6/28/93 Final Report 9/14/90 to 9/13/93

The Dual and Simultaneous Roles of Nucleophilic Delivery and Assistance of Leaving Group Departure by Metal Ions in Phosphate Transfer from Phosphate Diesters

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Our research has centered around the very difficult problem of the catalysis of hydrolysis of phosphate diesters. The study may be divided into two parts. The first deals with the design synthesis and study of new catalytic systems which incorporate a duality of functional groups designed to deliver a nucleophile, preferably metal bound water, and to stabilized the leaving alkoxide, preferably by metal cation. One completed study has been published and others are in progress (one nears completion). The second part of the problem is the design, synthesis, and study of compounds (which we call microgonotropens) which bind in the minor groove of dsDNA at A·T rich regions and direct a catalytic configuration of nucleophile and electrophile to particular dsDNA phosphodiester linkages. We have published one paper, three additional papers dealing with dien-microgonotropens will be published this summer and two on tren-microgonotropens approach completion. We are interested in how the microgonotropens change the shape of dsDNA and this has lead us to computer studies of the structures of peptide nucleic acid complexes with dsDNA. Two papers on this topic have been accepted for publication and will appear before the end of 1993.

11

DNA, microgonotropen, minor groove, major groove, hydrolysis phosphodiesters, metals, topoisomerase, group transfer, recognition

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FINAL REPORT QUESTIONNAIRE
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Principal Investigator Name: THOMAS C. BRUICE

Institution: UNIVERSITY OF CALIFORNIA, SANTA BARBARA

Project Title: THE DUAL AND SIMULTANEOUS ROLES OF NUCLEOPHILE DELIVERY AND ASSISTANCE TO LEAVING GROUP DEPARTURE BY METAL IONS IN PHOSPHATE TRANSFER FROM PHOSPHATE DIESTERS

Number of ONR supported

Papers published in refereed journals: 2 .(plus 4 accepted & in press and 3 submitted)

Papers or reports in non-refereed publications: 0 .

Books or book chapters published: 0 .

Number of ONR supported patents/inventions

Filed: 0 .

Granted: 0 . Patent name and number: N/A .

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No. Grad. Students:	1			
No. Postdoctorals:	7			5
No. Undergraduates:	0			

AWARDS/HONORS TO PI AND/OR TO MEMBERS OF PI'S RESEARCH GROUP (please describe):

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One of the 5 Most Cited Chemists in the University of California system.

Equipment purchased on grant (number and description of items costing >$1,500):

(1) UVI Optical & Control Unit {Pharmacia LKB Biotech Inc.}
(1) Add-on Memory Board and Memory Module for Power Series {Silicon Graphics}

FINAL REPORT ON CONTRACT
N00014-90-J-4132 R&T CODE 441n048

PRINCIPAL INVESTIGATOR: Thomas C. Bruice

CONTRACTOR: University of California at Santa Barbara, Santa Barbara, CA 93106

CONTRACT TITLE: The Dual and Simultaneous Roles of Nucleophilic Delivery and Assistance of Leaving Group Departure by Metal Ions in Phosphate Transfer from Phosphate Diesters

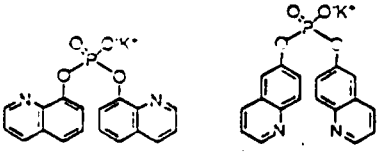
PERIOD OF PERFORMANCE: Sept. 14, 1990, to Sept. 13, 1993

RESEARCH OBJECTIVE: Our objectives are two-fold. The first is to design catalysts for phosphate transfer {hydrolysis} from phosphodiesters. The second is to design molecules which catalyze DNA hydrolysis at specific sequences. Both objectives are directed to a better understanding of the mechanism of phosphodiester hydrolysis and the practical application of the gained knowledge in the study of the chemistry of DNA.

PROGRESS REPORT:

In the 2.5 years of support by the present grant we have accumulated results that constitute five completed manuscripts and have almost completed three others. Our progress will be discussed in terms of these manuscripts. The first paper (A) represents a study of the hydrolysis of our first designed phosphodiester which is set up for competing metal ion catalysis and nucleophilic catalysis by a nitrogen base. Papers B-H describe the synthesis of dien- and tren-microgonotropens and studies of their interactions with DNA.

The pH dependence of the hydrolysis of bis(8-hydroxyquinoline) phosphate and bis(6-hydroxyquinoline) phosphate {**I** and **II**, respectively} was studied in the absence and presence of varying concentrations of Ni^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+}.A

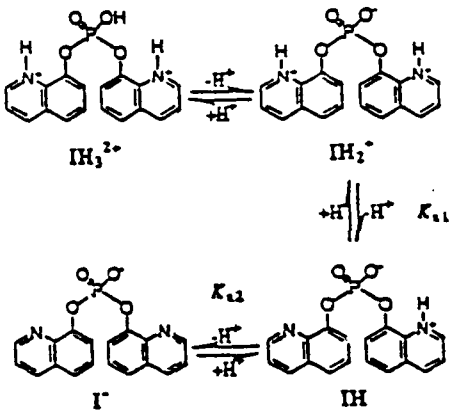


I

II

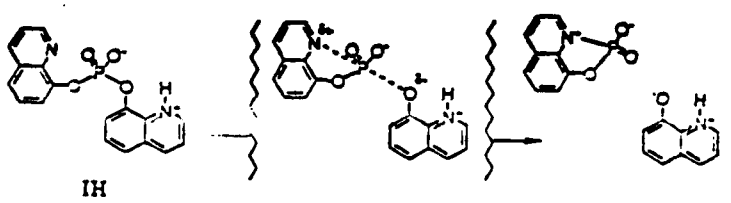
Since both diesters are characterized by having two quinoline nitrogens as basic centers and a negative phosphate, they exist, depending upon pH, as unprotonated, mono-, di-, and tri-protonated species {$\mathbf{I}^-$, $\mathbf{IH}$, $\mathbf{IH_2}^+$, and $\mathbf{IH_3}^{2+}$ for bis(8-hydroxyquinoline) phosphate {Scheme I}; $\mathbf{II}^-$, $\mathbf{IIH}$, $\mathbf{IIH_2}^+$, and $\mathbf{IIH_3}^{2+}$ for bis(6-hydroxy-quinoline) phosphate}. The partial charges and basicity of the hydroxyl groups of 6- and 8-hydroxyquinoline are comparable. The electronic effect on the rate of hydrolysis brought about by protonation or metal ion complexing to the quinoline nitrogens should be the same for both bis(6-hydroxyquinoline) phosphate and bis(8-hydroxyquinoline) phosphate. Electronic effects upon hydrolytic rates can, therefore, be differentiated from catalysis by proximity effects by comparison of the hydrolytic rate constants of

Scheme I



the two diesters. The bis(6-hydroxyquinoline) phosphate hydrolysis is: (i) quite slow (10^{-7} s^{-1}); (ii) pH independent from slightly acidic to neutral pH; and (iii) not catalyzed by the metal ions employed. On the other hand, the hydrolysis of bis(8-hydroxyquinoline) phosphate proceeds rapidly through spontaneous breakdown of **IH**. In accord with the mechanism of Scheme II, the hydrolysis of **IH** is: (i) not

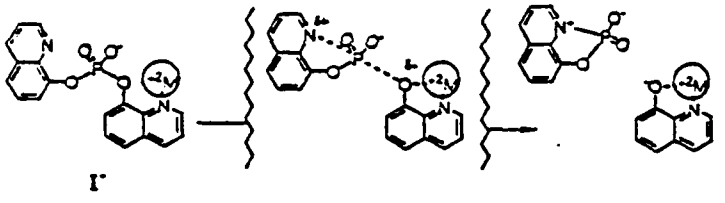
Scheme II



subject to buffer catalysis; (ii) exhibits no deuterium solvent kinetic isotope effect; and (iii) possesses a $\Delta S^{\dagger}$ of 12 e.u. This mechanism provides a rate enhancement over the hydrolysis of the bis(6-hydroxyquinoline) phosphate of 1.1 x 10^3. In the presence of metal ions the hydrolysis of the bis(8-hydroxy-quinoline) phosphate is first order in ester {**I**$^-$ species} and Ni^{2+}, Co^{2+} or Zn^{2+} (Mn^{2+} is not reactive). This mechanism provides, at 1 M in metal ion, rate enhancements over the spontaneous hydrolysis of **IH** of 4.7 x 10^4 for Ni^{2+}, 1.2 x 10^4 for Co^{2+}, and 1.3 x 10^4 for Zn^{2+}. The rate enhancement brought about by the concerted nucleophilic attack of quinoline nitrogen and metal ion assistance to departure of the leaving group is ~ 1 to 5 x 10^7 at 1 M metal ion.

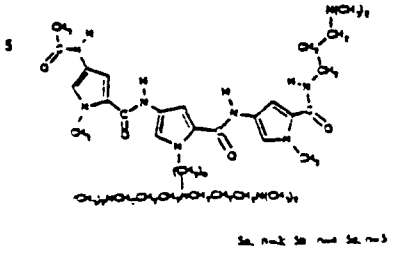
A rate enhancement of 10^7 M can explain most enzymatic reactions; however, the enzymatic hydrolysis of phosphodiesters is different. To explain the kinetics of restriction enzymes one requires effective molarities of 10^{12} M.[1]

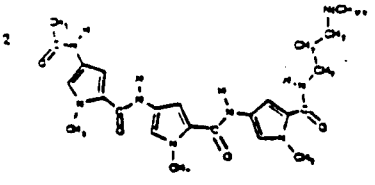
Scheme III



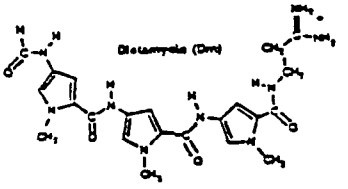
I$^-$

Exploration of the novel idea to employ a pyrrole nitrogen of a tri-pyrrole peptide minor groove binding agent to carry catalytic entities to the phosphates and major groove of DNA has been initiated with the synthesis of dien-microgonotropen-a, -b, and -c {**5a**, **5b** and **5c**}.[B,C] In **5a**, **5b** and **5c** the $-N[CH_2CH_2CH_2N(CH_3)_2]_2$





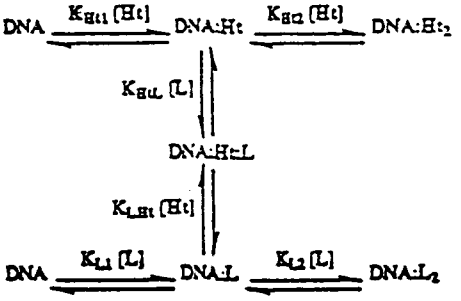
substituent should serve to hold a metal ion adjacent to a single phosphodiester linkage. The metal ions ligated by the $-N[CH_2CH_2CH_2N(CH_3)_2]_2$ moiety also may be bound to the PO^- of the phosphate ester or may act as a carrier of a nucleophilic hydroxyl group. In the absence of metal ions, the three (protonated) amino groups may interact by electrostatic forces and hydrogen bonding with the phosphodiester phosphates and also (as free base) play the role of nucleophile at the phosphate linkages. Replacing the carboxyl terminal amidine and amino terminal formyl functionalities of the natural product distamycin (**Dm**)[2] by $-CH_2N(CH_3)_2$ and acetyl substituents, respectively, provides **2**, which has greater stability in water than does **Dm**. The synthetic design allows the *N*-methyl



substituent on the central pyrrole of **2** to be replaced by connectors terminating in a dien ligand. The binding of **2**, **5a**, **5b** and **5c** to ctDNA, poly(dA-dT), and poly(dI-dC) has been explored. The specificity and affinity of binding of **5a**, **5b** and **5c** to the 5'-[^{32}P] 167 bp *Eco* RI/*Rsa* I restriction fragment of pBR322 was determined by DNase I footprint analysis of both 3'- and 5'-labeled strands. Specific inhibition of cleavage was observed at each of the four potential A+T-rich binding sites after pre-incubation with **5a**, **5b** and **5c** at concentrations as high as 50 μM. Electrophoretic migration of *Hae* III restriction digest fragments of ϕX-174-RF DNA after pre-incubation with **5a**, **5b** and **5c** was used to assess induction of gross conformational changes in DNA molecules. As the concentration of the agents increases, the effect of the agents in changing the conformation of DNA fragments decreases in the order **5c** > **5b** > **5a** >> **Dm** > Hoechst 33258. The dien-microgonotropens are much more effective in inducing changes than the sum of **Dm** and bis[3-(dimethylamino)propyl]methylamine parts. This is due to the unique relationship between the minor groove binding portion of the dien-microgonotropens and the electrostatic attraction of the covalently attached dien moiety to the phosphodiester backbone of DNA.

A quantitative methodology has been introduced to determine equilibrium constants for minor groove binding by dsDNA oligomers.[D] The method is dependent upon the fact that Hoechst 33258 (**Ht**)[3] fluoresces when bound in the minor groove of B-DNA while lexitropsins and dien-microgonotropens do not. Equilibrium constants were determined from competitive binding experiments with **Ht** at 35 °C. Equilibrium constants for the 1:1 and 1:2 complexing of the dsDNA hexadecamer d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) with dien-micro-gonotropen-a, -b and -c {**5a**, **5b**, and **5c**} have been compared to the same constants for the complexing of lexitropsins **2** and distamycin (**Dm**) as well as **Ht** {Scheme IV}. The equilibrium constants of Scheme IV were

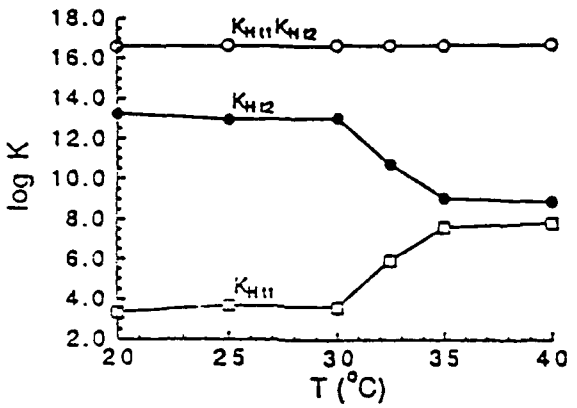
Scheme IV



determined. Anticooperativity for complexing of **2** is marked by K_{L2} being an order of magnitude less than K_{L1}. The first and second bindings of **2** to the hexadecamer are between one and four orders of magnitude weaker than the comparable bindings of **5a**, **5b**, **5c**, **Dm**, or **Ht**. For the latter, all second association constants {K_{Ht2}, K_{HtL}, K_{L2}, and K_{LHt}} are larger than the first association constants by ~ 1 to 3 orders of magnitude, indicating positive cooperativity. Although for **5a**, **5b**, **5c**, **Dm**, or **Ht** the equilibrium constants for stepwise complexation of one and two **L** or **Ht** species varied, the calculated equilibrium constants for formation of DNA:L_2 or DNA:Ht_2 species ($K_{L1}\cdot K_{L2}$ or $K_{Ht1}\cdot K_{Ht2}$) were similar {(1 to 20) x 10^{16} M^{-2}} and 10^4 greater than the comparable constant for **2**. The order of affinity is **5a** ~ **5b** ~ **5c** > **Ht** > **Dm** >> **2**. Replacement of the triamine substituents of **5a**, **5b**, **5c** with a methyl group provides **2**. Thus it can be seen that the triamine substituents contribute substantially to dsDNA complexation of **5a**, **5b**, and **5c**. The use of our methodology for determining sequential equilibrium constants for complexation to DNA is exemplified by the following experiment. The temperature dependence of **Ht** binding to the hexadecamer between 20 and 40 °C shows a critical temperature at ~ 32

°C. Cooperativity for **Ht** binding to hexadecamer duplex is 7 orders of magnitude greater below 30 °C (log K_{Ht1} = 3.9, log K_{Ht2} = 12.8) than above 30 °C (log K_{Ht1} = 7.6, log K_{Ht2} = 9.1) even though log $K_{Ht1}{\cdot}K_{Ht2}$ is unchanged {Figure 1}. These results are attributed to a marked

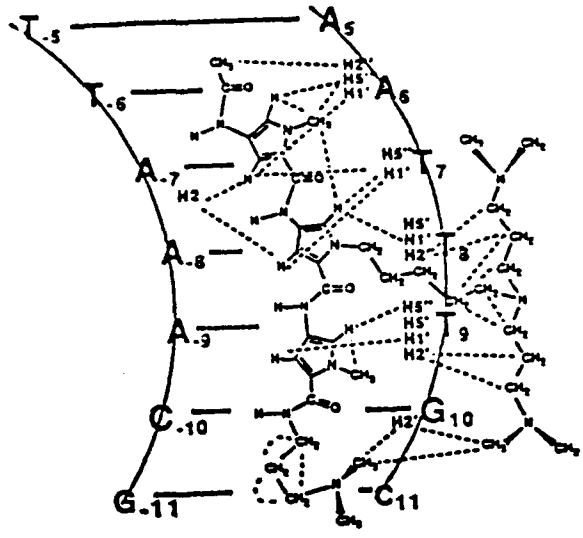
Figure 1



conformational change in the DNA:$\mathbf{Ht}_1$ species.

The solution structures of d(CGCAAATTTGCG)$_2$ and the 1:1 complex of d(CGCAAATTTGCG)$_2$ with dien-microgonotropen-c (**5c**) have been determined by 1D and 2D ^{1}H NMR spectroscopy and restrained molecular modeling using the X-ray structure of d(CGCAAATTTGCG)$_2$ as the point of departure.[E] One hundred and two resonances for the free DNA and 196 for the DNA bound to **5c** were assigned. The 1D (DNA imino protons) and 2D (NOESY) spectra of the 1:1 complex show that there is an asymmetric type of binding in the A+T-rich region involving 5 base pairs (5'-$A_6T_7T_8T_9G_{10}$). The most stable structure of the d(CGCAAATTTGCG)$_2$:**5c** complex has pyrrole rings A and B coplanar and in the minor groove, with pyrrole ring C out of plane by ~ 70° and out of the minor groove. A structure with pyrrole rings A, B coplanar and ring C out of plane by ~ 11° and in the minor groove {as in the structure of the distamycin DNA complex} is far less stable. This is due to the terminal dimethylpropylamino tail having a more favorable electrostatic interaction with phosphate (P11) of the phosphodiester backbone rather than in the minor groove. The energy barrier between the two bound **5c** conformations when removed from the DNA is but 2.5 kcal/mol in favor of the more coplanar structure. The dien polyamino

Figure 2



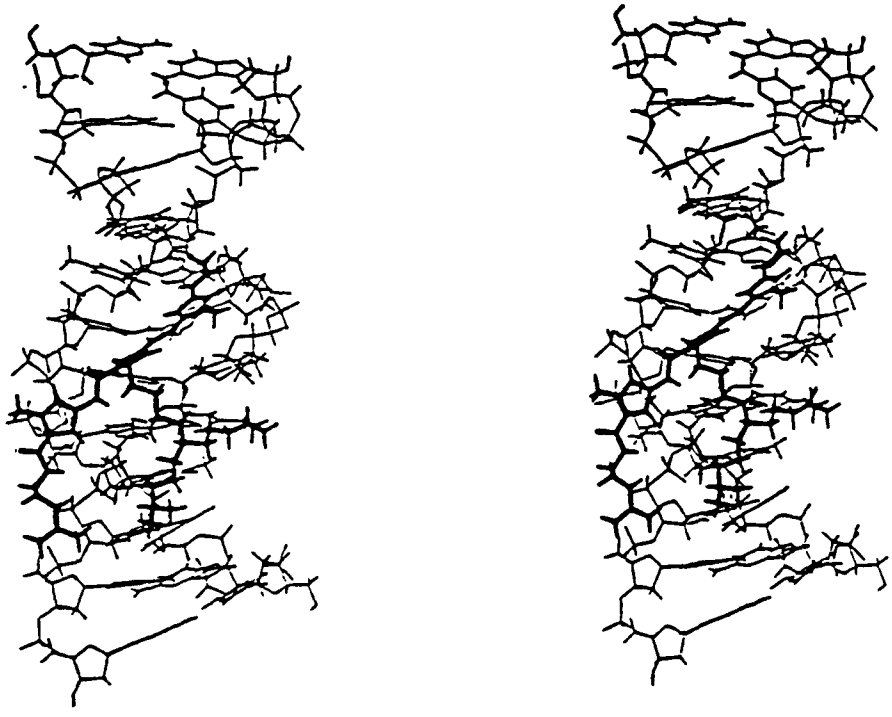
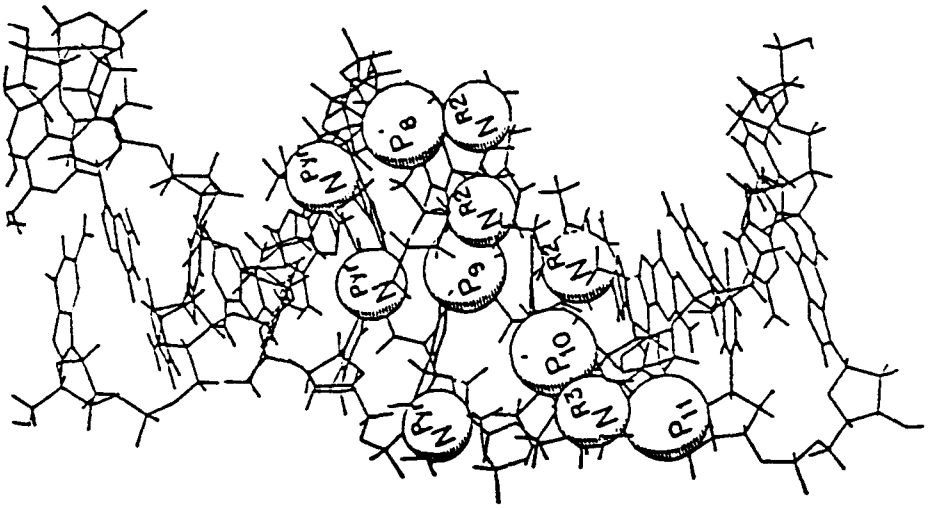


Figure 3

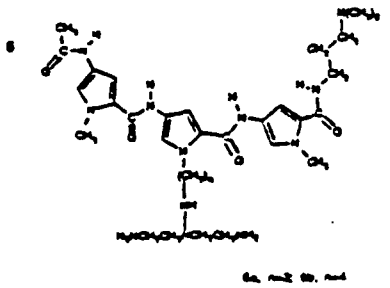
substituent residing on the nitrogen of pyrrole ring C runs above and along the phosphate backbone, towards the major groove. The protonated dien nitrogens reside on the edge of the major groove and pair with the phosphodiester linkages P_8, P_9, and P_{10} {Figure 4}.

Figure 4



The off-rate of **5c** from the 1:1 complex was found to be 1.3 $\pm$ 0.2 s^{-1}, corresponding to an activation energy of 17 kcal/mol. The relative positions of the DNA proton signals change as **5c** binds to the DNA. This is due, in part, to the widening of the minor groove (up to 3 Å) in the binding site. Compound **5c** binds 5-7 Å from the bottom of the groove and 5-6 and 4-6 Å distant from the (-) and (+) strand, respectively. Comparisons with the crystallographic data of the same DNA with and without distamycin were made. Molecular modeling of the free and **5c** bound DNA, based on NOE measurements, show that there is a break in the C_{2v} symmetry of the crystallized DNA as it goes into aqueous solution. A deviation of 1.8° from the two-fold axis of the crystallized DNA was found to occur at the A_6T_7 junction for the solution DNA, while only 0.3° was found for the solution DNA:**5c** complex. Upon solvation, the length of the duplex increases by 0.2 Å/bp for both the dodecamer and the **5c** complexed dodecamer compared to crystal structures of the free DNA and distamycin complexed DNA.

We are within a few weeks of having all the experimental data for this paper.[F] Tren-microgonotropen-a, and -b {which have been synthesized and characterized} differ in structure from the dien-microgonotropen-a, and -b in the replacement of the dien ligand of the latter by a tren ligand. The tren ligand has a much greater affinity for metal ions due to an additional secondary amino group. Thus, the tren-Ni^{2+} binding constant is about 10^{15} M^{-1} while the



dien-Ni^{2+} binding constant is about 10^8 M^{-1}.[4] The footprinting results with tren-microgonotropen-a and -b is much comparable to the results with the dien analogs. Gel shifting is much more marked with the tren analogs, and this is attributable to the more favorable equilibrium constants for association of tren- compared to dien-microgonotropen-a and -b with DNA. Of particular interest is the protection of supercoiled DNA from modification by tropoisomerase-I. Tren-microgonotropen-b at 30 μM provides complete protection to tropoisomerase-I for over 18 hrs.

All 1D and 2D ^1H NMR data has been collected.[G] We are about a month away from writing a paper. All data analysis and computer calculations are not yet complete.

Dien-microgonotropen-c (**5c**), tren-microgonotropen-b (**6b**) and distamycin (**Dm**) bind in the minor groove at the A=T rich region of $d(CGCAAATTTGCG)_2$ (oligo 12) to form 1:1 (with **5c** and **6b**) and 4:1 (with **Dm**) complexes.[H] A 10-20% concentration excess of **5c** and **6b** is a requirement for complete formation of their 1:1 binding complexes with oligo 12. Dien-microgonotropen-c binds 2:1 to an asymmetrical hexadecamer (oligo 16) containing the same A=T binding site as

d(GGCGC**AAATTT**GGCGG)/d(CCGCC**AAATTT**GCGCC)
(oligo 16)

oligo 12. On increase in [**Dm**] to 75% mol excess over oligo 12 there exists in solution a mixture of 4:1 complex, 2:1 complex, and free DNA. Hoechst 33258 displaces the two **5c** molecules bound to oligo 16 by binding simultaneously to the unoccupied A=T region of the opposite strand. Molecular modelling shows that there is a possibility for the A_6T_7 junction to completely lose its hydrogen bonding or to change from Watson-Crick to Hogsteen base pairing.

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