Mechanism for the Catalytic Activation of Ecteinascidin 743 and Its Subsequent Alkylation of Guanine N2

Bob M. Moore, II, Frederick C. Seaman,
Richard T. Wheelhouse, and Laurence H. Hurley*

Drug Dynamics Institute, College of Pharmacy
The University of Texas at Austin
Austin, Texas 78712-1074

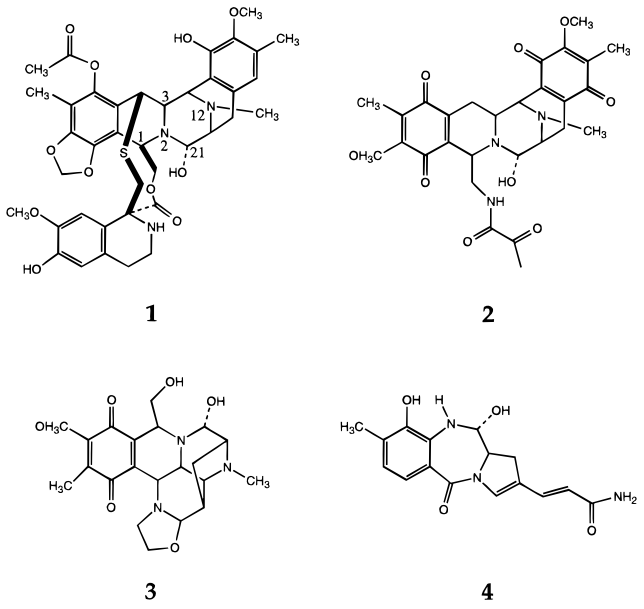
Received December 3, 1997

Ecteinascidin 743[1] (**1**), the first of a novel class of therapeutically significant marine alkaloids, has shown good activity against a variety of solid tumor types in phase 1 clinical trials.[2] For the design of novel synthetic ecteinascidins, it is important to identify the chemical and structural elements essential for activity. We have recently reported an NMR-based model of a **1** guanine N2 (GN2)−DNA adduct,[3] and herein we report the results of studies to determine the protonation state of the **1**−DNA adduct. Together, these data provide evidence for a specific mechanism for the catalytic activation of **1** in the minor groove of DNA and subsequent alkylation of GN2.

The alkylation of GN2 in duplex DNA is well established for a variety of carbinolamine-containing antibiotics, such as **1**,[4] anthramycin (**2**),[5] saframycin S (**3**),[6] and naphthyridinomycin (**4**).[7] The chemical reactivity of these agents has been proposed to reside in the iminium intermediate generated by the general acid-mediated dehydration of the carbinolamine moiety.[5,7] While the mechanism for the formation of the iminium intermediate in bulk solution is a general chemical reaction, both the source of general acid in the minor groove of DNA and the protonation state of the adduct remain unidentified. Examination of the carbinolamine antibiotic structures **1**−**4** shows that basic nitrogens, which should bear a proton at physiological pH, are in close proximity to the hydroxyl group and therefore could serve as a proton source when buried in the minor groove of DNA. Our approach to defining the mechanism of activation for **1** was to determine the protonation state of the drug and DNA in the covalent adduct and then utilize mass and charge balance to propose a mechanism leading to alkylation.

Our earlier studies demonstrated that a 1:1 drug−DNA adduct can be prepared by reacting **1** with the 12-mer oligonucleotide[8] $[d(CGTAAGCTTACG)]_2$ containing the preferred AGC alkylation site.[3] To fully assess the protonation state of the drug and DNA, an isotopically labeled (^{13}C, ^{15}N) 12-mer was synthesized according to the method of Crothers,[9] and the natural abundance duplex was prepared via phosphoramidite chemistry.[10] Both the labeled and natural abundance DNAs and the **1**−DNA adducts were investigated by high-field NMR spectroscopy.

Chart 1



1 **2** **3** **4**

Nitrogen atoms N2 and N12 of **1** and 6GN2 were the most likely candidates for protonation in the **1**−DNA adduct since they are centered around C21 of **1**, the site of nucleophilic attack of 6GN2. To assess the protonation of **1** in the adduct, the natural abundance **1**−DNA adduct was studied utilizing COSY, TOCSY, and NOESY experiments in an H_2O-D_2O (9:1) solvent system. The TOCSY (Figure 1) and COSY of **1**−DNA have a single cross-peak in the NH chemical shift range (8.05 ppm), showing a connection to 12NMe; additionally, this peak was absent in the D_2O spectrum, indicating that the proton was exchangeable. These data showed that 12N of **1** is protonated in the covalent adduct, and the relative intensity of the cross-peaks suggested that the proton was in slow exchange, i.e., probably hydrogen-bonded. The absence of cross-peaks in the NH region to H1, H3, and H21 of **1** in the TOCSY and COSY spectra indicates that N2 is not protonated in the adduct. This observation was consistent with the reported steric crowding around N2 in the crystal structure of Et 743 N12-oxide[11] and by the fact that a second protonation in a piperazine-like system is unlikely above pH 2.0.[12]

Heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) NMR spectroscopy in an H_2O-D_2O (9:1) solvent system was utilized to assess the protonation state of the DNA in the **1**−DNA adduct. Figure 2 shows the $^{15}N^1H$ chemical shift changes for 6GN2H associated with covalent bonding of **1** to DNA. Prior to drug modification, the NH cross-peak of 6G is in the usual range (proton 6.6−9.0 ppm, nitrogen 75 ppm) for duplex DNA;[9] however, covalent modification of 6GN2 by **1** shifts the 6GN2H 1.95 ppm downfield, while 6GN2 is only shifted 6.12 ppm.[13] The modest downfield shift in 6GN2, compared with a 40−60 ppm upfield shift expected

* To whom correspondence should be addressed. Tel.: (512) 471-4841. Fax: (512) 471-2746. E-mail: dg-dna@mail.utexas.edu.

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(13) The presence of a minor cross-peak in the 6GNH region most likely reflects the conformational flexibility of the C ring of **1** in the covalent adduct (manuscript in preparation).

S0002-7863(97)04109-7 CCC: $15.00
Published on Web 03/03/1998

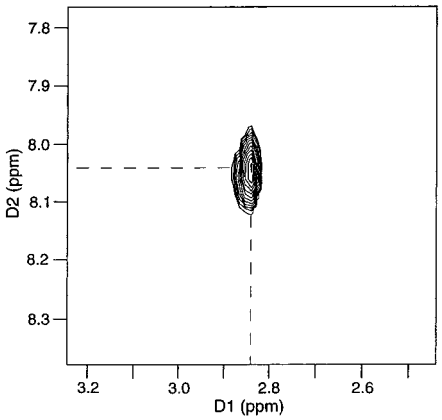


Figure 1. Partial 2D water TOCSY expansion contour plot of the cross-peak of 12NH into the 12NMe in the **1**−DNA adduct.

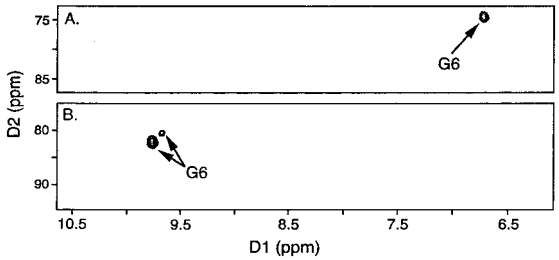
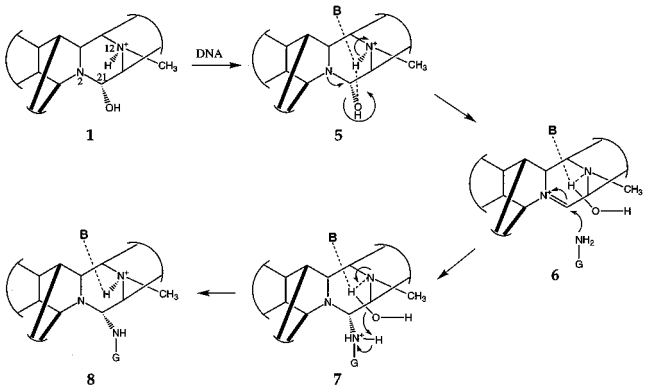


Figure 2. Proton−nitrogen correlations for the isotopically labeled DNA and the **1**−DNA adduct. (A) Partial HMQC expansion contour plot showing the 6GN2−H correlations of the duplex DNA. (B) Partial HMQC expansion contour plot showing the 6GN2−H correlations of the **1**−DNA adduct.

if 6GN2 were protonated,[14] clearly demonstrated that 6GN2 is not protonated.[15] The downfield shift in the hydrogen dimension therefore results from elimination of the time-averaged chemical shift of the 6GNHe and 6GNHb to a cross-peak arising from just the 6GNHb in the covalent adduct.

Scheme 1 shows a mechanism for the activation of **1** and covalent modification of 6GN that is consistent with the NMR data. The approach of **1** into the minor groove would be accompanied by desolvation of **1**, which in turn would increase the strength of the hydrogen bond between 12NH[16] to 21OH. The close proximity (2.54 Å) of 12NH to 21OH and the partial sp^2 character of 2N in **1**[11] should be sufficient to catalyze the dehydration of the carbinolamine (**5**). Since 12NH participates

Scheme 1. Mechanism for the Catalytic Activation of **1** and Alkylation of 6GN2[a]



[a] The dotted lines represent hydrogen-bond interactions, and **B** represents a DNA base accepting a hydrogen bond.

in hydrogen bonds in the covalent adduct and is believed to play a role in sequence recognition of DNA,[17] it is probable that the water remains hydrogen-bonded at this point in the mechanism (**6**). Nucleophilic attack at C21 of the iminium intermediate by 6GN2 results in a charged 6GN (**7**) having a proton with a pK_a expected to be below 0.[18] This proton could be transferred either to N2 or N12 of **1** or to a water molecule in the proximity of the protonated 6GN2. Considering the low pK_a of this proton, it could readily transfer to the transiently bound water molecule, setting up a proton shuttle by which the original protonation state of 12N of **1** would be restored and the water molecule expelled as the transition state collapses to the final covalent adduct (**8**). The final structure of the **1**−DNA adduct is consistent with the NMR data and an earlier theoretical activation mechanism of **4**;[5] furthermore, the essential hydrogen bonds for DNA recognition and binding are preserved throughout the mechanism.

In this Communication, we have proposed a chemical mechanism that gives rise to the alkylation of 6GN2 by **1** in duplex DNA. These data indicate that in the development of ecteinascidin analogues, a proton source should be retained in close proximity to the carbinolamine to catalyze the dehydration of this moiety. This result implies that nature has developed a mechanism to ensure the reactivity of the carbinolamine antibiotics **1**−**4** by the inclusion of an internal catalytic proton adjacent to the leaving hydroxyl group. Finally, we believe these results not only impact on the development of ecteinascidins but also aid in the *de novo* design of new DNA-reactive reagents.

Acknowledgment. We thank Pharma Mar for a sample of Et 743. We are grateful to Steve Sorey for NMR technical assistance. Research was supported by a grant from the National Institutes of Health (CA-49751). We also thank Oleg Fedorov for insightful discussions and David Bishop for proofreading and editing the manuscript.

JA974109R

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(15) The lack of any DNA protonation was evidenced by the absence of 40−60 ppm chemical shift changes in the ^{15}N spectra (data not shown) and the absence of thermal strand breakage noted in ref 4.

(16) Et 743 (3 mg) was stirred in NMR buffer (footnote 8) containing 30% DMSO for 1 h. The mixture was extracted three times with CH_2Cl_2, and the organic phase was dried over magnesium sulfate then evaporated. The residue was taken up in 500 μL of $CDCl_3$. The 1D ^{1}H spectrum contained five exchangeable protons. The DQF-COSY spectrum showed an exchangeable proton at 10.80 ppm having a connectivity to the 12NMe, indicating that 12N is protonated under the conditions of the drug−DNA reaction.

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