

The Reactions of Mitomycin C with Dithiols

II. Formation of Dithiol Cross-Links

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Abstract

We report that the clinically used antitumor drug mitomycin C reacts with excess dithiols to give dithiol cross-links as major products. Mechanistic studies reveal that three dithiol molecules participate at different stages of the reaction: in the reductive activation of mitomycin C, in an alkylation at C1, and in an additional reduction that activates C10 for the second-arm alkylation by the dithiol. We hypothesize that the reactions reported here indicate that mitomycin C could act as a mechanism-based inhibitor of enzymes containing a dithiol active site.

Introduction

Mitomycin C (MC, **1**) is an antitumor antibiotic used clinically in cancer chemotherapy¹ and in ophthalmologic procedures.² MC is a “smart” prodrug, inert towards nucleophiles in its original structure, but transformed into a highly reactive bis-electrophile after reduction.³ The mechanism of activation of MC is initiated by the reduction of the quinone ring of MC to form hydroquinone **2**, that spontaneously eliminates methanol to form leucoaziridinomitosenone **3** (Scheme 1), a bis-electrophile that alkylates cellular nucleophiles such as phosphate,⁴ bicarbonate,⁵ glutathione⁶ and DNA.⁷ Sequential alkylation of DNA at the 1 and 10 positions of **3** generates a cytotoxic interstrand crosslink that specifically links N2 of opposite deoxyguanosine residues at CpG sites of double stranded DNA.

Recent reports indicate that glucose regulatory protein (GRP58) plays a significant role in the cellular activation of MC,⁸ and that the activity of this protein resides in its two thioredoxin-like domains.⁹ The involvement of proteins containing a dithiol active site in the biological activity of MC is not restricted to the above mentioned activation by GRP58. We recently reported a successful activation of MC using simple dithiols as chemical models for the dithiol functional group present in the active site of proteins of the thioredoxin family.¹⁰ Continuing with our investigation of simple thiols as a chemical model for dithiol-containing proteins in their reactions with MC we report here that dithiols, in addition to perform the reductive activation of MC, are alkylated by activated MC to form *S,S'*-crosslinks as the almost exclusive end products.

Results and discussion

The initial reactions were performed using DTT, and the product profile observed in the HPLC trace (Figure 1) consisted of four mitosenes with identical UV and MS, that we identified as the four possible diastereomeric mitosenes resulting from alkylation of DTT by a single MC molecule at its 1- and 10- positions. The HPLC analysis of reactions between the oxidoreductase cofactor DHLA and MC (Figure 1) revealed the formation of at least seven of the eight expected isomers, resulting from bis-alkylation of MC with one molecule of racemic DHLA.

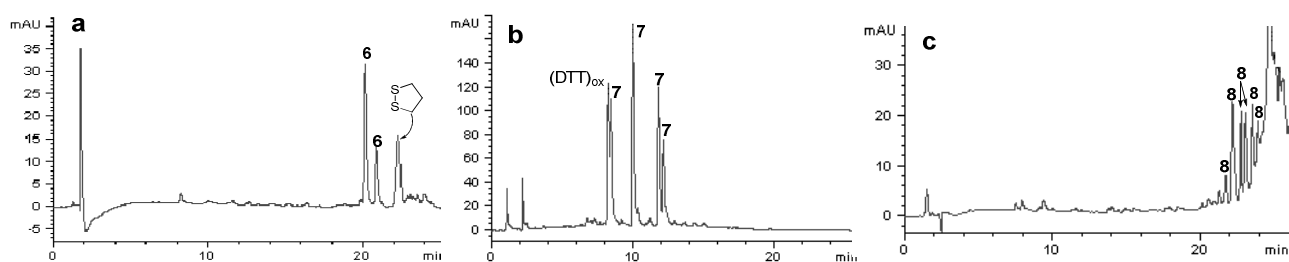


Figure 1. HPLC traces for the reaction of MC with dithiols: (a) 1,3-propanedithiol; (b) DTT; (c) DHLA. Reactions contained a molar ratio dithiol/MC = 5, and were performed at 20 °C for 8 hours, quenched by acidification and analyzed by LC/MS ($\lambda = 310$ nm).

LC-HRMS gave exact masses that confirmed the molecular formula of the proposed DTT crosslinks **7** and DHLA crosslinks **8** (Chart 1). For a full characterization of the crosslinks of MC with dithiols, we used 1,3-propanedithiol as a means to simplify the composition of the reaction mixture. As expected, the reaction of MC with 1,3-propanedithiol (Figure 1) gave two isomers with UV and MS supporting structure **6**. We should note here that the simple chromatograms obtained after the reaction of MC with dithiols (Figure 1) do not imply that all MC is converted to MC-dithiol crosslinks. Side reactions do occur but, as we will discuss later, they result in the formation of unidentified insoluble mitosenes that do not appear on the HPLC trace. The two isomers of **6** could be separated and fully characterized using NMR spectroscopy. The main evidence for the stereochemistry was obtained using circular dichroism, a well established technique to assign the stereochemical configuration at C-1 of 2-aminomitosenes.¹¹ In our case, the isomers from the reaction of MC and 1,3-propanedithiol gave CD spectra that were almost symmetrical (Figure 3), and confirmed the expected stereochemistry at C-1, with the major isomer bearing a *cis*-configuration.

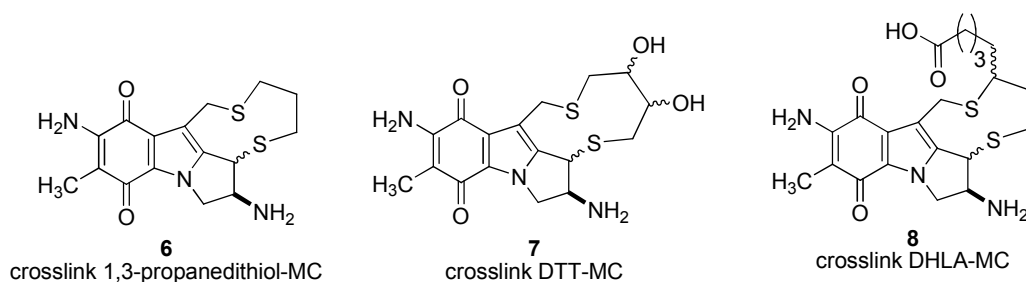
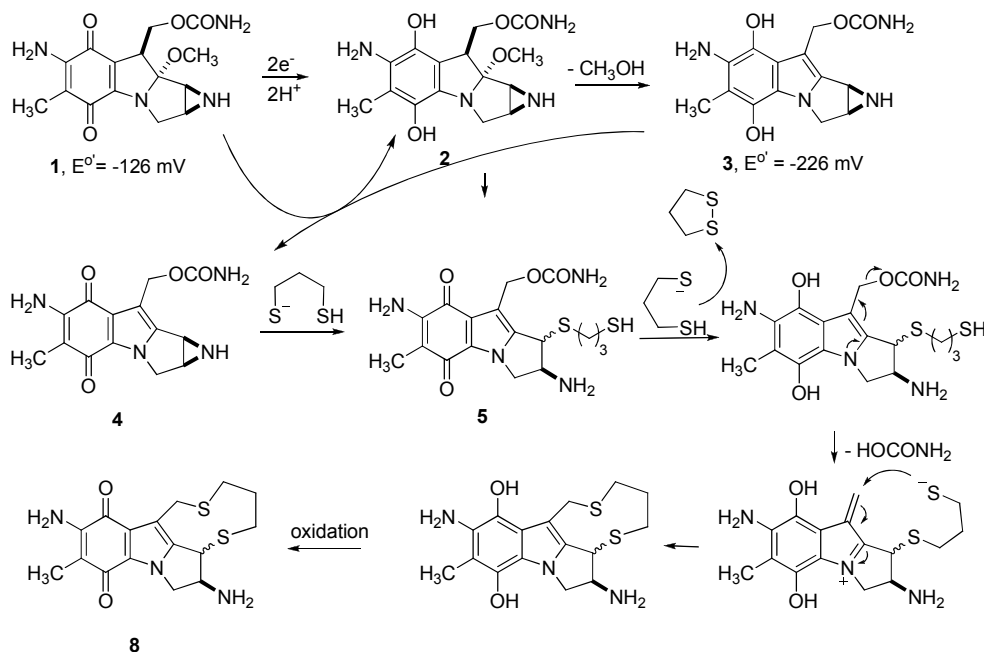


Chart 1. Structures of mitomycin C-dithiol crosslinks identified in this work.

A study of the dependence of the reaction on dithiol concentration provided insight in the mechanism for the formation of **8**. The proposed global mechanism for the reaction of MC with dithiols involves a cascade of reactions where the dithiol intervenes in three different stages (Scheme 3): the first step is the autocatalytic reaction of mitomycin C with substoichiometric dithiol to form aziridinomitosene **4**; in the second step, a nucleophilic addition of thiolate to C-1 of **4** forms monoadduct **10**; the third step involves the reduction of the mitosene-dithiol monoadduct **5** by a third molecule of dithiol to the hydroquinone form, with the subsequent elimination of the carbamoyloxy group of the C-10 position; in the fourth step a nucleophilic addition of the second arm of the dithiol to the C-10 position generates the crosslink adduct **6** in the reduced form; in the final step, oxidation to the quinone form generates the end product **6**.

The ease of formation of MC crosslinks with simple dithiols raises the question if biological dithiols could be a substrate for MC-induced *S,S'*-crosslinking. The thiol group of one of the cysteine residues in thioredoxin superfamily is known to be deprotonated at physiological pH and to react as an excellent nucleophile,¹² and a similar reactivity has been observed for the selenol group in thioredoxin reductase (TrxR).¹³ Alkylation reactions of Trx and TrxR with electrophiles that target DNA, such as cisplatin,¹⁴ and 4-hydroxy-2-nonenal,¹⁵ have been reported, and bis-electrophilic mustards alkylated a single cysteine in the dithiol active site of trypanothione reductase.^{16,17} The alkylation of TrxR with the bis-electrophile curcumin has been reported to give bis-adducts containing two curcumin molecules, but not crosslinks.¹⁸ The distance between the two sulfur atoms in reduced human thioredoxin is 3.1 Å while in *E. coli* thioredoxin is 3.8 Å.¹⁹ These values are similar to the 3.5 Å distance between the exocyclic 2-amino groups of opposite deoxyguanosine residues of CpG sequences, that are the target for DNA-DNA crosslinks generated by MC.²⁰ It is also comparable to the C1-C10 distance calculated for an energy-minimized bis-electrophilic mitosene (3.38 Å), and to the C1-C10 distance of 3.4 Å measured from a solution structure of an oligonucleotide modified by MC.²¹ Consequently, based on the results presented here, on the known reactivity of Trx and TrxR, and on geometric considerations, we consider that the formation of *S,S'*-crosslinks by MC could be reproduced in proteins containing a dithiol

active site. We consider that the potential formation of dithiol crosslinks in proteins constitutes an exciting prospect, as the only reported precedents of such chemistry are arsenic derivatives.^{22 23 24} We should also remark that prospective studies in the reductive activation of MC by dithiol-containing proteins must bear in mind that the activation reaction may result in inhibition, as a result of alkylation reactions in the active site of the enzyme by activated MC.



Scheme 2. Proposed mechanism for the formation of MC - 1,3-propanedithiol crosslinks.

Conclusions

Crosslink adducts formed by three dithiols with MC are reported, including the biological cofactor dihydrolipoic acid. The formation of the mitomycin-dithiol crosslink requires excess dithiol, and we propose that it is needed for a second quinone reduction that activates the initially formed dithiol-mitosene monoadduct for nucleophilic addition at C10. A study of the time course for the reaction of MC with 1,3-propanedithiol showed an initial fast formation of an aziridinomitosene, that was slowly converted to dithiol crosslinks. The biological target of MC is considered to be DNA, but the biological activity of other electrophiles that target DNA has also been linked to the formation of covalent protein adducts, occurring predominantly at cysteine residues.²⁵ Our work with small dithiols has shown that they are oxidized and crosslinked by MC. Further work should clarify if proteins containing a dithiol active site could be a target for oxidation and/or formation of covalent crosslinks by this drug.

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