upon the orientation of the chemical shift tensor with respect to the molecular frame, and on the mean orientation with respect to the field of both the peptide plane containing the site and the axis about which the dispersion occurs. The data in Figure 1 support a qualitative interpretation for an orientational distribution about the C_α-C_α axis of ±10 to 20° for both of the isotopically labeled peptide linkages.

Several possible explanations for these observed results can be considered. Substantial tilting of the channel axis away from the magnetic field caused by distortions of the bilayer surface upon cooling through the phase transition is unlikely. Such distortions are anticipated to be small and consequently could not account for the broad dispersion of chemical shift frequencies observed at the Gly_2 site at low temperatures. Another possible explanation is that significant structural changes of the polypeptide backbone have occurred upon lowering the temperature. This would be expected to have a profound effect on channel conductance, but the gramicidin dimer is an effective ion conducting channel below the phase transition temperature.[15] The most probable explanation for the observation of the orientational dispersion at low temperature is that the peptide linkages are trapped in local potential energy minima. These minima represent conformational substates that have the same overall structure of the gramicidin channel but differ only in subtle structural details including the orientation of the isotopically labeled site with respect to the channel axis. This interpretation requires that the transition rate from one conformational substate to another is much faster than the NMR time scale (3.5 kHz) when the sample is at 23 °C, resulting in a narrow motionally averaged chemical shift resonance. At 8 °C, the transition rate is slower than the NMR time scale, allowing the chemical shift spectrum to reflect the orientation of each conformational substate. Such a change in transition rate could be based on differences in the environment for the amino acid side chains as the phase of the sample is changed.[16] Finally, it should be noted that a continuous motion of a peptide plane within a smooth and shallow potential energy well is unlikely, since, for such a model, a decrease in frequency requires a dramatic increase in amplitude, which is unreasonable considering the dynamic behavior of the lipid at low temperature.

A large number of experiments and computational studies have implicated the existence of conformational substates in protein systems.[18,19] For the gramicidin channel, conductance studies have detected functional substates,[16,20] suggesting that a variety of similar channel conformations may exist. Despite maximal hydrogen bonding in the model structure of the peptide backbone of gramicidin, computations have indicated that the backbone is very flexible[2-4] and the existence of helically librated states has been suggested.[2] From the experimental results presented here, the computationally predicted flexibility is due to transitions over a wide range of conformational substates. For the first time in the study of gramicidin, direct experimental evidence for these substates has been achieved. Moreover, solid-state NMR has for the first time been used to provide evidence for conformational substates. These ^{15}N chemical shift spectra of uniformly aligned samples at low temperature have provided a window through which to view the orientational dispersion of conformational substates, opening an exciting avenue for obtaining a detailed spatial description of the internal motions not only of the gramicidin channel but also of other bilayer bound systems.

Acknowledgment. The authors gratefully acknowledge Professor D. W. Urry and K. U. Prasad for the synthesis of ^{15}N-Gly_2-gramicidin, support for which was provided by NIH Grant GM 26898. Their generous gift of this molecule enabled us to have a first glimpse at the narrow resonance arising from an oriented sample of gramicidin containing a single labeled site. We further acknowledge fruitful discussions with M. T. Brenneman, who suggested the idea of conformational substates for the interpretation of the NMR data. We also express our appreciation to Richard Rosanski and Thomas Gedris for their skillful maintenance, modification, and repair of the NMR spectrometer purchased with the aid of NSF Grant DMB-8504250. This work is supported by NIH Grant AI-23007 and NSF Grant DMB-8451876 with Procter and Gamble through a Presidential Young Investigator Award to T.A.C.

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Synthesis of a Bacteriophage DNA Containing a Site-Specific Cis-Syn Thymine Dimer

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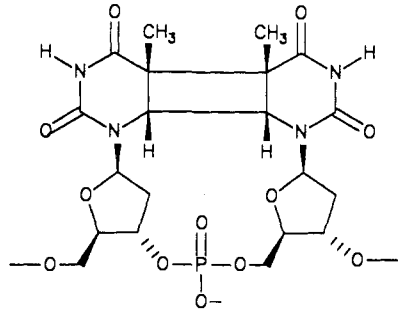
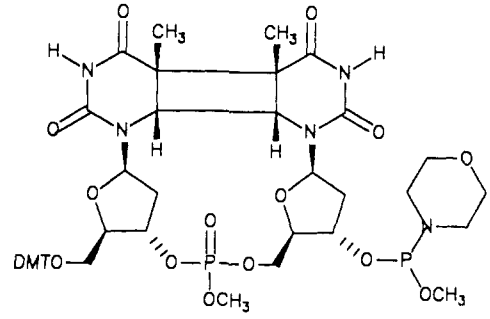
Received July 22, 1988

The cis-syn thymine dimer is the major sunlight-induced photoproduct of DNA[2] whose production has been linked with mutations and skin cancer.[3] The lethality, mutagenicity, and mutation spectrum of the cis-syn thymine dimer as a function of its location in a genome is unknown.[4] Attempts to derive such structure-activity relationships have been hampered by the lack of general methods for the preparation of DNA containing site-specific cis-syn thymine dimers for study. Recently, we reported the synthesis of the building block **1** for the site-specific incorporation of cis-syn thymine dimers into oligonucleotides by solid phase DNA synthesis technology.[5] Herein we report the synthesis and characterization of a bacteriophage DNA containing a site-specific cis-syn thymine dimer suitable for in vivo repair, replication, and mutagenesis studies.



cis-syn



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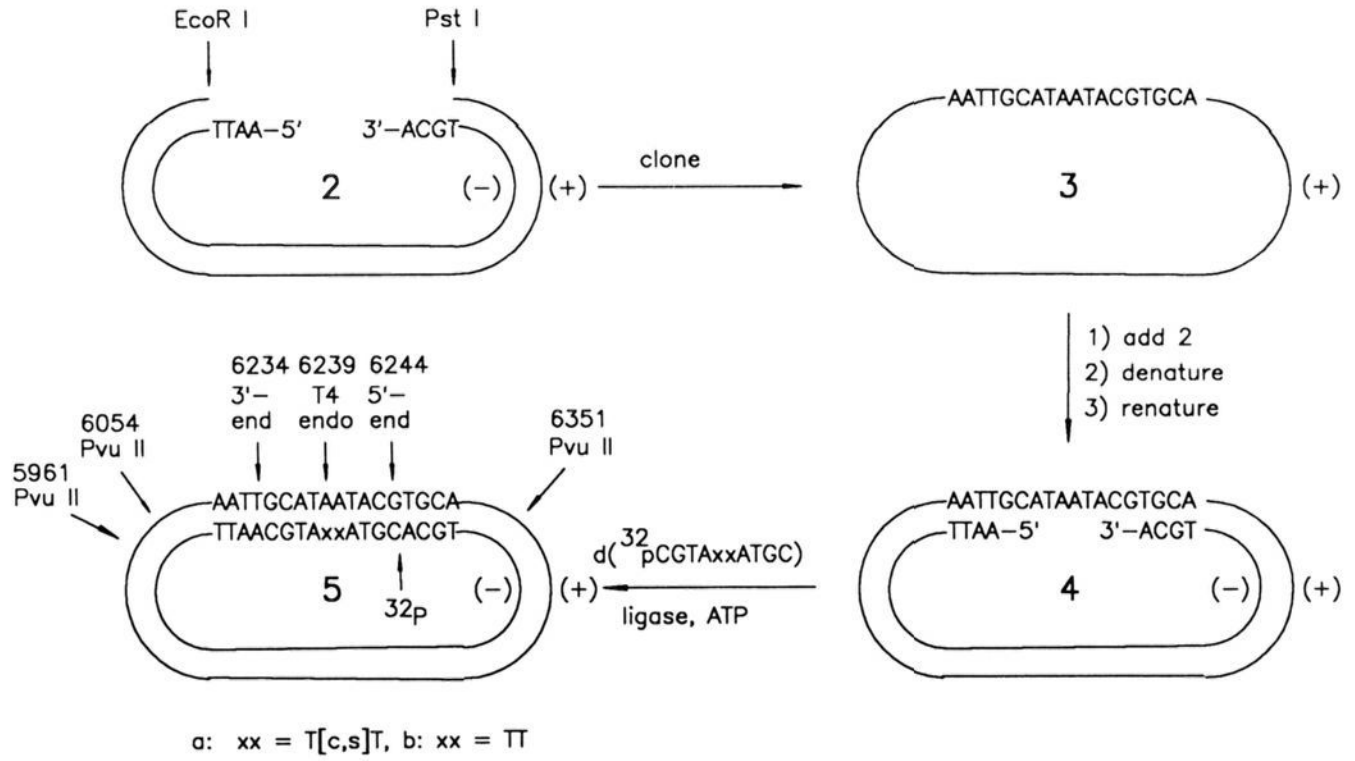
Scheme I



a: xx = T[c,s]T, b: xx = TT

The route used to construct the bacteriophage DNA containing a thymine dimer (Scheme I)[6] is similar to that recently reported for the construction of a *cis*-diammineplatinum(II) adduct-containing M13 clone.[7] Thus d(pAATTGCATAATACGTCGA)-d(pCGTATTATGC) was cloned into Eco RI, Pst I restricted M13mp18[8] replicative form DNA, compound **2**, by standard techniques.[9] Four equiv of the (+) strand of this clone, compound **3**, were renatured in the presence of **2** by dialysis against decreasing concentrations of formamide. The resulting mixture, containing the gapped duplex **4**, was then incubated with 30 equiv of d(^{32}pCGTAT[c,s]TATGC)[10] in the presence of T4 DNA ligase and ATP at 4 °C overnight. d(^{32}pCGTATTATGC) was similarly ligated for comparison purposes.

Agarose gel electrophoresis of the ligation reaction mixtures in the presence of ethidium bromide followed by autoradiography (Figure 1, left) clearly established the presence of closed circular bacteriophage DNA containing the cis-syn thymine dimer (form IV DNA), compound **5a**. In addition, open circular DNA (form II DNA) resulting from incomplete ligation and nicking was detected along with linear DNA (form III DNA). The radiolabeled form IV:II:III bacteriophage DNA was produced in ratios of 17:75:8 for the dimer and 18:74:8 for the nondimer.[11]

The contributions of incomplete ligation and nicking to the low yields of the form IV DNA were determined by Pvu II restriction digestion of the ligation mixtures followed by denaturing gel electrophoresis and autoradiography (Figure 1, middle). Radiolabeled bands corresponding to fragments resulting from complete 5′ and 3′ (297-nt), incomplete 5′ (190-nt), and incomplete 3′ (117-nt) ligation of the decamers were detected in ratios of 38:47:15 for the dimer and 42:38:17 for the nondimer.[12] The fact that similar ratios were obtained for both decamers suggests that incomplete ligation was due to a common cause, such as partial degradation of the ends of the gapped duplex **4** prior or during ligation, rather than to the presence of the cis-syn dimer.

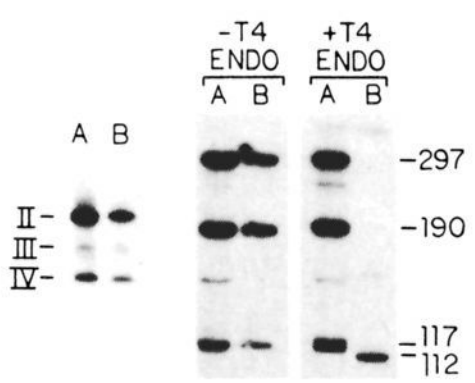


Figure 1. Left: Autoradiogram of a 0.5 μg/mL ethidium bromide, 1% agarose electrophoresis gel of the ligation reaction mixture of the gapped duplex, **4**, and (a) d(^{32}pCGTATTATGC) and (b) d(^{32}pCGTAT[c,s]TATGC). Middle and right: Autoradiogram of a 7 M urea 10% acrylamide electrophoresis gel of the same ligation reaction mixtures restricted with Pvu II with (+) and without (–) subsequent treatment with T4 denV endonuclease V.

To demonstrate the integrity and location of the cis-syn thymine dimer after the series of synthetic and enzymatic steps, the Pvu II restricted ligation mixtures were subjected to the action of the cis-syn pyrimidine dimer-specific T4 denV endonuclease V.[3,13] As expected for an intact cis-syn thymine dimer located at nucleotides 6239-40, all the original bands disappear and a band corresponding to a 112-nt fragment is observed (Figure 1, middle).

In conclusion, we have successfully incorporated the major sunlight-induced photoproduct of DNA, the cis-syn thymine dimer, into a unique site of a form IV bacteriophage DNA via a combined synthetic, enzymatic, and recombinant DNA approach. The lethality, mutagenicity, and mutation spectrum of this cis-syn thymine dimer is currently under study.

Acknowledgment. This investigation was supported by PHS Grant No. R01-CA40463, awarded by the National Cancer Institute, DHHS.

(6) Locations shown for **5** refer to the coordinate of the nucleotide to the 3′ side of a cleavage or ligation site in the (–) strand. Ligation sites are labeled as 3′ and 5′ with respect to the ends of the decamers.

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(10) The decamer containing the cis-syn thymine dimer, d(CGTAT[c,s]-TATGC), was prepared by solid phase synthesis utilizing the building block **1**[5] and 5′-phosphorylated with T4 polynucleotide kinase and γ-^{32}P-ATP to give d(^{32}pCGTAT[c,s]TATGC). The synthetic material was characterized by 500 MHz ^{1}H NMR and by denaturing gel electrophoresis of the ^{32}P-endlabeled material. Both techniques indicated that the samples were of greater than 95% purity.

(11) The average ligation efficiencies (calculated as described in ref 7b) of two other ligation reactions of the decamers containing the cis-syn dimer and a nondimer were 78% and 73%, respectively, with average radiolabeled form IV:II:III ratios of 37:58:5 and 32:62:6 respectively.

(12) The origin of the minor bands (<3% in lanes A and <1% in lanes B, Figure 1, right) is not known. The fact that the intensities of the minor bands vary between lanes suggest that they may be due to alternate secondary structures that the major fragments adopt during electrophoresis. Similar results were obtained when the two other ligation reactions described in footnote 11 were analyzed with Pvu II and the combinations Pvu II/Hind III and Nar I/Pvu I.

(13) Purchased from Applied Genetics Inc., Freeport, NY, 11520.