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COVALENT ALKYLATION OF DNA WITH DUOCARMYCIN A. IDENTIFICATION OF ABASIC SITE STRUCTURE

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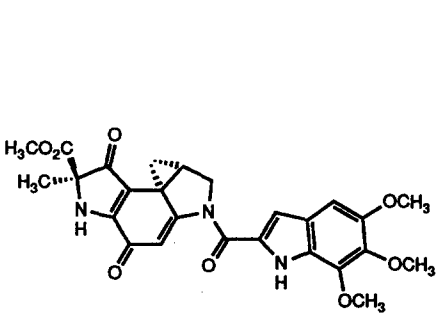
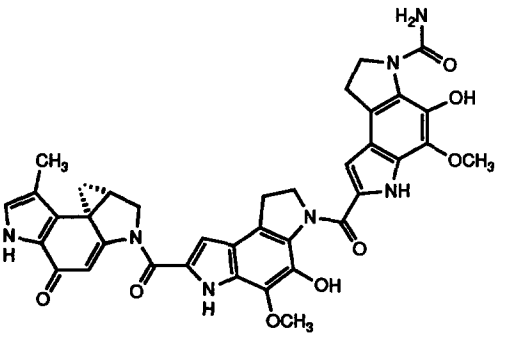
Summary: *Alkylation of d(CGTATACG) by antitumor antibiotic duocarmycin A was investigated. It was found that N3 of adenine$_6$ (A_6) attacks the cyclopropane subunit of duocarmycin A to produce covalently alkylated adduct 5. Upon heating (90 °C, 5 min) the adduct 5 decomposed to modified oligomer 1 with concomitant release of adenine adduct 2.*

There has been much current interest in the molecules that induce covalent alkylation of DNA in a sequence specific fashion, as they may provide information useful for the design of novel chemotherapeutic agents and artificial DNA-cleaving molecules.[1] Duocarmycins are a new class of antitumor antibiotics produced by *Streptomyces sp* and effective against murine lymphocytic Leukemia P388 and murine Sarcoma 180 in mice.[2] The structural similarities between duocarmycin A and CC-1065[1c,d] suggest that this drug may be acting by a related mechanism involving covalent alkylation of DNA. Herein described is the first demonstration of the chemistry of DNA covalent alkylation with duocarmycin A, by showing the structures of the heat-induced abasic site on DNA and the covalently alkylated adenine adduct.



Duocarmycin A



CC-1065

According to the sequence specificity reported for the DNA alkylation with duocarmycin A,[3] we selected self-complementary octanucleotide d(CGTATACG)$_2$ as a substrate to investigate the chemistry of the DNA damage. A typical reaction mixture (50 μL) containing duocarmycin A (0.1 mM) and d(CGTATACG)$_2$ (1 mM base concentration) in 50 mM sodium cacodylate buffer (pH 7.0) was incubated at 0 °C for 1 h. Direct analysis of the reaction mixture by reverse phase HPLC indicated that approximately half of d(CGTATACG) was consumed with the formation of one major

product eluted at 12.5 min (Figure 1a). The product obtained from the collection of the HPLC peak showed a very similar UV absorption with that of 1:1 mixture of duocarmycin A and d(CGTATACG), suggesting that this product is a covalently alkylated adduct. In fact, upon heating at 90 °C for 5 min, the product was immediately converted to two peaks eluted at 8.3 min and 26.2 min (Figure 1b). The structure of the product obtained from the collection of the later peak was assigned as duocarmycin A-adenine adduct **2** on the basis of the comigration on HPLC under several different conditions with the product isolated from a large scale reaction of calf thymus DNA with duocarmycin A.[4] The structure of the product with retention time of 8.3 min was characterized as follows. Treatment of the fraction with hot alkali (0.1 N NaOH, 90 °C, 5 min) produced d(CGTATp) and d(pCG), suggesting that this modified oligomer possesses an alkali labile site at A_6 of $d(C_1G_2T_3A_4T_5A_6C_7G_8)$. Therefore, independent synthesis of the oligomer containing an alkali labile site at A_6 from methyl-protected oligomer **3** was carried out.[5] By treatment with 0.05 N HCl (0 °C, 27 h), **3** was converted to **1** (*ca.* 40%) which showed identical HPLC behaviors with those of the product obtained from the duocarmycin A reaction under three different conditions. For further confirmation of the structure, $NaBH_4$ reduction of the reaction mixture gave rise to the formation of **4**.[7] These results clearly indicated that N3 of adenine$_6$ attacks the cyclopropane subunit of duocarmycin A to produce alkylated adduct **5** which upon heating is converted to abasic site-containing oligomer **1** with concomitant release of adenine adduct **2** (Scheme 1).

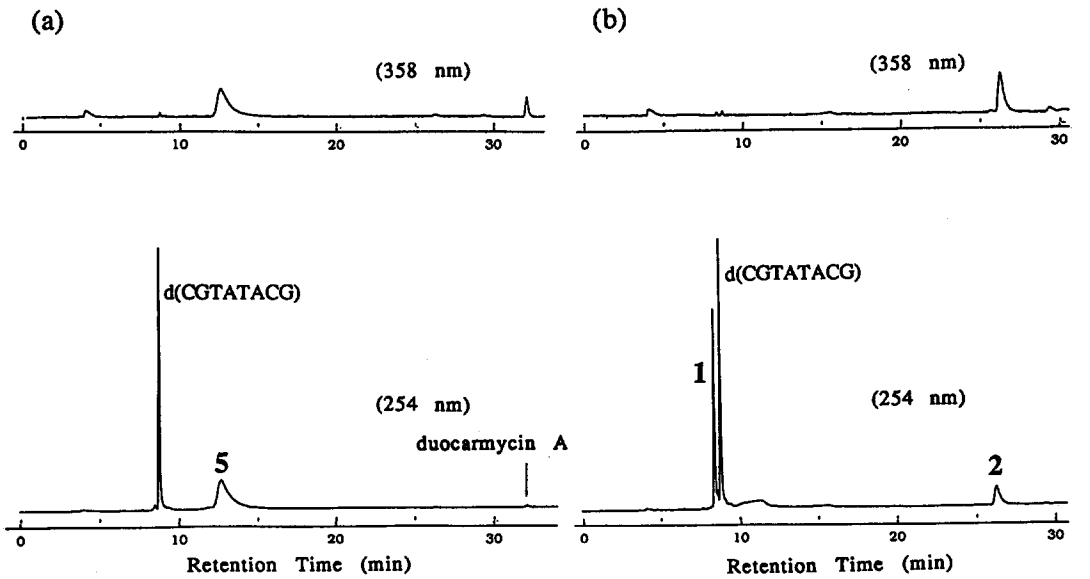
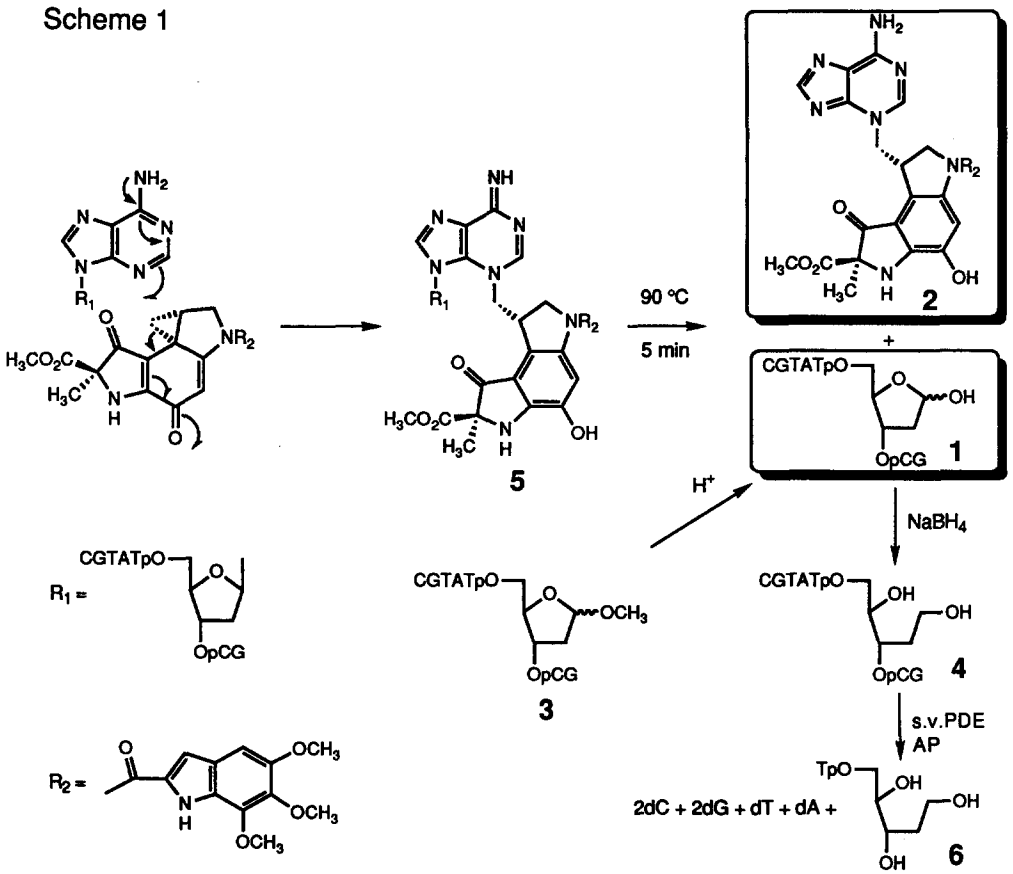


Figure 1. HPLC analysis of duocarmycin A-treated d(CGTATACG). The reaction mixture was incubated at 0 °C for 1 h and then analyzed (a) directly and (b) after heating at 90 °C for 5 min. Analysis was carried out on a Cosmosil 5 C_{18} column (4.6 x 150 mm). Elution was with 0.05 M ammonium formate, 0-50% acetonitrile linear gradient (40 min), at a flow rate of 1.5 mL/min. Detection was carried out at 254 nm (lower) and 358 nm (upper) as indicated.

Scheme 1



This oligomer alkylation proceeded efficiently at 0 °C, and duocarmycin A was almost quantitatively utilized for the alkylation as judged by HPLC. At 37 °C the yield of **5** was dropped to 55% presumably due to the increased decomposition rate of duocarmycin A itself under the conditions.[8] Alkylation of A_6 of d(CGTATACG) with duocarmycin A has a first-order kinetic with the rate constant of $k = 6.8 \times 10^{-5}\ s^{-1}$ at 0 °C and $k = 2.8 \times 10^{-4}\ s^{-1}$ at 37 °C. Covalently alkylated adduct **5** was fairly stable at 0 °C and no decomposition was observed after 2 days. At higher temperatures **5** was converted to **2** with $t_{1/2}$ of 134 h at 37 °C and 1.1 h at 60 °C.

Of particular interest is the clean formation of oligomer **1** from **5** upon brief heating (90 °C, 5 min) at neutral pH. By contrast, under the prolonged heating condition (100 °C, 30 min) employed in the previous studies[1,3] **1** was no more stable and completely decomposed to d(pCG) and d(CGTATp) bearing multiple modified sugar ends as revealed by HPLC.[9] Therefore, it is suggested that the thermal cleavage of alkylated DNA reported for CC-1065[1c,d] and duocarmycin A[3] may result from the further degradation of the abasic sites such as **1**. In conclusion, by using d(CGTATACG) the alkylation with duocarmycin A was directly observed on HPLC and the structures of the heat-induced abasic site **1** and adenine adduct **2** were unambiguously characterized.

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4) Preparation of **2** was carried out as follows. Duocarmycin A (20 mg, 39 μmol) in 10 mL of DMF was mixed with calf thymus DNA (200 mg) in 200 mL of 10 mM sodium phosphate buffer, pH 7.0. The reaction mixture was incubated at 35 °C for 8 h. The solution was extracted with ethyl acetate to remove unreacted duocarmycin A. The aqueous layer was heated at 100 °C for 1 h under stirring. The resulting solution was extracted with ethyl acetate, and the organic layer was concentrated to dryness to afford crude product, which was further purified by silica gel preparative TLC ($CHCl_3$-CH_3OH, 8:1) to give **2**; yield 10.3 mg (41.1%). ^1H NMR (DMSO-d_6, TSP) δ 1.41 (s, 3 H), 3.58 (s, 3 H), 3.79 (s, 3 H), 3.81 (s, 3 H), 3.92 (s, 3 H), 4.22 (m, 1 H), 4.41 (t, 1 H, J = 10.5 Hz), 4.49 (dd, 1 H, J = 13.5, 6.9 Hz), 4.64 (dd, 1 H, J = 13.5, 5.4 Hz), 4.67 (dd, 1 H, J = 10.5, 2.4 Hz), 6.83 (d, 1 H, J = 2.1 Hz), 6.90 (s, 1 H), 7.24 (s, 1 H), 7,65 (s, 1 H), 7.78 (br, 2 H), 7.92 (s, 1 H), 7.95 (br, 1 H), 10.15 (brs, 1 H), 11.20 (brs, 1 H) ; SIMS (m/z) 643 $(M+1)^+$. Tautomeric form of an analogous N3-alkylated adenine was found to exist in the amino form.[1a]

5) Synthesis of oligomer **3** was carried out as follows. Cyanoethyl phosphoramidite of 1-*O*-methyl-5-*O*-dimethoxytrityl-2-deoxy-D-ribose was prepared by the reported procedure[6] and used for DNA synthesis without further purification. Synthesis of oligomers was performed on an Applied Biosystem 381A DNA synthesizer using the phosphoramidite method with 1 mmol column. For preparation of abasic site-containing oligomers by a different method, see, Iocono, J. A.; Gildea, B.; Mclaughlin, L. W. *Tetrahedron Lett.* **1990,** *31*, 175.

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7) Enzymatic digestion with snake venom phosphodiesterase (s. v. PDE) and alkaline phosphatase (AP) of the synthetic **4** and the product obtained from the duocarmycin reaction gave exactly the same result, producing 2-deoxycytidine, 2-deoxyguanosine, thymidine, and 2-deoxyadenosine in a 2:2:1:1 ratio together with **6**. **6**; ^1H NMR (D_2O, TSP) δ 1.47 - 1.54 (m, 1 H, 2"), 1.72 -1.79 (m, 1 H, 2"), 1.74 (s, 3 H, $5CH_3$), 2.23 - 2.31 (m, 1 H, 2'), 2.40 (ddd, 1 H, J = 2.7, 6.6, 14.5 Hz, 2'), 3.54 - 3.72 (m, 4 H, 5', 1"), 3.74 - 3.81 (m, 1 H, 5"), 3.86 - 3.92 (m, 1 H, 5"), 4.04 - 4.08 (m, 1 H, 4'), 4.52 - 4.68 (m, 3 H, 3', 3", 4"), 6.17 (t, 1 H, J = 6.8 Hz, 1'), 7.51 (s, 1 H, 6).

8) Duocarmycin A was slowly decomposed to ring-opened products in aqueous neutral solution.

9) Treatment of the mixture with hot alkali (0.1 N NaOH, 90 °C, 5 min) resulted in a clean formation of d(CGTATp).

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