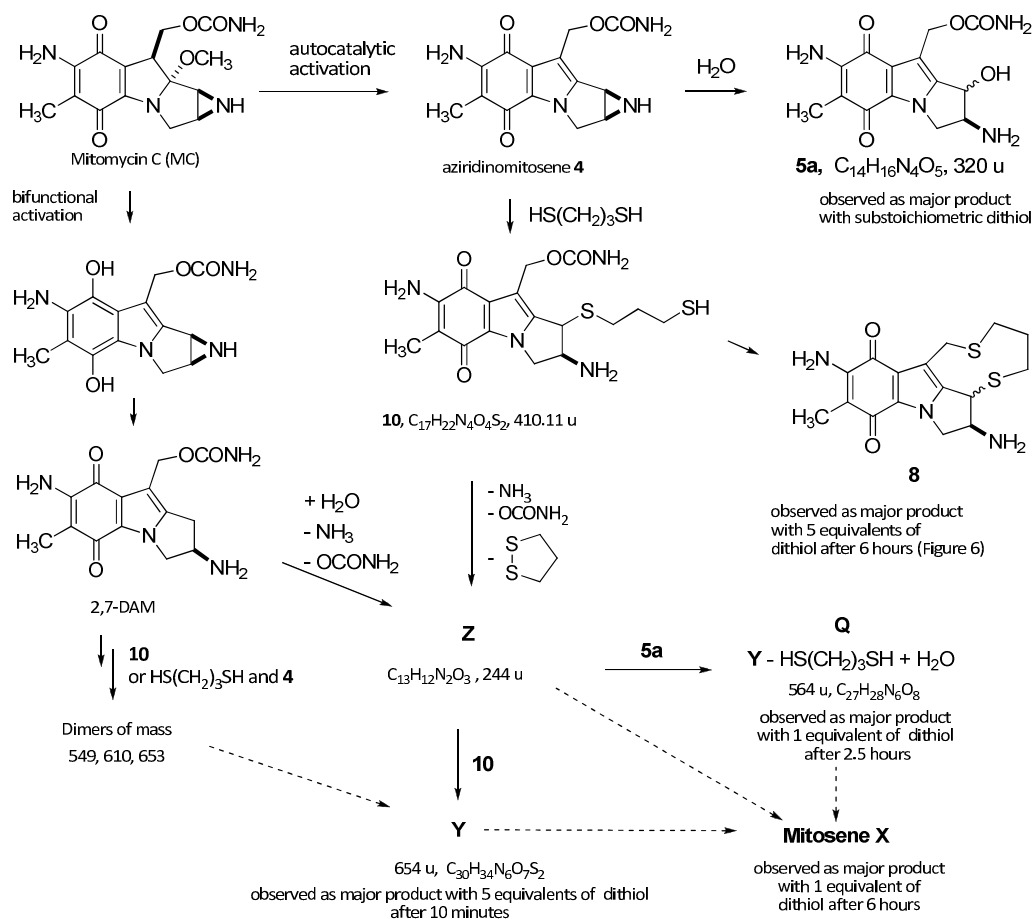
Scheme 1. Summary of our hypothesis for the formation of mitosenes **Q, X, Y, Z** during the reactions of MC with 1,3-propanedithiol.

We performed a number of experiments aimed at detecting transient intermediates formed during the reaction of MC with 1,3-propanedithiol. Aliquots of a reaction mixture of MC and propanedithiol were removed at time intervals and quenched by lowering the pH to 5-6. LC/MS analysis showed the formation of 1-hydroxymitosenes **5a** in aliquots quenched immediately after the autocatalytic reaction, and the formation of cross-links **8** after prolonged times (Figure 2). Aliquots quenched after intermediate times (5-20 min) showed the presence of a transient major product, thereafter termed **Y**, with an apparent mass of 654 amu. Peaks corresponding to monoadduct **10** were also observed, but they were formed in relatively small quantities (Figure 3a). When aliquots taken after 5-10 minutes of the autocatalytic reaction were quenched with the thiol trapping reagents maleimide or iodoacetate, adducts corresponding to maleimide or iodoacetate adducts of **Y** were observed (termed **Y**-MI and **Y**-IA respectively), together with minor peaks corresponding to the addition of maleimide or iodoacetate on **10** (Figures 3b, 3c). An exact mass for **Y** could be obtained using LC/HRMS, and the only plausible formula fitting the observed ions was $C_{30}H_{34}N_6O_7S_2$,[4] indicating the formation of a dimeric structure containing one molecule of dithiol.

A structure consistent with this molecular formula can not proposed using known MC reactivity.The proposed structure for **Y** is based on the following considerations:

a. It must contain one free SH group, as it reacts with maleimide and iodoacetate to give a maleil- or carboxymethyl-monoadducts.
b. From (a), it follows that **Y** must incorporate (at least) one molecule of dithiol in its structure. If two are incorporated, only one of them contains a free SH group.
c. It contains one carbamate group: the EI-MS of **Y**, **Y**-IA and **Y**-MI shows ions for the corresponding fragment.
d. It must also contain one hydroxyl group: EI-MS of **Y** and **Y**-MI show a fragment corresponding to loss of carbamate and H_2O.

Based on the HRMS of **Y** (Figure S19), the only plausible molecular formula is $C_{30}H_{34}N_6O_7S_2$. Requirements to deduce the formula were:

a. at least 5 oxygen atoms (four for two quinones, one for one carbamate)
b. at least 5 nitrogen atoms (four for two mitosene cores and one for carbamate)
c. two or four sulfur atoms
d. Other formulas fitting the expected mass were $C_{31}H_{38}N_6O_2S_4$, $C_{33}H_{40}N_3O_3S_4$, $C_{32}H_{36}N_3O_8S_2$, and $C_{33}H_{32}N_7O_4S_2$.[4]
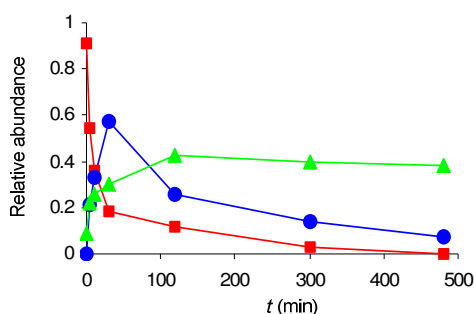

Figure 2. Time course of the reaction of MC with 1,3-propanedithiol. Aliquots of a reaction mixture containing 5 mM MC and 25 mM propanedithiol at pH 10.0 (dithiol as internal buffer) were removed at time intervals after the autocatalytic reaction was observed, quenched by addition of phosphate buffer pH 6.0, and analyzed by HPLC (λ = 310 nm). The relative abundance of each compound was determined from the area of the observed peaks: **5a** (red squares); transient intermediate **Y** (blue circles); cross-links **8** (green triangles). The abundance of each compound was normalized (1 for the sum of mitosenes observed at t = 0).
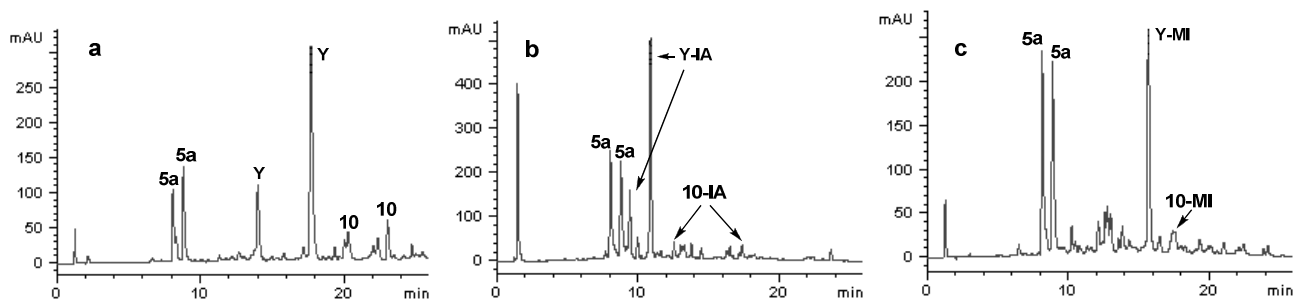

Figure 3. Detection of intermediates formed during the reaction of MC with 1,3-propanedithiol: Aliquots from the reaction of MC (4 mM) with propanedithiol (20 mM) were quenched after 10 minutes with NH_4Ac (A), iodoacetate (B) or maleimide (C) and analyzed by HPLC (λ = 310 nm). **Y** represents the unknown intermediates with a mass of 654 amu. **Y**-IA and **Y**-MI represent iodoacetate and maleimide adducts of **Y**. **10**-IA and **10**-MI represent iodoacetate and maleimide adducts of **10**.

Wwe propose that the intermediate **Y** and the intermediate with mass 564 amu (mitosene **Q**) result from a coupling reaction of a common mitosene intermediate **Z** of molecular formula $C_{13}H_{12}N_2O_3$ with **10** and **5a** respectively (Scheme 1).

In addition to **Y** other unusual compounds with dimeric structure were observed in the HPLC trace:

- Compounds with mass 594 amu, that can be attributed to a dimer linking two units of 2,7-DAM by one dithiol molecule, and also to coupling products of 2,7-DAM derivatives with C-10 reduced derivatives and one molecule of dithiol in different combinations (Chart 1).
- Compounds with mass 610 amu, that can be attributed to coupling products of decarbamoyl-**10** with 2,7-DAM, among other possibilities (Chart 1).
- Compounds with mass 653 amu, that can be attributed to carbamoylated versions of the compounds with mass 610 amu (Chart 1).

The proposed structures for the observed dimeric mitosenes (Chart 1) are derived (at least in one of the mitosene portions) from 2,7-DAM or a C10 reduced mitosene. That would mean that in the reaction mixtures of MC and dithiols generate bifunctionally activated MC (from which 2,7-DAM or C10 reduced mitosenes are formed).

We propose that the reaction of MC with dithiols generates an intermediate **Z** that is converted to compounds **Q** and **Y** by addition of **5a** or **10** respectively (Scheme 1). These structures would fit the observed mass (564 and 654 amu) and the molecular formulas derived for **Y** and **Q**. In essence, the required removal of ammonia could occur by hydrolysis of N-7, but this reaction requires much more drastic conditions than the ones employed in our experiments, were the 7-amino group has consistently shown to be stable. As the 7-hydroxy-2-aminomitosene hypothesis is very unlikely we considered other options, and the only alternative hypothesis we envision is a deaziridination reaction. This reaction is unprecedented to our knowledge, but reasonable mechanisms for such transformation can be proposed, and they are presented in Scheme 2.
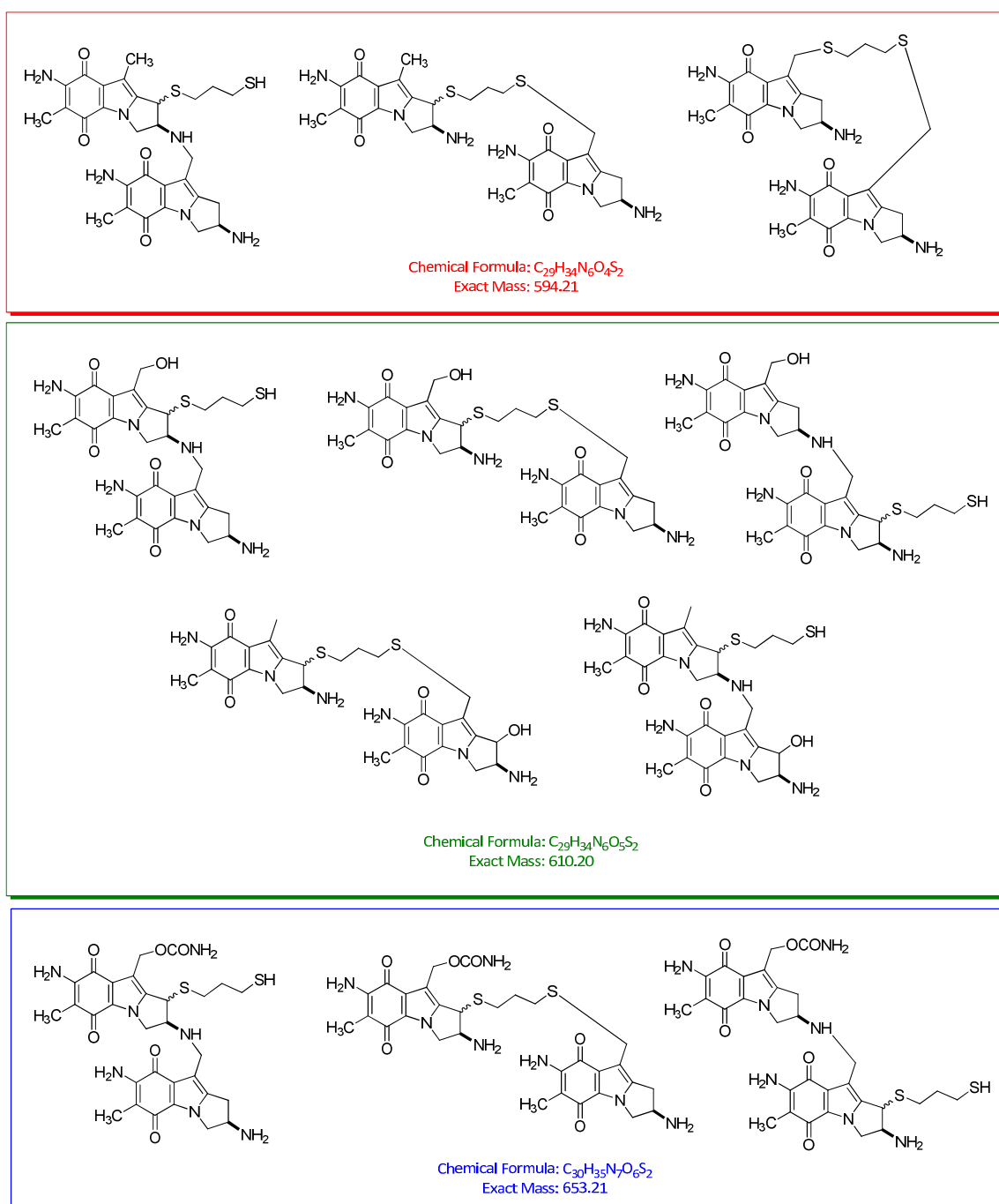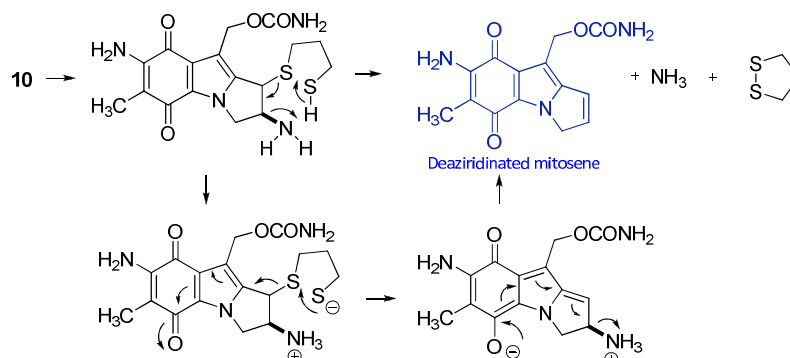

Chemical Formula: $C_{29}H_{34}N_6O_4S_2$
Exact Mass: 594.21

Chemical Formula: $C_{29}H_{34}N_6O_5S_2$
Exact Mass: 610.20

Chemical Formula: $C_{30}H_{35}N_7O_6S_2$
Exact Mass: 653.21

Chart 1 . Some hypothetical dimeric mitosenes fitting a mass of 594, 610 or 653 amu.

Scheme 2. A mechanistic hypothesis to explain how deaziridinated mitosenes could be formed.

Addition of H_2O to deaziridinated mitosene provides structures for mitosene **Z** (Chart 3) that, after coupling with **5a** or **10**, would explain the mass spectra observed for mitosene **Y** and other compounds detected during the reaction of MC with dithiols.
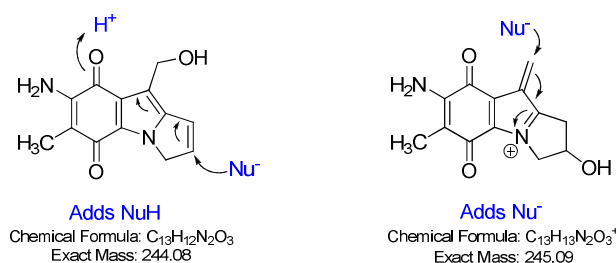

Chart 3. Proposed structures for mitosene Z

While precedents for deaziridination reactions by dithiols are unknown to us a mechanistically related reaction is known: the reduction of epoxides by dithiols. An analogous mechanism to the one proposed here for the deaziridination of mitosenes has been postulated by Silverman[5] for the reduction of vitamin K epoxide to vitamin K by dithiols.[6] Further research will be necessary to confirm that dithiols can indeed reduce aziridines to alquenes.

Reference

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[2] Paz, M. M. Reductive Activation of Mitomycin C by Thiols: Kinetics, Mechanism, and Biological Implications.. *Chem. Res. Toxicol.* **2009**, *22*, 1663–1668.

[3] Paz, M. M. Cross-Linking of Dithiols by Mitomycin. *Chem. Res. Toxicol.* **2010**, *23*, 1384–1392.

[4] Calculated using Molecular Fragment Calculator, version 1.0 (James E. Deline, 1995) using a tolerance of 0.003 u.

[5] Silverman, R. B. (1981) Chemical Model Studies for the Mechanism of Vitamin K Epoxide Reductase. *J. Am. Chem. Soc. 103*, 5939-5941.

[6] Jin, D. Y., Tie, J. K., and Stafford, D. W. (2007) The conversion of vitamin K epoxide to vitamin K quinone and vitamin K quinone to vitamin K hydroquinone uses the same active site cysteines. *Biochemistry 46*, 7279-7283