We can compare the present results with those reported by Scaiano and co-workers,[16] viz., $k_a = (2.04 \pm 0.73) \times 10^6$ $M^{-1}$ $s^{-1}$ in benzene at 27 °C and $k_\beta = 1.0 \times 10^6$ $s^{-1}$ in chlorobenzene[17] (as calculated from their Arrhenius equation). Walling[10,11] was the first to suggest that solvents will influence bimolecular reactions to a lesser extent than unimolecular reactions. Taken together, our results and Scaiano's lend support to this idea. However, the difference between our direct measurement of $k_\beta$ and Scaiano's value cannot be accounted for if the usual assumption[10,11] is made that $k_a$ is solvent independent. That is, combining our value of $k_\beta$ with Walling and Wagner's[11] values for $k_a/k_\beta$ at 25 °C, viz., 6.42 $M^{-1}$ in $CCl_4$[18] and 4.22 $M^{-1}$ in chlorobenzene, and assuming a solvent-independent $k_a$ gives $k_\beta$ in chlorobenzene = $3.45 \times 10^5$ $s^{-1}$. The most probable reason for the disagreement in the values of $k_\beta$ is the indirect method used by Scaiano and co-workers to measure this quantity.[19]

We conclude that the LFP/TRIR technique provides a direct and reliable method for measuring the rate constants for β-scission of alkoxyl radicals. Structural and solvent effects on this reaction will be explored.

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(17) It proved difficult to study the β-scission of cumyloxyl in chlorobenzene because of this solvent's absorption at 1690 $cm^{-1}$. Preliminary experiments indicate that $k_{expt} \sim 3 \times 10^5$ $s^{-1}$, which gives an upper limit for $k_\beta$ in chlorobenzene.

(18) Our absolute values of $k_a$ and $k_\beta$ in $CCl_4$ yield $k_a/k_\beta = (4.20 \pm 0.84)$ $M^{-1}$.

(19) These workers relied on product studies to partition the rate constant for β-scission from the rate constants for competing bimolecular reactions.

## Determination at Single-Nucleotide Resolution of the Sequence Specificity of DNA Interstrand Cross-Linking Agents in DNA Fragments

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Interstrand cross-linking of DNA is believed to account for the acute cytotoxicity of many bifunctional alkylating agents, including important antitumor substances.[1] Determining the structural details of cross-linked DNAs is complicated by both molecular size and the inefficiency of cross-linking, with monofunctional binding to DNA usually greatly exceeding the bifunctional, cross-linking mode. One strategy, exhaustive hydrolysis of the phosphodiester backbone of cross-linked DNA followed by conjugate isolation and structure elucidation, can effectively define *sites* of covalent linkage between drug and DNA (e.g., guanine N-7), but is not generally useful for determining preferred base *sequence* at the cross-link site. We describe herein a simple and general chemical method for determining the base sequence preferences of DNA interstrand cross-linking drugs at single-nucleotide resolution. The method is demonstrated by using DNAs crosslinked with a psoralen, mitomycin C, and an analogue of a pyrrolizidine alkaloid metabolite.

The sequence dependence of noncovalent protein-DNA[2a] and drug-DNA[2b] interactions has been studied by "footprinting", in which the complexed DNA is randomly cleaved and the fragment size distribution analyzed.[2c] Analogous treatment of a cross-linked DNA might be expected to define cross-link location. Single-hit, random cleavage to the radiolabeled (*) 3'-side of the cross-link

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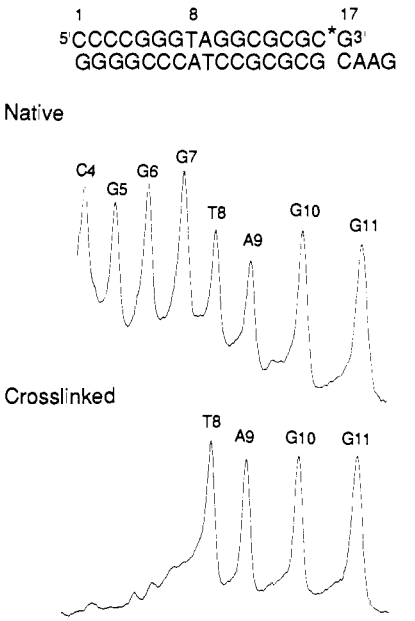
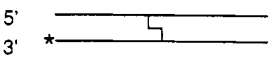



**Figure 1.** Partial fragmentation patterns for radiolabeled ((*) = $^{32}P$) native and HMT cross-linked DNA duplexes. Lettering indicates residue cleaved.

in the DNA shown schematically below should provide short radiolabeled fragments. Cleavage at any other site on either strand should afford a much larger radiolabeled fragment. Electrophoretic separation of the fragment mixture should provide a discontinuity diagnostic for the cross-link site.




A DNA duplex containing a single 5'-d(TA) cross-linkable sequence was cross-linked by using 4'-(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT), a substance known to bridge covalently two thymine moieties.[3] The product was selectively end radiolabeled ($^{32}P$), and the cross-linked DNA was separated from residual single-stranded DNA by denaturing polyacrylamide gel electrophoresis (PAGE).[4] Radiolabeled native and cross-linked materials were subjected sequentially to iron(II)/EDTA cleavage,[5] single-base-resolution PAGE,[6] autoradiography, and one-dimensional scanning densitometry.[7] As predicted by the above analysis,

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(7) DNA was synthesized (Applied Biosystems Model 380A) and was purified by denaturing PAGE (20%, 95:5 acrylamide/bis(acrylamide)). A 1:1 ($OD_{260}$) mixture of strands (see Figure 1), 17 μM in duplex DNA, 300 μM in HMT, and containing 10 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris (pH 8.0), 0.1 mM EDTA, total volume 650 μL, was nutated for 1.5 h at 25 °C and then irradiated at 4 °C in a silanized Pyrex test tube at 351 nm (100 mW, Spectra-Physics argon laser model 2025-05) for 0.5 h. An aliquot was 3'-end labeled by using Klenow fragment (Boehringer Mannheim)/[α-$^{32}P$]dGTP (New England Nuclear), and cross-linked DNA was isolated by denaturing PAGE (25%).[4] Iron(II) EDTA[5] cleavage reactions were carried out in 50 μM $(NH_4)_2Fe(SO_4)_2$, 100 μM EDTA, 1 mM sodium ascorbate, 10 mM $H_2O_2$, 10 mM NaCl, 10 mM Tris (pH 8.0), 1 min, 25 °C, and were stopped with excess thiourea. Samples were lyophilized, suspended in 90% formamide, 10 mM Tris (pH 8.0), 0.1% xylene cyanol, 0.1 mM EDTA, heat denatured at 90 °C for 1 min, cooled to 0 °C, and subjected to electrophoresis on a 25% polyacrylamide gel (95:5 acrylamide/bis(acrylamide), 50% urea, 0.35-mm thick, 41 × 37 cm) using a Hoeffer thermojacketed Poker Face gel stand at ca. 1500 V and 70 °C until the dye had traveled 19 cm. The gel was dried (Bio-Rad Model 583) onto Whatman 3MM paper and autoradiographed on Kodak XAR-5 film. Cleavage site was assigned by reference to a Maxam-Gilbert G-lane.[6a] The autoradiogram was scanned and plotted by using the program Spectra Calc (Galactic Industries Corporation, Salem, NH).

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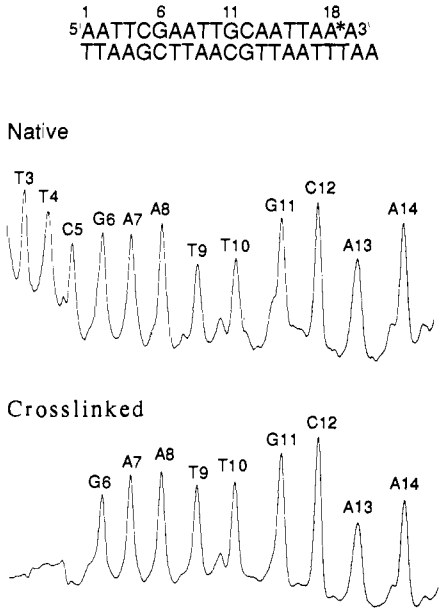



**Figure 2.** Partial fragmentation patterns for radiolabeled ((*) = $^{32}P$) native and mitomycin C cross-linked DNA duplexes. Lettering indicates residue cleaved.

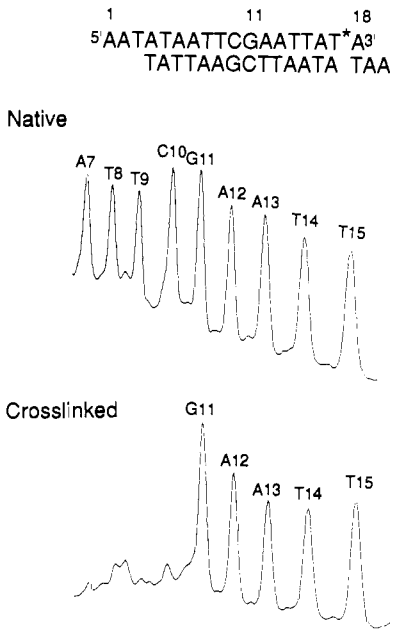
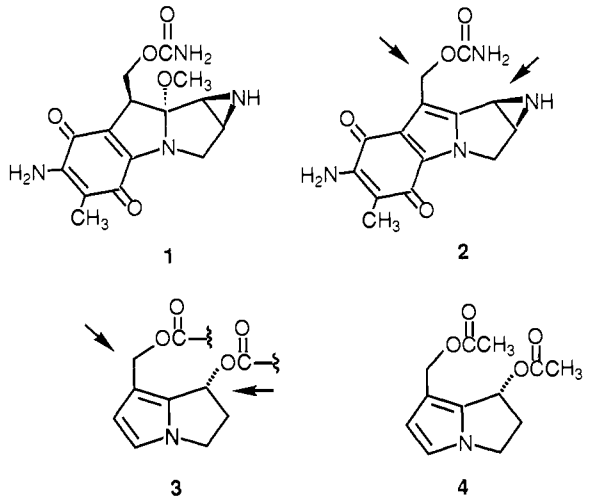



**Figure 3.** Partial fragmentation patterns for radiolabeled ((*) = $^{32}P$) native and dehydroretronecine diacetate (**4**) cross-linked DNA duplexes. Lettering indicates residue cleaved.

the cross-linked sample (Figure 1) yielded fragments representing cleavage from the radiolabeled end to T8. The absence in the cross-linked sample of fragments corresponding to cleavage of the native sample at nucleotides G7–C1 defines T8 as the cross-linked residue, conclusively demonstrating the ability of the assay to detect sites of cross-linkage.[8]

The biological target of the antibiotic and antitumor substance mitomycin C (**1**) is believed to be DNA.[9] Reductive activation, either in vivo or in vitro, results in the covalent modification of DNA, including interstrand cross-linking, which has been proposed to be the primary mechanism of cytotoxicity.[10] The recent isolation of a conjugate derived from one mitomycin C and two deoxyguanosine residues suggests that the dinucleotide units 5′-d(CG) and 5′-d(GC) in B-DNA might form interstrand cross-links with minimal distortion.[11,12] A radiolabeled DNA duplex containing these sequences admixed with excess mitomycin C was treated with sodium dithionite.[13,14] The least electrophoretically mobile product was isolated and processed as above. Short radiolabeled fragments were obtained only upon cleavage from the radiolabeled end through G6 (Figure 2), pinpointing G of the sequence 5′-d(CG) as cross-linked by reductively activated mitomycin C in strong preference to 5′-d(GC).[12,15] Confirming this conclusion, radiolabeling of the 5′-terminus of this same strand afforded an analogous result (data not shown).




The pyrrolizidine alkaloids are widely distributed, plant-derived toxins, some of which after metabolic activation act as bifunctional electrophiles.[17] DNA cross-linking has been observed for members of this family;[18] several mononucleoside adducts are known,[19] but any sequence preference for cross-linking is unknown. The pro-

(8) Analysis of a cross-linked DNA containing more than one cross-linkable site has also been achieved and will be reported elsewhere.

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(12) Chawla, A. K.; Lipman, R.; Tomasz, M. In *Structure and Expression, Volume 2: DNA and Its Drug Complexes*; Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: Albany, NY, 1987.

(13) The efficiency of cross-linking is low, rendering statistically insignificant the probability of an appreciable population of doubly cross-linked duplexes.

(14) Cross-linking conditions are a modification of those of Tomasz et al.[3] Labeled DNA was added to 0.7 mM (base pairs) duplex DNA and 1.4 mM mitomycin C (Sigma, from a 20 mM stock in 33% aqueous methanol containing 2.7 M NaCl) in 15 mM Tris buffer (pH 7.5), total volume 110 μL. Samples were incubated at 37 °C for 1 h prior to bubbling argon through the solution for 20 min. Three equal aliquots of freshly prepared sodium dithionite (Baker, 50 mM stock solution in deoxygenated water) were added at 15-min intervals to the DNA/mitomycin C mixture at 37 °C (total: 0.14 μmol, 1:1 molar ratio of dithionite to mitomycin C). Fifteen minutes following the final addition, samples were ethanol precipitated and then subjected to 20% denaturing PAGE.

(15) While this manuscript was in preparation, Crothers et al.[16] reported the results of a related study. Seventeen DNA duplexes were subjected to reductively activated mitomycins. On the basis of electrophoretic mobility, it was concluded that 5′-d(CG) was required for cross-linking. Their results are therefore complementary to and in agreement with those reported here. Taken together, the results of Crothers et al.[16] and Tomasz et al.[11,12] and those herein provide compelling evidence that reductively activated mitomycin C, and likely other mitomycins as well, generate interstrand cross-links in duplex DNA predominantly at 5′-CG sequences by bridging deoxyguanosine residues through N2.

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(17) Mattocks, A. R. *Chemistry and Toxicology of Pyrrolizidine Alkaloids*; London: Academic Press, 1986.

(18) (a) White, I. H. N.; Mattocks, A. R. *Biochem. J.* **1972**, *128*, 291. (b) Petry, T. W.; Bowden, J. T.; Huxtable, R. J.; Sipes, I. G. *Cancer Res.* **1984**, *44*, 1505. (c) Reed, R. L.; Ahern, K. G.; Pearson, G. D.; Buhler, D. R. *Carcinogenesis* **1988**, *9*, 1355.

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nounced structural congruence of the electrophilic sites (arrows in **2** and **3**) of reductively activated mitomycin C (mitosene **2**) and oxidatively activated pyrrolizidine esters (dehydropyrrolizidine alkaloids, **3**) suggests that deoxyguanosine residues of 5′-d(CG) might likewise be cross-linkable by activated pyrrolizidine alkaloids.[20] Dehydroretronecine diacetate (**4**) was incubated with a radiolabeled DNA duplex containing a single 5′-d(CG) sequence.[21] Processing of the least electrophoretically mobile product as above afforded short, radiolabeled fragments for cleavage from the radiolabeled end through G11 (Figure 3). This is consistent with cross-linkage predominantly at the deoxyguanosine of 5′-d(CG).

These experiments demonstrate conclusively that sequence-random cleavage of cross-linked DNAs can be used to define sites of interstrand cross-linkage in DNA at single-nucleotide resolution.[22]

**Acknowledgment**. We thank the Sloan Foundation and the National Institutes of Health (AG00417, GM35466, GM32681) and National Science Foundation (DIR-8220099) for financial support, Susan Ribeiro for synthetic DNA, Ug-Sung Kim and Gary Anderson for technical assistance, Dr. John C. Amedio, Jr., and Professor James D. White for the generous gift of dehydroretronecine, and Professor Stanley Raucher for helpful discussions.

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(21) Labeled DNA (1.7 mM base pairs) in 50 μL of 50 mM sodium citrate buffer (pH 5.0), 5 mM NaCl, 5 mM $MgCl_2$ was vortexed at 25 °C with 0.4 mL of a $CDCl_3$ solution of dehydroretronecine diacetate. After 35 min, the DNA solution was ethanol precipitated and subjected to 25% denaturing PAGE. Cross-linked material was identified by comparison to the mitomycin cross-linked DNA of the same sequence. The specific activity of the cross-linked DNA was enhanced by reexposure to Klenow fragment and [α-$^{32}$P]-dATP prior to fragmentation.

(22) This chemical-based method complements existing methods involving inhibition of enzymatic reactions (polymerase,[23] restriction endonuclease,[3b] exonuclease[3c,24]).

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## Enzymatic Peptide Synthesis via Segment Condensation in the Presence of Water Mimics

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Convergent condensation of peptide segments (e.g., prepared by the stepwise solid-phase methodology[1]) is a powerful strategy for the synthesis of biologically active polypeptides, in particular if many homologous sequences are needed.[2] Enzymatic methods, being mild and selective, have proven attractive for peptide synthesis.[3] However, the use of enzymes as catalysts of peptide segment coupling has been rare[4] due to their propensity to concomitantly hydrolyze the growing polypeptide chain.[3] Thus the versatile protease thermolysin[5] has been widely used for the synthesis of dipeptides (including the commercial production of the sweetener aspartame[6]) but not polypeptides.[7]

**Table I.** Initial Rates of the Reaction between Z-Gly-Gly-Phe and Phe-$NH_2$ Catalyzed by Thermolysin in *tert*-Amyl Alcohol Containing Various Cosolvents[a]

| cosolvent, % (v/v) | reactn rate,[b] μM $h^{-1}$ (mg of enzyme)$^{-1}$ |
|---|---|
| none | 0 |
| 1% water[c] | 19 |
| 4% water[c] | 3500 |
| 1% water[c] + 9% formamide[d] | 3800 |
| 1% water[c] + 9% ethylene glycol[d] | 1500 |
| 1% water[c] + 9% glycerol | 820 |
| 1% water[c] + 9% ethylene glycol monomethyl ether | 140 |
| 1% water[c] + 9% methanol | 130 |
| 1% water[c] + 9% ethylene glycol dimethyl ether | 60 |
| 1% water[c] + 9% dimethylformamide | 51 |
| 1% water[c] + 9% tetrahydrofuran | 25 |

[a] Conditions: 10 mM Z-Gly-Gly-Phe, 25 mM Phe-$NH_2$, 0.5 mg/mL thermolysin,[5] 45 °C, shaking at 300 rpm. The enzyme (Sigma) was prepared by lyophilization of a 5 mg/mL aqueous solution, pH 7.2, containing 10 mM $Ca(CH_3COO)_2$; the resultant powder was placed in a substrate solution and sonicated for 5 s. No peptide-bond formation was detected without thermolysin. [b] The initial rate was measured by HPLC (Waters' μBondapak $C_{18}$ column, $CH_3CN/H_2O/CF_3COOH$ (45:55:0.1) as a mobile phase) by following the formation of the tetrapeptide product. [c] In all cases, the term "water" refers to an aqueous solution, pH 7.2, containing 10 mM $Ca(CH_3COO)_2$. [d] No reaction was observed when *all* of the water was replaced with formamide or ethylene glycol.

However, the efficient synthesis of the Phe–Phe peptide bond catalyzed by thermolysis in *tert*-amyl alcohol containing 4% of water is accompanied by a substantial secondary hydrolysis: e.g., after 6

Many of these thermolysin-catalyzed reactions have been carried out in aqueous–organic mixtures in order to shift the thermodynamic equilibrium toward peptide-bond formation.[3] This reaction medium should also diminish the unwanted secondary proteolytic cleavage. Attempts to maximize these benefits by completely replacing water with organic solvents,[8] however, result in the loss of thermolysin activity: e.g., as one can see in the first line of Table I, the enzyme fails to form a (favored in water[9]) Phe–Phe peptide bond in anhydrous *tert*-amyl alcohol.[10] This enzymatic reaction becomes noticeable at 1% of water and very fast at 4% (lines 2 and 3, respectively, in Table I). Unfortunately,

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