Covalent Structure of the DNA–DNA Interstrand Cross-Link Formed by Reductively Activated FR66979 in Synthetic DNA Duplexes

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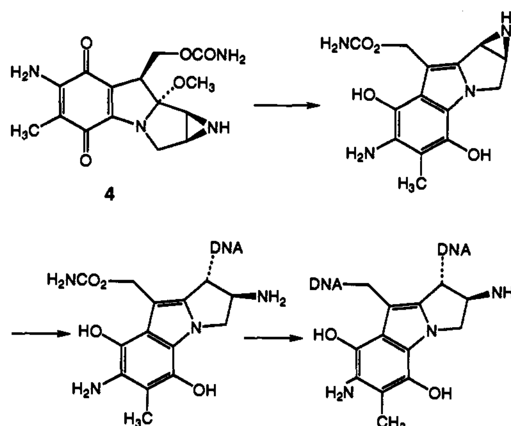
Abstract: The lesion responsible for the dG-to-dG, DNA–DNA interstrand cross-link formed at the duplex dinucleotide sequence 5′-d(CG) by reductively activated FR66979 (**2**) was isolated from the cross-linked synthetic oligonucleotide duplex [5′-d(TATACGTATA)]$_2$. The lesion and a peracetylated derivative of this substance were studied by mass, UV, and ^{1}H NMR (2D PS COSY and 1D NOE) spectroscopies. The data overwhelmingly support the proposal that this lesion possesses the mitosene-like structure **8**, the analog of the lesion **5a** formed in DNA by reductively activated mitomycin C. This finding offers in vitro support for the proposal of in vivo bioreductive activation of these substances as the prelude to DNA–DNA interstrand cross-linking.

Introduction

Scientists at Fujisawa Pharmaceutical have isolated and characterized a family of antitumor agents similar in structure to the mitomycins. The initial reports described FR900482 (**1**).[1] Other members of this family include FR66979 (**2**),[2] a dihydro derivative of FR900482 isolated from the same fermentation broth, and FK973 (**3**), a synthetic triacetate of FR900482.[3] All of these substances exhibited promising antimicrobial and antineoplastic activity at approximately micromolar concentrations, with FK973 being suggested as a potential clinical candidate. FR900482 and FK973 share with mitomycin C the ability to create interstrand cross-links in DNA in vivo, a property which may contribute to the biological activity of these compounds.[4]

The structural similarity of the mitomycins (e.g. mitomycin C, **4**) to **1–3** strongly suggests that the DNA–DNA interstrand cross-linking activity common to all of these substances may share mechanistic and structural features. Much is known about the cross-linking reaction of mitomycin C, the salient features of which were proposed some 30 years ago by Iyer and Szybalski (Scheme 1).[5] The essential features of this scheme are supported by observations in many laboratories, but particularly concisely by the isolation and characterization of **5a** from enzymatic digests of mitomycin C-treated DNA.[6] Important in the present context is the fact that mitomycin C is relatively inert toward nucleophiles at pH 7, but forms a reactive electrophile following reductive activation. The revelation of the pyrrole function (Scheme 1) is expected on mechanistic grounds to be critical in promoting the formation of these electrophiles, consistent with the fact that even simple pyrroles such as **6** mimic some features of the mitomycin C cross-linking reaction.[7]

Scheme 1. Alkylation of DNA by Reductively Activated Mitomycin C



Compounds **1–3** lack the pyrrole functional group of reductively activated mitomycin C. Fukuyama and Goto recognized that reductive scission of the N–O bond of FR900482 would in principle permit formation of the critical pyrrole (Scheme 2).[8] A related mechanism in which an attacking nucleophile at C5 cleaves the key N–O bond by an S_N2' reaction was proposed by Danishefsky and McClure.[9] Finally, Williams and Rajski have noted that FR66979 at approximately millimolar concentrations possesses DNA interstrand cross-linking activity in the *absence* of added reductant or nucleophiles, and they suggested that analogy to the mitomycins might not pertain, i.e. that a mitosene analog might not be involved in this reaction.[10]

We have recently reported on the DNA–DNA interstrand cross-linking reactions of FR900482 and FR66979 using synthetic DNA duplexes.[11] In common with the corresponding reactions of mitomycin C, we found that the efficiency of cross-linking was greatly enhanced by the addition of sodium dithionite, and that

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(6) Tomasz, M.; Lipman, R.; Chowdary, D.; Pawlak, J.; Verdine, G.; Nakanishi, K. *Science* **1987**, *235*, 1204.
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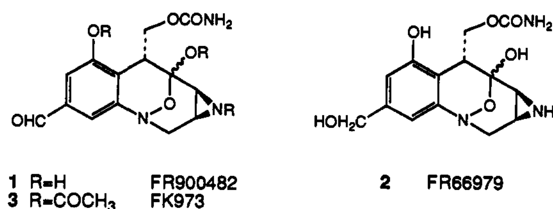
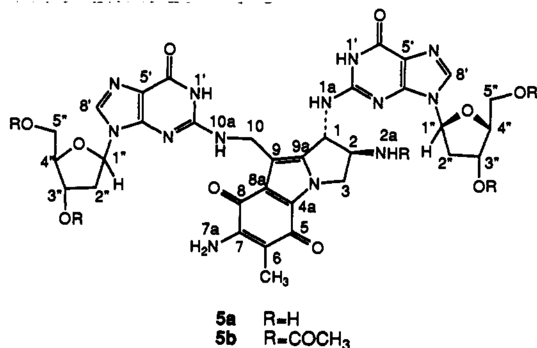
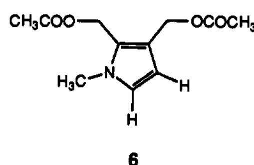
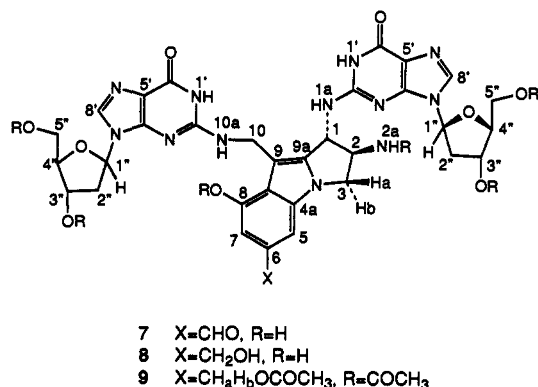
Chart 1

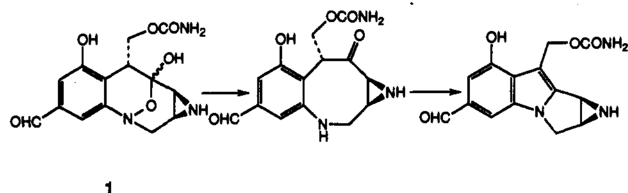






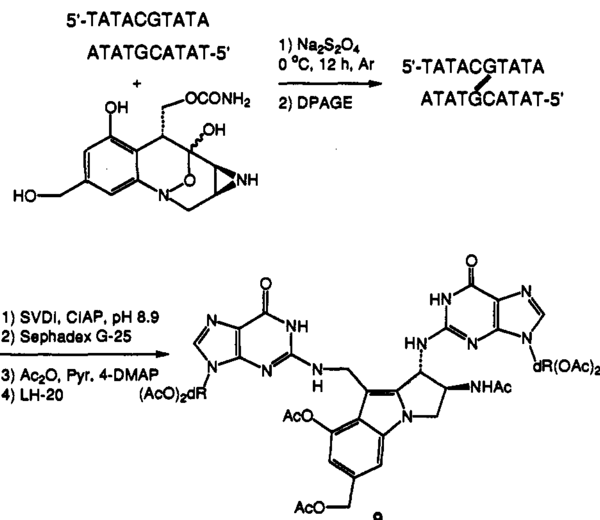


Scheme 2. Goto and Fukuyama's Proposal for Reductive Activation of FR900482 to a Mitosene Analog



DNAs containing the duplex sequence 5′-d(CG) were maximally cross-linked. The presence of both exocyclic amino groups of the two deoxyguanosine residues at this duplex sequence was required for cross-linking. Microgram quantities of the lesions responsible for the cross-links by both FR900482 and FR66979 were isolated from enzymatic digests. The molecular weights were fully consistent with reductive activation/pyrrole formation as in the proposal of Fukuyama and Goto (Scheme 2) followed by linkage to deoxyguanosine residues as implied by **7** and **8**. The UV spectral properties of **7** were consistent with the presence of the indole chromophore not present in **1**. These observations did not constitute a rigorous structure proof.

Scheme 3. Preparation of Peracylated Derivative **9**



We report herein the isolation on larger scale and structural characterization primarily by MS and ^{1}H NMR measurements on the peracetylated derivative **9** of the putative **8**, the nucleus of the DNA–DNA interstrand cross-link formed by reductively activated FR66979. Critical to the success of this effort was derivatization of this substance by peracylation to afford **9**,[6] which circumvented an otherwise complicating oxidative decomposition of the lesion. The data are fully consistent with this lesion possessing the structure **8**, the analog of **5a**.

Results

Preparation and Characterization of 8. Although FR900482 is the most readily available member of this family, it has previously been shown that FR66979 is the more efficient DNA interstrand cross-linking agent.[11] For this reason, FR66979 was chosen for the majority of these studies. A series of preliminary experiments in which the relative amounts of FR66979, dithionite, and DNA were varied along with temperature and time established conditions under which ca. 40% of the synthetic DNA [5′-d(TATACGTATA)]$_2$ could be isolated as the interstrand cross-linked product following denaturing polyacrylamide gel electrophoresis (DPAGE). Accordingly, aliquots of this DNA admixed with FR66979 (1:8 molar ratio) at pH 7.5, 0 °C, were treated sequentially with 5 aliquots of aqueous sodium dithionite (Scheme 3). The resulting mixture of single strands and cross-linked DNAs was separated by DPAGE to afford the low-mobility, interstrand cross-linked product.

The interstrand cross-linked product was digested with a mixture of snake venom phosphodiesterase I and calf intestinal alkaline phosphatase at pH 8.9. Reverse phase HPLC analysis with detection at 260 nm (Figure 1) revealed the return of dA, dT, and dC, but the virtual absence of dG, as expected given its consumption in forming the cross-link. Instead, a high retention volume peak was returned. In small-scale experiments, the signal for this last substance was only ca. 70% of that which would be anticipated for two dG residues; the actual recovery of the lesion was thus probably even less than 70%, because the FR66979-derived component of the lesion would be expected to contribute to its UV absorbance at this wavelength. HPLC on larger scales returned proportionately less of this material. This poor recovery was attributed to the tendency of derivatives of dG, especially those containing more than one guanyl moiety, to aggregate.[12] The quantity of pure material isolated by this method was sufficient for its characterization by MS and UV, described in

(12) (a) Rink, S. M.; Solomon, M. S.; Taylor, M. J.; Rajur, S. B.; McLaughlin, L. W.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 2551. (b) Private communication, Prof. M. Tomasz.

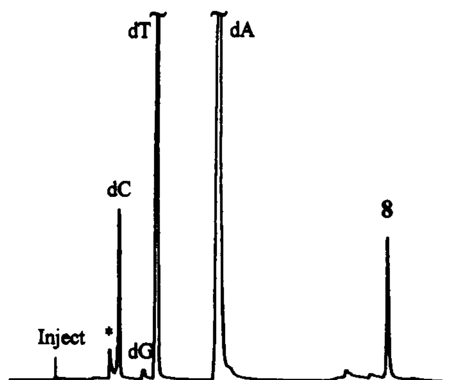


Figure 1. HPLC of the enzymatic digest of FR66979-cross-linked DNA. Retention times increase to the right. An asterisk denotes DMSO added to solubilize **8**.

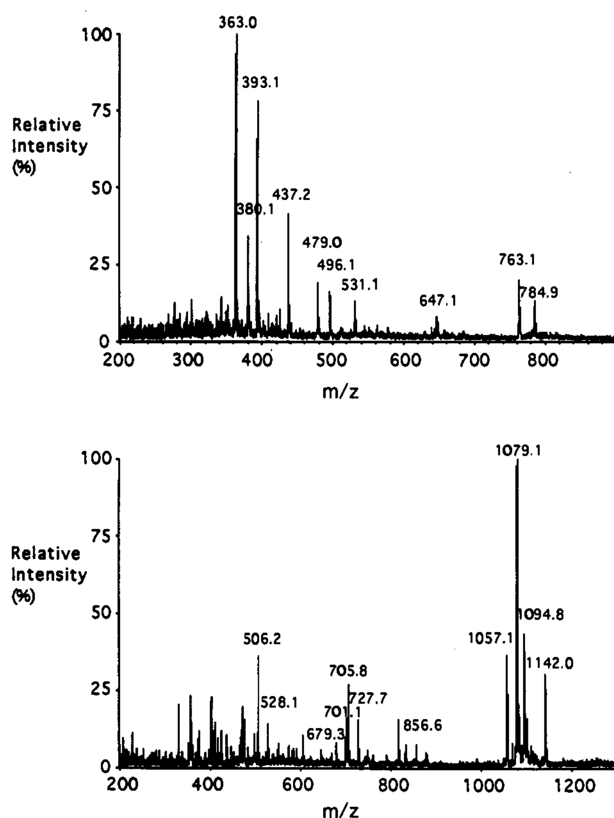
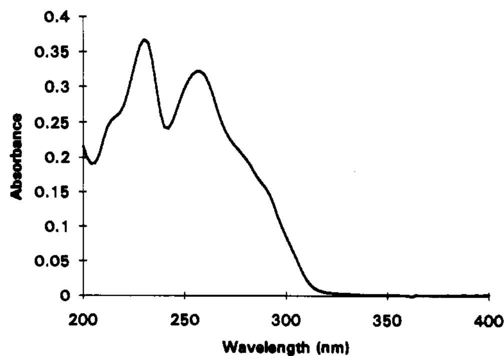


Figure 2. Positive ion ESIMS of **8** (upper) and its peracylated derivative **9** (lower).

the following paragraphs, but was insufficient for convenient study by NMR, necessitating development of an alternate scheme. Because the quality of the structural studies on this lesion would ultimately depend on the quantity of this lesion which could be isolated, a more efficient isolation procedure was developed, as described below.

The electrospray ionization mass spectrum (ESIMS) of the HPLC isolate (Figure 2, upper) gave a molecular ion consistent with its formulation as **8** ($M + H^+$, m/z 763; $M + Na^+$, m/z 785). The appearance of fragment ions corresponding to exchange of one (m/z 647) and two (m/z 531) deoxyribosyl groups for protons assured that the connection point of drug to DNA was through the base rather than sugar. Fragment ions were prominent corresponding to sequential loss from protonated **8** of one deoxyguanosine (m/z 496), followed by either loss of ammonia (m/z 479), or replacement of one deoxyribosyl with a proton (m/z 380), or both (m/z 363). The masses of all of these ions were thus fully consistent with a scenario as shown in Scheme 2 in which relative to the sum of the masses of FR66979 and two deoxyguanosines, the lesion resulted from the net introduction of two hydrogen atoms, loss of two water molecules, and loss of one molecule of carbamic acid.



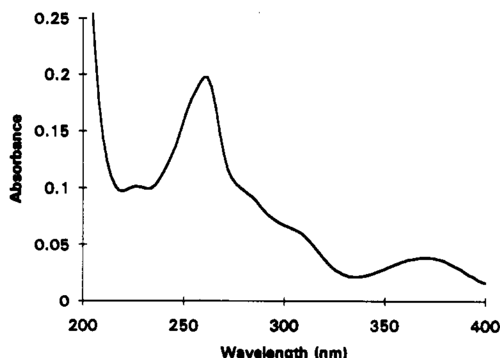


Figure 3. UV spectra of **7** (lower) and **8** (upper) in H_2O.

The UV spectra of **7**, the isolation of which on a small scale has been previously reported,[11] and **8** both offered support for the putative changes in the aromatic chromophores of FR900482 and FR66979 from a substituted phenol to a substituted hydroxyindole. Particularly diagnostic in the UV spectrum of **7** (Figure 3, lower) was a long wavelength absorbance of λ_{max} 372 nm which we attribute to the hydroxyindolecarboxaldehyde $n \rightarrow \pi^*$ transition. This band appears at λ_{max} 330 nm in FR900482 itself.[1a] This bathochromic shift of 40 nm is similar to the difference between a benzaldehyde (λ_{max} 290 nm) and 2-naphthaldehyde (λ_{max} 343 nm) or indole-6-carboxaldehyde (λ_{max} 345 nm),[14a,b] and thus supports the formation of the pyrrole ring as shown in **7**. Analysis of the UV spectrum of the putative **8** was complicated by overlap of the FR66979-derived chromophore with the purines, but was likewise in qualitative support of the indole substructure. Whereas FR66979 possesses an intense $\pi \rightarrow \pi^*$ transition at 215 nm (ϵ 20 000 $M^{-1}\,cm^{-1}$),[2] the UV spectrum of the putative **8** (Figure 3, upper) shows this band bathochromically shifted to 230 nm. A similar bathochromic shift is seen in comparing phenol itself (λ_{max} 211 nm) to indol-4-ol (λ_{max} 220 nm, ϵ 35 000 $M^{-1}\,cm^{-1}$).[14c]

Preparation and Characterization of Peracetylated Derivative 9. An alternate procedure for the isolation of **8** from the enzymatic digest was sought which would overcome not only the low recovery of the HPLC protocol but also the tendency of **8** to undergo oxidation reactions in solution.[13] Improved recovery of this

(13) Reanalysis by HPLC of a solution of **8** isolated from G-25 chromatography and stored for several days revealed two new, less hydrophobic peaks. Parent ions in the ESIMS were consistent with formulation of these as oxidation products, one being two units of mass lighter than **8** ($M + H^+$, m/z 761) and the other having lost these two units as well as having exchanged deoxyguanosyl for hydroxyl ($M + H^+$, m/z 512). The small quantities of these substances available to us precluded further structural studies.

(14) (a) *UV Atlas of Organic Compounds*; Butterworths: London, 1967. (b) Weast, R. C.; Graselli, J. G. *Handbook of Data on Organic Compounds*; CRC Press: Boca Raton, FL, 1985. (c) Stoll, A.; Troxler, F.; Peyer, J.; Hoffman, A. *Helv. Chim. Acta* **1955**, *38*, 1452.

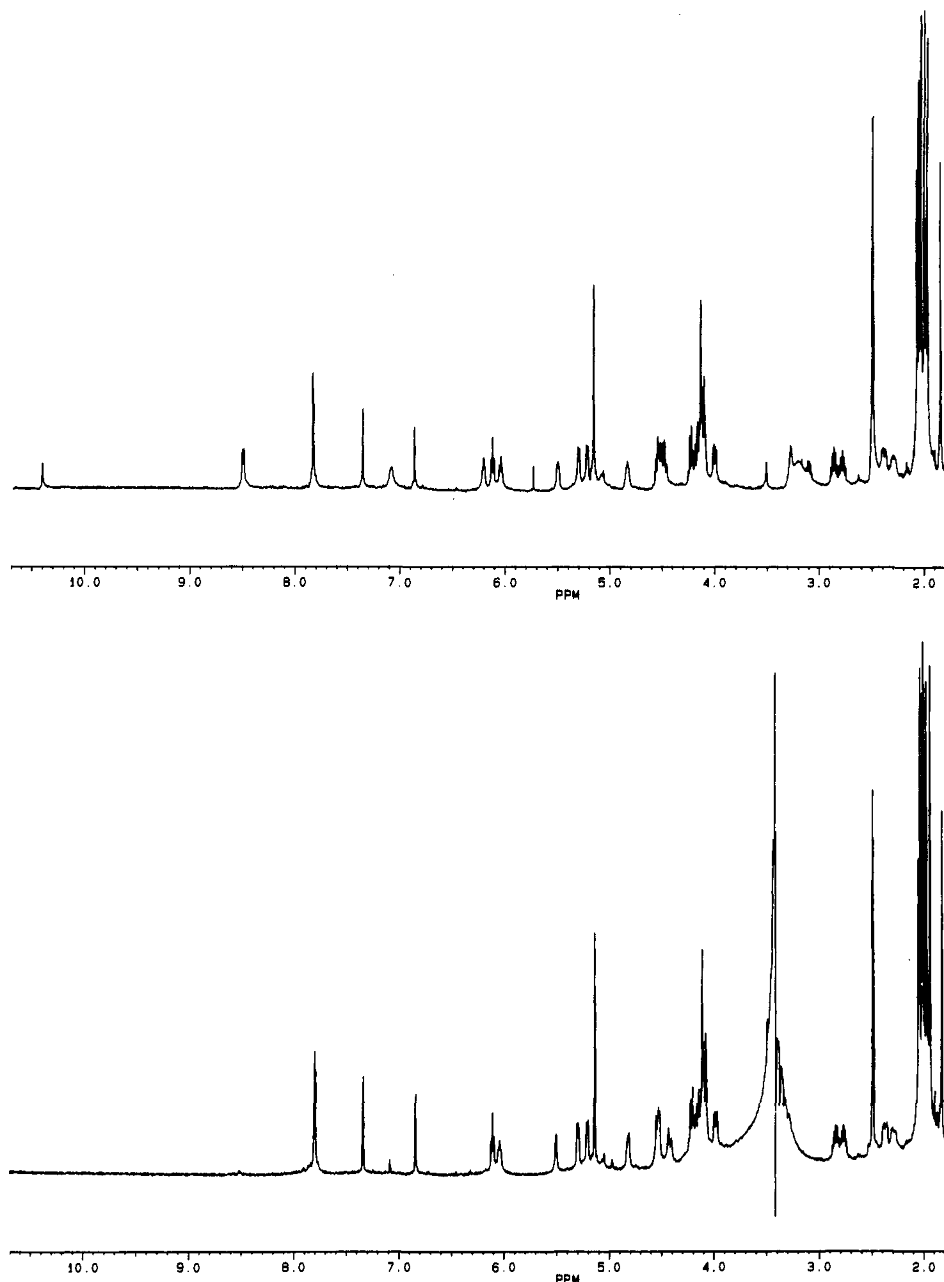


Figure 4. 1D ^1H NMR spectra (500 MHz) of peracylated derivative **9** at 37 °C in DMSO-d_6 (upper) and in DMSO-d_6/D_2O (lower). A resonance for one N1'H at δ 10.65 was present in some samples but is absent here, presumably due to rapid exchange with residual H_2O.

substance was achieved following chromatography of the enzymatic hydrolysate on Sephadex G-25,[6] which separated the putative **8** from the residual deoxynucleosides (Scheme 3). On the assumption that the oxidative decomposition of this substance was initiated by the phenolic function, this substance was peracetylated with acetic anhydride, pyridine, and 4-(dimethylamino)pyridine, an approach used by Tomasz, Nakanishi, et al.[6] The product of this reaction was purified by passage through lipophilic sephadex (LH-20), and it showed no further tendency to undergo oxidation. HPLC analysis of this product revealed a single peak; based upon the evidence described below, we formulate this substance as **9**.

The ESIMS of the peracetylated derivative of **8** (Figure 2, lower) was consistent with the introduction of seven acetyl functions (m/z 1057, M + H^+; m/z 1079, M + Na^+), as formulated in **9**. In one run, a hexaacetylated form of this same substance was inadvertently prepared, as evidenced by the ESIMS parent ion of m/z 1015 (M + H^+) (data not shown). This was probably the result of hydrolysis of the phenolic acetate under mild conditions, providing further evidence for this substructure.

The ^1H NMR spectrum of the putative **9** at 500 MHz in DMSO-d_6 (Figure 4) was readily assigned (Table 1) primarily on the basis of the results of a phase-sensitive (PS) COSY experiment (Figure 5, Table 2). As described below, several 1D NOE experiments (Figure 6, Table 2) aided in resolving residual ambiguities. At 500 MHz these NOEs were negative. The chemical shift assignments (Table 1) are strikingly similar to those made by Tomasz, Nakanishi, et al. in their work with the mitomycin-derived analog, **5b**,[6] despite the fact that the present assignment was made fully independently. The PSCOSY clearly revealed the connectivity of the proton-rich regions of the molecule through ^1H–^1H, 2-bond, and 3-bond scalar couplings. Important in this regard were the assignments of both of the deoxyribosyl groups, the iminomethylene (C10/N10a) and the substituted propylene chain (C1/C2/C3). One cross-peak critical to the assignment, the C1H/C2H cross-peak, was quite weak, but this scalar coupling was confirmed by reciprocal 1D decoupling

Table 1. NMR Assignments for Peracetylated Derivatives **9** and **5b** in DMSO-d_6

A. ^{1}H NMR of **9** and **5b**

proton	chemical shift (δ) 9	5b[a]	proton	chemical shift (δ) 9	5b[a]
C1H	5.49	5.16	N7aH_2		6.63
C2H	4.83	4.68	N10aH	6.20	6.49
C3H_a	4.00	4.00	N(CO)CH_3	1.84	1.79
C3H_b	4.54	4.51	C8′H	7.82, 7.83	7.81, 7.89
C5H	7.35		N1′H	10.40, 10.65[b]	10.54, 10.77
C6aH_a	5.15		C1″H	6.12, 6.04[c]	6.02, 6.09
C6aH_b	5.15		C2″H_a	2.86, 2.78[c]	2.70–2.95
C6aH_3		1.75	C2″H_b	2.39, 2.30[c]	
C7H	6.86		C3″H	5.30, 5.22[c]	5.16–5.28
C10H_a	4.47	4.41	C4″H	4.09–4.24[d]	4.05–4.25
C10H_b	4.52	4.65	C5″H_2	4.09–4.24[d]	4.05–4.25
N1aH	7.07	7.13	OCOCH_3	2.07, 2.05, 2.03	2.01, 1.99, 1.97
N2aH	8.49	8.52		2.02, 2.00, 1.97	

B. ^{13}C NMR of **9**

carbon	Chemical Shift (δ)	Carbon	Chemical Shift (δ)
C1	53.5	C8′	134.6
C2	58.2	C1″	82.3, 82.0[e]
C3	47.6	C2″	34.5, 35.3[e]
C5	112.4	C3″	73.4, 73.4[e]
C6a	64.9	C4″, C5″	63.0, 80.9[f]
C7	107.4		
C10	35.0	O(CO)CH_3	19.3–20.6
N(CO)CH_3	21.8		

[a] Data for **5b** taken from ref 6. [b] The δ 10.40 proton is N1′H of the deoxyguanosyl moiety attached to C1, as shown by its NOE on irradiation of C1H. [c] Protons bound to C1″, C2″, and C3″ were revealed by COSY as two separate spin systems for the two deoxyribosyl moieties. Those listed first constituted one spin system, and those listed second the other. [d] Spectral overalp precluded distinction of C4″H and C5″H_2 from one another or their assignment to the independent spin systems (see footnote *c*). [e] Carbons C1″, C2″, and C3″ were correlated with the proton spin systems described in footnote *c*. The first carbon resonance listed in each case was correlated with the first listed of the proton resonances in part A. [f] Spectral overlap in the proton spectrum precluded distinction of resonances for C4″ and C5″ from one another, or their assignment to the independent spin systems (see footnote *c*).

experiments involving these hydrogens. On the basis of the changes in line shape of the resonance for C1H upon irradiation of C2H, this coupling was shown to be ca. 2-3 Hz. The ^{1}H NMR assignment was fully consistent with the results of proton-detected, heteronuclear multiple quantum correlation of the protons with their attached carbons. The ^{13}C resonance positions of all proton-bearing carbon atoms are included in Table 1 and are themselves fully consistent with structure **9**.

Critical to the structure assignment was attribution of the five resonances for the exchangeable, nitrogen-bound hydrogens. Of the five, two were assigned as deoxyguanosyl N1 (1′ in **9**) hydrogens based upon their chemical shifts and lack of scalar coupling to other protons. One of these (δ 10.65) was in rapid chemical exchange with the medium, as evidenced by its breadth or even absence in some spectra. The observation of an NOE to one of these from N1aH further confirmed this assignment. Of the remaining three exchangeable protons, only one showed an NOE to the highest field acetylmethyl, and was assigned as belonging to the acetamide. The PSCOSY results mandated that this acetamide was bonded to the central carbon (C2) of the propylene chain. The remaining two exchangeable hydrogens were revealed by the PSCOSY to be adjacent one each to a methine and a methylene. The NH–methylene pair constituted an independent spin system; the methine of the NH–methine was C1H of the propylene (C1/C2/C3) spin system (Figure 5). Importantly, the finding of these two presumably deoxyguanosyl-derived NH's connected directly to the FR66979-derived portion of the molecule assured that alkylation of dG was at N2 (N1a and N10a in **9**) because only alkylation of an amino group could leave a residual proton on the alkylated nitrogen atom. This was fully consistent with the observation that deoxyinosine, which

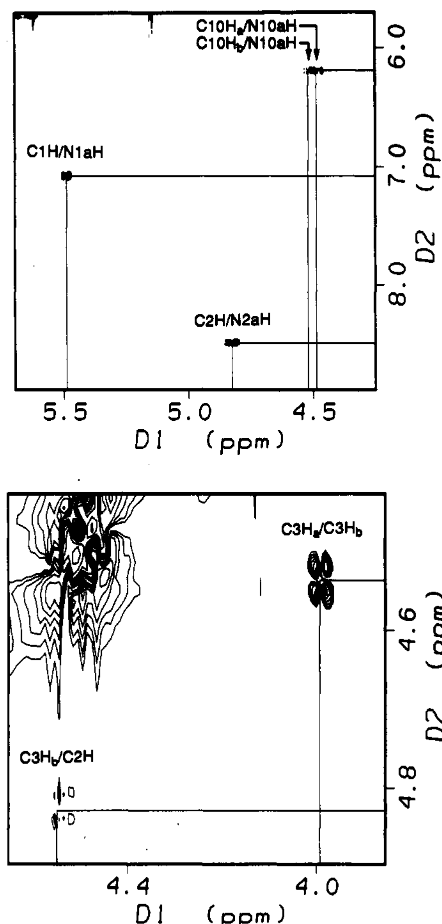


Figure 5. Sections of COSY data for peracylated derivative **9** in DMSO-d_6 at 37 °C identifying exchangeable hydrogens (upper) and C2/C3-bound protons (lower).

Table 2. Non-Deoxyribosyl, Scalar- and Dipolar-Coupled Protons in **9**

		A. Scalar Coupled Protons (from COSY)
C1H	→	N1aH, C2H[a]
C2H	→	C1H,[a] N2aH, C3H_b
C3H_a	→	C3H_b
C3H_b	→	C2H, C3H_a
C10H_a	→	N10aH
C10H_b	→	N10aH
		B. Dipolar Coupled Protons (from 1D NOE)[b]
C1H	→	C2H (m), C10H_a (w), C10H_b (m), N1aH (m), N2aH (m)
C2H	→	C1H (m), C3H_a (m), C3H_b (s), N1aH (m), N2aH (m)
N1aH	→	C1H (m), C2H (m), N1′H (m)[c]
N2aH	→	C1H (m), C2H (m), C3H_a (m), N1(CO)CH_3 (s)

[a] Very weak cross-peak in COSY confirmed by 1D decoupling (see text). [b] w, weak; m, medium; s, strong. [c] δ 10.45.

lacks this amino group, could not substitute for deoxyguanosine in these cross-linking reactions.[11]

The observations of two aromatic singlets (C5H and C7H) as well as a methylene group (C6aH_2) were consistent with the return of the benzene ring of FR66979 in essentially unaltered form in **9**. The near chemical shift equivalence of the methylene protons C6aH_2 was expected, based upon the large distance of this group from the elements of asymmetry localized at C1 and C2.

The final issue addressed was that of the relative stereochemistry of the substituents at C1 and C2. The difficulty of this task has been previously discussed.[15,16] The absolute stereochemistry at C2 was not an issue, because the indicated configuration is defined

(15) Tomasz, M.; Jung, M.; Verdine, G.; Nakanishi, K. *J. Am. Chem. Soc.* **1984**, *106*, 7367.

(16) Taylor, W. G.; Remers, W. A. *J. Med. Chem.* **1975**, *18*, 307.

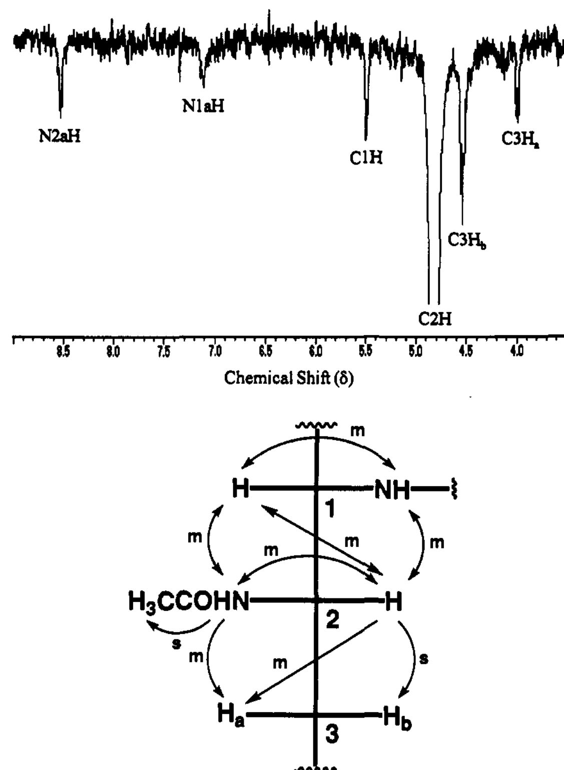


Figure 6. 1D difference NOE results for peracylated derivative **9**. Difference spectrum on irradiation of C2H (upper) and summary of results from irradiation of CH1, CH2, NH1a, and NH2a (bottom—w, weak; m, medium; s, strong; the absence of a connecting line indicates the absence of an observable NOE), with the stereochemistry at C1/C2/C3 shown in the Fischer projection. Because neither $C3H_a$ nor $C3H_b$ were irradiated, any NOE relationship between these two protons was not determined.

by the observation that D-glucosamine is a biosynthetic precursor of FR900482.[17] The relative stereochemistry at C1/C2 in the mitomycin series was assigned previously by the sign of a Cotton effect of a chromophore at long wavelength (λ_{max} 570 nm).[6] This technique is applicable only when the observed chromophore is widely separated from other chromophores,[15,18] a situation not present in **7**, **8**, or **9**. In the present case, the trans-stereochemistry at C1/C2 was tentatively assigned on the basis of several NOE observations (Figure 6, Table 2) in which C1H, C2H, N1aH, and N2aH were irradiated. Two lines of reasoning based upon these data independently support this assignment. Firstly, medium intensity NOEs were observed between the pairs C1H/N2aH, C2H/N1aH, and $C3H_a$/N2aH, but *not* $C3H_b$/N2aH. The simplest interpretation of these data places these dipolar coupled pairs on the same side of the five-membered ring, as shown in **9**, where Dreiding models indicate a closest approach of ca. 2 Å is possible. A model of the alternative cis-isomer reveals C1H/N2aH and C2H/N1aH closest approach distances of ca. 3 Å. Secondly, C2H showed only a medium intensity NOE to C1H, consistent with a trans relationship of these two protons. For reference, C2H showed one strong and one medium intensity NOE to the adjacent C3 hydrogens (Figure 6, upper), which were thus assigned as the cis- and trans-oriented hydrogens H_b and H_a, respectively.

Discussion

The experiments described herein establish unequivocally that the covalent connectivity of the FR66979-induced DNA interstrand cross-link formed at the duplex sequence 5′-d(CG) under conditions of reductive activation is as shown in **8**. The stereochemistry at C1/C2 is tentatively assigned as trans. These results offer an in vitro model for the mechanism proposed by Goto and Fukuyama[8] for the interstrand cross-linking reactions of this family, establishing directly the close parallel of the reactions of reductively activated mitomycin C[6] and FR66979, and by analogy those of FR900482 and FK973.

These results address only indirectly the proposal of McClure and Danishefsky that the creation of the mitosene nucleus in FR900482 might be initiated by nucleophilic attack at C5.[9] We have previously shown in synthetic oligonucleotides that the cross-linking reaction is greatly stimulated by sodium dithionite, and both mass spectroscopic and proton NMR evidence preclude incorporation of a nucleophile at C5. Therefore, the attack of a nucleophile at this position is not a prerequisite to cross-linking. However, we have not studied the reactions of FR900482 in the presence of strong nucleophiles, and cannot rule out the possibility that this mechanism might constitute a competing pathway.

Williams and Rajski[10] have studied the activity of FR66979 as an interstrand cross-linking agent in a plasmid-based assay, finding measurable interstrand cross-linking in the absence of an added reducing agent and little enhancement on addition of equimolar 2-mercaptoethanol. In contrast, in an oligonucleotide-based assay, we found no measureable cross-linking in the absence of reducing agent and relatively efficient cross-linking following addition of sodium dithionite.[11] These results are not necessarily incompatible, as the plasmid-based assay is several orders of magnitude more sensitive to cross-linking than the oligonucleotide-based assay. Inspection of Figure 1 in ref 10 suggests that at a concentration somewhere between 0.1 and 1.0 mM FR66979 an average of one cross-link per plasmid (63% cross-linked, 1 cross-link per ca. 4400 bp) was achieved in the absence of reducing agent. That a comparable concentration of FR66979 (ca. 1 mM) showed no detectable cross-linking in the oligonucleotide-based assay is not necessarily inconsistent, as 1 cross-link per 4400 bp corresponds to a yield of only 0.4% of cross-linked 16-mer duplex, which is near the detection limit of the assay. The oligonucleotide-based assay unequivocally proves that the efficiency of cross-linking is enhanced at least 100-fold in the presence of added sodium dithionite,[11] and a structural rationale for this is provided by the present structure proof. While the high-efficiency cross-linking enabled by reductive activation and shown herein to result in a mitosene analog as the causative lesion seems well suited to account for the antimicrobial and antitumor activity of these substances at micromolar concentrations, a parallel pathway not involving a mitosene analog cannot be ruled out by these experiments. An alternative interpretation of the plasmid-based experiments is that cross-linking in the absence of reductant by FR66979, which is prepared by reduction of FR900482 by catalytic hydrogenation or treatment with sodium borohydride,[10] is actually caused by an over-reduced substance present at the part-per-hundred to part-per-thousand level and thus far undetected by routine analysis. A further alternative is intra- or intermolecular oxidation–reduction reactions which, albeit less efficiently than an exogenous reductant, lead to mitosene-like intermediates. These ambiguities remain unresolved.

Experimental Section

Materials and Methods. Materials and their sources were as follows: DNA synthesis reagents, Applied Biosystems; phosphodiesterase I (Crotalus adamanteus venom), Pharmacia; alkaline phosphatase (calf intestinal), Boehringer Mannheim; Sephadex G-25 (superfine) and LH-20, Pharmacia. FR900482 was a gift from Fujisawa Pharmaceutical Co., Ltd. of Japan. FR66979 was prepared from reduction of FR 900482 by $NaBH_4$ as reported.[10] Water was purified on a Millipore Milli-Q deionizer. All other reagents were commercial and were used as received except for pyridine, which was distilled under nitrogen from calcium hydride. Unless otherwise specified, solutions were aqueous. Samples

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(18) Hirada, N.; Nakanishi, K. *Circular Dichroic Spectroscopy—Excitation Coupling in Organic Stereochemistry*; University Science Books: Mill Valley, CA, 1983.

were concentrated on a Savant Speed Vac concentrator. The Tris buffer for cross-linking reactions was 200 mM Tris/Tris·HCl (pH 7.5). The loading buffer was 90% aqueous deionized formamide containing 10 mM Tris/Tris·HCl (pH 7.5) and 1 mM Na_2EDTA. The 1 × TBE buffer was 90 mM Tris/90 mM boric acid (pH 8.9) and 1.8 mM Na_2EDTA. The elution buffer was 500 mM NH_4OAc, 10 mM $Mg(OAc)_2$, and 1 mM Na_2EDTA. The hydrolysis buffer was 50 mM Tris/Tris·HCl (pH 8.9) and 10 mM $MgCl_2$. Sonication was carried out on a G112SP1G sonicator. UV spectra were measured on a Hewlett-Packard 8452A or a Perkin-Elmer Lambda 3A spectrophotometer. Nuclear magnetic resonance spectra (^1H NMR) were measured on a Bruker AM500 (500 MHz) spectrometer and are reported in parts per million relative to DMSO-d_5 at δ 2.49. Electrospray ionization mass spectra (ESIMS) were measured on a Sciex Atmospheric Pressure Ionization triple quadrapole mass spectrometer. Operations, data display, and analysis were performed using Sciex' Tune (v. 2.3), MacSpec (v. 3.21), and Canvas (v. 3.0.4) sofware operating on a Macintosh IIfx. Except where otherwise noted, selected ions, diagnostic for the substance of interest, are reported. HPLC was performed on a Alltech, 5 mm, C18, 250 mm × 4.6 mm Econosphere column using an SSI 200B/220B dual pump system with an SSI controller and SSI 500 UV/vis (output to a Linear 255/M recorder and an HP 3390A electronic integrator) detector. Solvent gradients were run at 1 mL/min. Gradient A: solvent A—100 mM ammonium acetate (pH 7.0); solvent B—CH_3CN; isocratic 92% A for 7 min, 13 min linear gradient to 70% A, 10 min linear gradient to 60% A, then 10 min linear gradient to initial conditions. Gradient B: solvent A—100 mM ammonium acetate (pH 7.0); solvent B—CH_3CN; 10 min linear gradient from initial 75% A to 65% A, 10 min linear gradient to 50% A, 10 min linear gradient to 40% A, then 10 min linear gradient to initial conditions. Gradient C: the same as gradient B except solvent A is 5 mM ammonium acetate (pH 7.0).

Preparation of Cross-Linked DNA. The oligodeoxynucleotide was synthesized on an Applied Biosystems Model 392 synthesizer. Crude, self-complementary DNA 5'-$[d(ATATCGATAT)]_2$, 620 OD (2.5 mM), and FR 66979 (20 mM) were dissolved in 1.2 mL of 200 mM Tris buffer (pH 7.5). The mixture was divided into 4 microfuge tubes. The solutions were cooled to 0 °C in an ice–water bath and deaerated by bubbling Argon gas for 10 min. A 40-μL portion of a pre-cooled (0 °C), deaerated solution of freshly made 150 mM $Na_2S_2O_4$ in the same buffer was added to each reaction mixture in five equal increments at 10-min intervals under Argon bubbling. After 12 h at 0 °C under constant Argon bubbling, each cross-linking mixture was further divided into two samples. Each of these samples was ethanol precipitated, the supernatants removed, and the pellets dried. Each pellet of DNA was dissolved in 160 μL of 1:1 water and loading buffer and purified by DPAGE (ca. 77.5 O.D. of crude DNA, 25% polyacrylamide; 19:1 acrylamide:bis-acrylamide and 8 M urea in 1 × TBE buffer, 1.5 mm thick, 14 × 16 cm, using a five-toothed comb), run until the xylene cyanol dye had traveled 6–7 cm from the origin. Native and cross-linked DNA were visualized by UV shadowing[19] and isolated from the gel by a crush and soak procedure: the cross-linked DNA was cut from the gel, crushed with a glass rod into fine particles, and nutated at 25 °C for 2 h in elution buffer. The supernatant was removed and the gel twice nutated with fresh elution buffer for 1 h. The supernatants were combined and passed through a Waters Sep-Pak C_{18} cartridge (previously washed with 10 mL of acetonitrile and 10 mL of water) followed by 10 mL of 10 mM aqueous ammonium acetate and 10 mL of water, then the DNA was eluted with 3 mL of 25% aqueous acetonitrile. Cross-linked DNA (250 O.D.) was recovered by concentration of the acetonitrile/water eluent (40% yield from starting native DNA).

Conjugate 8 from the Hydrolysate of Cross-Linked DNA. Cross-linked DNA (250 O.D.) was hydrolyzed using 125 μL of hydrolysis buffer, 94 units of snake venom phosphodiesterase I, and 375 units of calf intestinal alkaline phosphatase (total volume 1.25 mL). HPLC analysis was carried out using gradient A to determine the extent of hydrolysis. HPLC analysis showed completion of hydrolysis, returning the nucleosides and a strongly retained substance with a retention time of 20.8 min after 18 h at 37 °C. The hydrolysate was mixed with an equal volume of DMSO and the resulting mixture was sonicated for 2 min. The mixture was chromatographed over a column of Sephadex G-25 (superfine) (1.6 × 53 cm) and eluted with 20 mM NH_4HCO_3 (pH 8.5). The free nucleosides were eluted at ca. 85 mL for dT and dC and 105 mL for dA and dG. The conjugate **8** was eluted at ca. 185 mL. HPLC analysis (Gradient A) showed 95% (before concentration) and 90% (after concentration) purity of conjugate **8**. Conjugate **8**, 23 O.D., was recovered. The desired fractions (~50 mL) were pooled and concentrated in ca. 17-mL aliquots on a rotary evaporator to a total volume of 1 mL. The concentrated sample was transferred to a microfuge tube and then further concentrated to dryness in a Speed-Vac concentrator.

Peracetylated Derivative 9. Product **8** from the previous experiment was sequentially dried 3 times from a small amount of pyridine and then dissolved in 400 μL of pyridine, 70 μL of acetic anhydride, and 11 μL of 5 mM 4-(dimethylamino)pyridine in pyridine. After the mixture was sonicated for 30 min and then stirred for 50 min at 25 °C, 50 μL of water were added and the mixture was stirred an additional 5 min. After concentration to dryness, the sample was dissolved in 300 μL of 1:1 DMSO and 1 mM methanolic NH_4OAc. The solution was sonicated and then chromatographed over a column of LH-20 (1.2 × 52 cm), eluted with 1 mM methanolic NH_4OAc. The peracetylated derivative **9** was eluted at the 21 mL volume. Each fraction with appreciable UV absorption was analyzed by HPLC (Gradient B). The fractions containing pure **9** (>95% purity by HPLC, total volume 10.5 mL) were pooled and concentrated to dryness on a Speed-Vac concentrator and stored at –78 °C (10 O.D.). This sample in 0.35 mL of DMSO-d_6 (100 atom%) was used for the 1D ^1H COSY spectra. The sample for 1D NOE and heteroatom correlated spectra contained roughly twice this concentration of **9**, with the additional material obtained by repetition of the preparation of **9** on the described scale. The sample for ESIMS was further purified by HPLC using Gradient C. The peak with a 21-min retention time was collected and concentrated to dryness and the residue was then dissolved in methanol and analyzed by electrospray ionization mass spectrometry. ESIMS (180 V inlet voltage, 4400 V needle voltage, 3 μL/min, sum of 10 scans): *m/e* 1095 ($M + K^+$), 1079 ($M + Na^+$), 1057 ($M + H^+$), 857 ($M + 2H^+$ – deoxyribosyl$^+$), 728 ($M + 2Na^+$ – deoxyguanosine), 706 ($M + H^+$ – deoxyguanosine), 701 ($M + H^+ + 2Na^+$ – 2 deoxyribosyl$^+$), 679 ($M + 2H^+ + Na^+$ – 2 deoxyribosyl$^+$), 528 ($M + H^+ + Na^+$ – deoxyribosyl$^+$ – deoxyguanosine), 506 ($M + 2H^+$ – deoxyribosyl$^+$ – deoxyguanosine).

The COSY data were acquired in the phase-sensitive mode using 512 increments of 64 scans each with a sweep width of 5050 Hz in both dimensions (f_1 and f_2). The transient NOE experiments employed a mixing time of 600 ms and consisted of 4608 scans each. The proton detected heteronuclear multiple quantum correlation experiment (HMQC) was performed in the phase-sensitive mode using 128 scans for each of 300 increments in a sweep width of 25000 Hz in f_1. These data were then extended by linear prediction and zero filled to 1024 points prior to Fourier transformation.

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