DNA Interstrand Cross-Link Formation Induced by Bioxalomycin α_2

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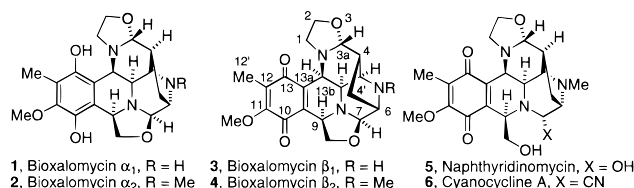
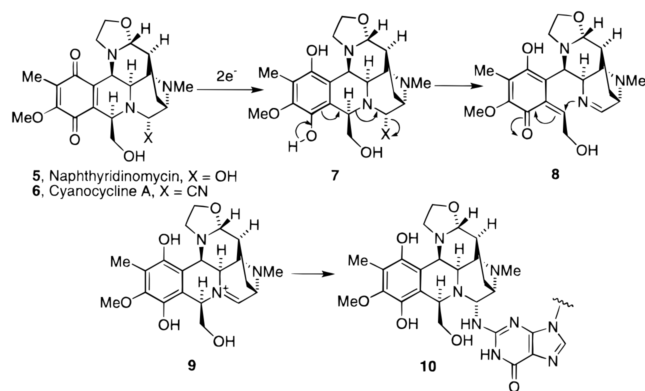
The Bioxalomycins (**1**−**4**) are antitumor antibiotics isolated from *Streptomyces viridostaticus* subsp. "*litoralis*".[1] Bioxalomycin α_2 (**2**), the main component of the mixture, possesses activity against Gram-positive and Gram-negative bacteria, including potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA).[2] Subsequent biological evaluation demonstrated that these compounds also exhibit activity against a panel of tumor cell lines. Ellestad and co-workers at Lederle Laboratories have demonstrated that bioxalomycin β_2 is identical with the well-known antibiotic naphthyridinomycin.[3] The antitumor activity of this class of compounds is believed to arise from their ability to inhibit DNA synthesis.[4] Previous work on the antibiotic naphthyridinomycin (**5**) and cyanocycline A (**6**)[3] demonstrated that these substances inhibit DNA synthesis via alkylation of DNA in the minor groove in GC-rich regions.[4d] In addition, the obligate reductive activation of these substances through reduction of the quinone moiety to the semiquinone radical anion species results in redox cycling of molecular oxygen with the production of superoxide; downstream Fenton−Haber−Weiss redox cycling culminates in oxidative damage and DNA strand scission.[5]



Remers and co-workers,[4c] as well as Cox and co-workers,[4b,f] have carried out molecular mechanics calculations on the interaction of naphthyridinomycin with DNA and have postulated a mode for covalent monoadduct formation in the minor groove (Scheme 1). Two-electron reduction of the quinone moiety to the hydroquinone (**7**) was proposed by Zmijewski[4d] to facilitate expulsion of the cyano group (in the case of cyanocycline) or water (in the case of naphthyridinomycin) from C7 to form the iminium species **9**; subsequent nucleophilic capture by the exocyclic amine of guanine provides the monoalkylation adduct **10** with the R-stereochemistry at C7.

Scheme 1



Additional modeling of the possible formation of covalent adducts at C3a and C7 forced these workers to conclude that: *"The geometry of naphthyridinomycin does not permit interstrand cross-linking involving both C3a and C7, but formation of cross-link to protein appears possible"*.[4c] Herein, we describe the first experimental evidence for DNA interstrand cross-link formation mediated by this class of compounds.

Incubating the 5′-^{32}P end-labeled A−B oligomer (Figure 1) with bioxalomycin α_2 at 37 °C for 12 h resulted in band-shifted products of slower mobility for the cross-linked DNA (Figure 1, C, lane 2).[6,8] The cross-linked DNA product was separated by denaturing polyacrylamide gel electrophoresis (DPAGE) (Figure 1, A, lane 2 and Figure 1, B, lane 2). Following isolation, both native and cross-linked materials were subjected to Fe(II)/EDTA footprinting reactions[7] (Figure 1, A and B). As expected, native DNA subjected to Fe(II)-EDTA digestion yields an equimolar assortment of all fragment sizes up to and including the full-length strand (Figure 1, A, lane 5 and Figure 1, B, lane 5). On the other hand, analogous treatment of the cross-linked product yields short fragments corresponding to cleavage at or to the radiolabeled side of the alkylated residue (Figure 1, A, lane 6 and Figure 1, B, lane 6). The observed cleavage patterns show that, for cross-linking of the native duplex, the drug spans dG10 (oligo A) to dG25 (oligo B) demonstrating a $^{5'}$CG$^{3'}$ specificity. Further evidence for this specificity was obtained by using duplex bearing inosine substituted at the dG25 on oligomer B. Incubation of the 2′-deoxy inosine-substituted duplex, where dG25 is substituted with 2′-deoxy inosine, abolished cross-link formation (Figure 1, C, lane 3). This result implicates that the alkylation events occur at the exocyclic amine at C2 of guanosine in the minor groove of DNA.

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(2) Singh, M. P.; Peterson, P. J.; Jacobus, N. V.; Maiese, W. M.; Greenstein, M.; Steinberg, D. A. *Antimicrob. Agents Chemother.* **1994**, *38*, 1808−1812.

(3) The structure previously portrayed in the literature as naphthyridnomycin (**5**) is actually incorrect. Ellestad et al.[1] have shown that structure **5** is an artifact of isolation where the oxazolidine ring has been hydrolytically ring-opened. Naphthyridinomycin is therefore identical to bioxalomycin β_2. For isolation and structural elucidation of naphthyridinomycin and the related compound cyanocycline, see: (a) Sygusch, J.; Brisse, F.; Hanessian, S. *Tetrahedron Lett.* **1974**, 4021−4023. (b) Sygusch, J.; Brisse, F.; Hanessian, S. *Acta Crystallogr.* **1976**, *B32*, 1139−1142. (c) Kluepfel, D.; Baker, H. A.; Piattoni, G.; Sehgal, S. N.; Sidorowicz, A.; Singh, K.; Vezina, C. *J. Antibiot.* **1975**, *28*, 497−502. (d) Hayashi, T.; Noto, T.; Nawata, Y.; Okazaki, H.; Sawada, M.; Ando, K. *J. Antibiot.* **1982**, *35*, 771−777. (e) Zmijewski, M. J., Jr.; Goebel, M. *J. Antibiot.* **1982**, *35*, 524−526.

(4) (a) Singh, K.; Sun, S.; Kluepfel, D. *Dev. Ind. Microbiol.* **1976**, *17*, 209−221. (b) Cox, M. B.; Arjunan, P.; Arora, S. K. *J. Antibiot.* **1991**, *44*, 885−894. (c) Hill, G. C.; Wunz, T. P.; MacKenzie, N. E.; Gooley, P. R.; Remers, W. A. *J. Med. Chem.* **1991**, *34*, 2079−2088. (d) Zmijewski, M. J., Jr.; Miller-Hatch, K.; Mikolajczak, M. *Chem.-Biol. Interact.* **1985**, *52,* 361−375. (e) Zmijewski, M. J., Jr.; Miller-Hatch, K.; Goebel, M. *Antimicrob. Agents Chemother.* **1982**, *21*, 787−793. A partial intercalative approach of naphthyridinomycin has been considered; however, the unnatural enantiomer was modeled in this study, see: (f) Arora, S. K.; Cox, M. B. *J. Biomol. Struct. Dynam.* **1988**, *6*, 489−502. For related studies on the covalent alkylation of DNA by saframycin A, see: (g) Ishiguro, K.; Takahashi, K.; Yazawa, K.; Sakiyama, S.; Arai, T. *J. Biol. Chem.* **1981**, *256*, 2162−2167.

(5) Breen, A. P.; Murphy, J. A. *Free Radical Biol. Med.* **1995**, *18*, 1033−1076.

(6) The doubling seen for the cross-link product is presumed to be due to orientational isomerism of the drug with respect to the cross-linkable site. For a related example of this phenomenon, see: Williams, R. M.; Rajski, S. R.; Rollins, S. B. *Chem. Biol.* **1997**, *4*, 127−137.

(7) Weidner, M. F.; Millard, J. T.; Hopkins, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 9270−9272.

(8) (a) Tomasz, M. *Chem. Biol.* **1995**, *2*, 575−579. (b) Verdine, G. L. Ph.D. Thesis, Columbia University, 1986. (c) Tomasz, M.; Lipman, R. *J. Am. Chem. Soc.* **1979**, *101*, 6063−6067.

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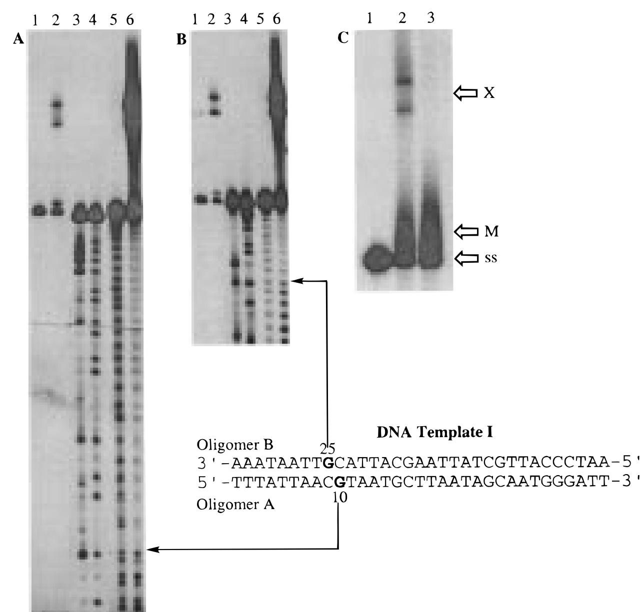

Figure 1. Autoradiograms A and B: Fe(II)-EDTA footprinting of cross-linked template 1 (^{32}P-labeled at the 5′ terminus of oligo A and B, respectively). Lanes 1 and 2, standard DNA, cross-linked template 1; lanes 3 and 4, Maxam−Gilbert G, G+A, respectively; lane 5, 1.5 mM Fe(II)-EDTA control; lane 6, cross-linked product after 1.5 mM Fe(II)-EDTA digestion. Autoradiogram C: Bioxalomycin α_2 reactions with dG25 substitution in oligomer B. Lane 1 is the native DNA template control. Lane 2 is the cross-linked DNA product. Lane 3 is the reaction of template 1 with bioxalomycin α_2 where inosine has been substituted at dG25. The cross-linked product at X is where the drug spans dG10 (A) to dG25 (B); ss refers to single-stranded DNA; M is monoalkylated DNA.

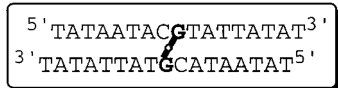

Figure 2.

Since earlier molecular modeling suggested that the complete naphthyridinomycin molecule could not cross-link DNA, we endeavored to secure experimental evidence for the molecular mass of the cross-linked DNA. We have isolated the gel-purified cross-link from the self-complementary DNA substrate depicted in Figure 2 and have obtained electrospray mass spectral data for the product; the observed mass was 10732 $\pm$ 5.5. The calculated mass for a bioxalomycin cross-link was 10766; the difference in the calculated and observed mass corresponds to a loss of the hydroxymethyl moiety at C9. This facile fragmentation has been observed in related hydroxymethyl-substituted isoquinolines.[9] In addition, the electrospray mass spectrum of cyanocycline (**6**) observed under the same conditions gave the molecular ion peak (calcd mass 426.2) minus the CH_2OH fragment (obsd mass 395.1) without detection of the parent ion peak.[10]

Several bis-electrophilic species can be envisioned to arise from the bioxalomycin framework. Zmijewski proposed a mechanism (Scheme 1) that accounts for alkylation at C3a or C7 of bioxalomycin α_2.[4d] Another mechanism for DNA cross-linking by naphthyridinomycin was postulated by Moore[11] wherein it was proposed that a quinone methide, formed from the deprotonation of the dihydroquinone, is the alkylating agent. This would place the alkylation sites at C13b and C9 of bioxalomycin α_2. We have found that cyanocycline (**6**) cross-links, in low yield, a similar DNA template, but *only in the presence of dithiothreitol* (which reduces the quinone to the dihydroquinone). This experimental result lends further support for the importance of the dihydroquinone moiety in activating the electrophilic sites. Based on this experimental observation, it can be envisioned that an *ortho*-quinone methide species would result in alkylation at C13b and C7 via a partial intercalative presentation of the drug.[12] Previous modeling work in this area[4b,c,f] apparently only considered approach of the drug from the right-hand sector toward the minor groove in a "face on" approach and did not consider a partial intercalative approach. Positions C3a and C9 are also possible but seem unlikely in view of the well-established importance of the carbinolamine (C7 for bioxalomycin) or functionally equivalent derivatives of the carbinolamine in DNA alkylation by these drugs.[4g] Identification of the exact molecular structure of the bioxalomycin α_2-mediated cross-link is currently being pursued in this laboratory.

These results point to the possible significance of benzylic (C13b) oxidation in this family of antitumor antibiotics and that similar DNA interstrand and/or DNA−protein cross-linking behavior might be anticipated for the structurally related marine antitumor antibiotics, the ecteinascidins.[13] Efforts are underway to examine these issues and to determine the exact molecular structure of the cross-linked species and if such a reaction occurs in vivo.

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Supporting Information Available: Experimental procedures for cross-link formation, digestion, and mass spectral analysis of the isolated cross-linked product including data for a 7-deazaguanosine-substituted DNA substrate (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(10) The electrospray mass spectrum of bioxalomycin α_2 yielded a molecular ion (*m/z* 418.0 (M−H)). Based on the observation that cyanocycline, which is in the quinone form, loses CH_2OH as the major fragmentation pathway, we suspect that the cross-linked adduct has undergone a dihydroquinone to quinone autoxidation.

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(12) We have carried out preliminary molecular mechanics calculations (Silicon Graphics Indigo IIzx, Biosym Insight/Discover) on bioxalomycin noncovalently bound to DNA in an effort to determine likely modes of association of the drug with DNA that would result in an appropriate presentation of two electrophilic sites that would culminate in DNA cross-linking. We have found that there is excellent presentation of the putative electrophilic sites at C7 and C13b if the aromatic portion of the drug partially *intercalates* into the DNA helix.

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