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The binding of dinucleoside phosphonates in the active site of the metallo-enzyme, Staphylococcal nuclease: synthetic, solution and preliminary crystallographic studies ☆

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Abstract

Staphylococcal nuclease is a Ca^{2+}-activated, extracellular phosphodiesterase which degrades DNA or RNA ot 3′-nucleotides. The enzyme is strongly inhibited by deoxythymidine 3′,5′-diphosphate (pdTp). The high resolution crystal structure of the nuclease–pdTp-Ca^{2+} complex has facilitated a proposed mechanism of action. However, attempts to test this mechanism in solution have been frustrated by the lack of non-hydrolyzable dinucleotide analogues. Here we report a novel synthesis route for several isoteric phosphonate analogues of pdTpdT. Binding and kinetic experiments show that the phosphonate derivatives competitively inhibit enzymatic activity while stabilizing the protein's tertiary conformation. A description of the active site as seen in the nuclease–dinucleoside phosphonate (pcdTpcdT)-Ca^{2+} complex at 2.0 Å resolution is presented.

Keywords: Dinucleoside phosphonate; Metallo-enzyme; Staphylococcal nuclease

1. Introduction

Because of the relative simplicity of its structure, the Staphylococcal (micrococcal) nuclease (Ribonucleate (deoxyribonucleate) 3′-nucleotide-hydrolase, EC3.1.4.7) has served as a model system for protein structure and metallo-enzyme function (reviewed in Refs. [1–3]). The enzyme is a Ca^{2+}-activated extracellular phosphodiesterase of 16 807 daltons which degrades both RNA and DNA to 3′-nucleotides. This latter activity has been extensively exploited to probe the nucleosome structure of chromatin (reviewed in Ref. [4]). With synthetic deoxyribonucleotide substrates of the type R–pdT–R [1], hydrolysis results in the release of R-phosphate (cleaving the P–O bond in the 5′ C–O–P ester linkage). But in the absence of an R substitution there is a strong competitive inhibition (e.g. pdTp) [5,6]. From studies of binding affinity versus length of pdT oligonucleotide inhibitors, maximal inhibition was reached with a trinucleotide having a terminal 5′-phosphate [7], suggesting a model (Fig. 1) in which three binding subsites are involved: the two to the 'right' (P2 and P3) lending binding stability and the first on the 'left' (P1) promoting catalysis.

The high resolution structure of the nuclease–pdTp-Ca^{2+} complex [8,9] has shown the geometry of one of the sites, the non-productive binding at P1 (Fig. 1(b)). However, due to the high rate of hydrolysis of phosphodiester bonds on the crystallographic time scale even at low temperature, typical di, tri, and longer natural oligonucleotides are not suitable for mapping of the active site. The susceptible phosphodiester linkage must be rendered stable to cleavage but gross alteration of its stereochemistry must be avoided for this approach to succeed.

The utility of synthetic nucleotide analogues which are resistant to phosphodiesterase attack has been demonstrated in a variety of diverse biological problems. Rammler and co-workers [10] compared the effects of a small series of polynucleotides, derived from 5′-dT-5′-5′-methylene carbon, on the action of the staphy-

☆ This paper is dedicated to Professor F.A. Cotton on the occasion of his 65th birthday.

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[1] R and R′, respectively, represent the 5′ and 3′ moieties of deoxythymidine 5′-monophosphate, pdT. Other abbreviations are dT, deoxythymidine; pdTp, deoxythymidine 3′,5′-diphosphate; dTp, deoxythymidine 3′-monophosphate; UpA, uridylyl-(5′→3′)-adenosylate; dTpdT, deoxythymidylyl-(5′→3′)-thymidylate; nitrophenyl-pdT, deoxythymidine-5′-*p*-nitrophenyl-phosphate.

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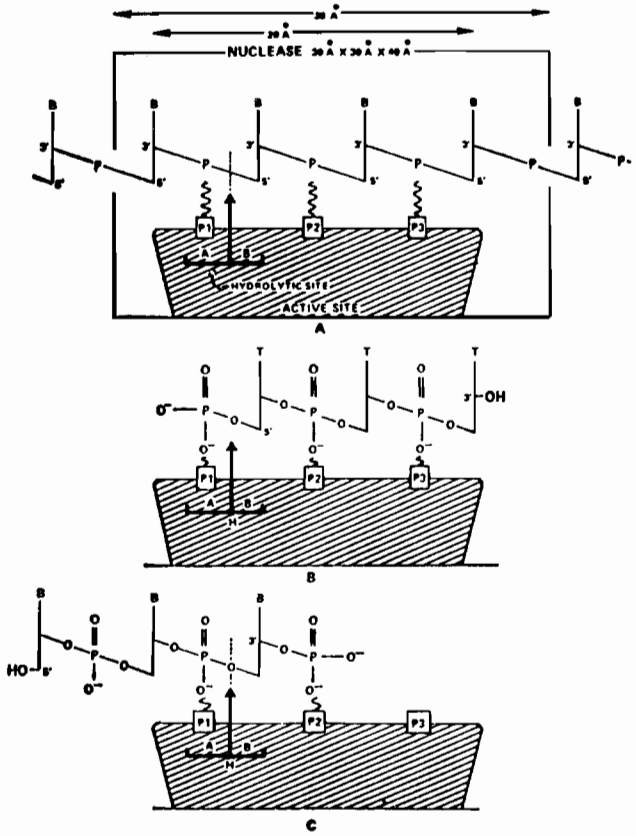


Fig. 1. Hypothetic representation of the nuclease active site. Complex formation between the Staphylococcal nuclease and polynucleotide substrates (a), inhibitory oligonucleotides bearing 5′-phosphoryl termini (b). The major substrate binding regions ('phosphate subsites') are indicated as P1, P2 and P3. The hydrolytic site (H) consists of a region closely related to P1 which recognizes the phosphodiester bond (A) and a region which recognizes the sugar-base moiety whose 5′-OH is linked to the phosphate group (B).

lococcal nuclease. The oligomers were resistant to hydrolysis because they lacked the C–O–P bond at the C-5′ of the analogue nucleotide. A particularly useful derivative, employed by Richards et al. [11,12] in the crystallographic assessment of the binding site in ribonuclease-S, is the isosteric phosphonate analogue UpcA. UpcA is identical to the excellent substrate of ribonuclease, UpA, except that the 5′-oxygen of adenosine has been replaced by a methylene group.

The availability of nucleoside phosphonates has been limited because of the complexities of their synthesis. We have previously described a general route for the preparation of the isosteric phosphonate analogs of mononucleotides via condensation of suitably protected nucleoside 5′-aldehydes with diphenyl triphenylphosphoranylidenemethylphosphonate followed by reduction and cleavage of protecting groups [13]. We subsequently converted these compounds into a variety of phosphonate analogs of biologically interesting phosphodiesters [14].

In this paper we describe the conversion of pcdT (**3b**) [2,3] into pcdTpcdT (**7b**, the isosteric phosphonate analogue of pdTpdT), dTpcdT (**5**) and pcdTpcdTp (**7c**). Data are presented suggesting that these substrate analogues bind tightly in the active site of the staphylococcal nuclease, stabilize its native conformation in the presence of Ca^{2+}, and competitively inhibit its enzymatic activity. We also include preliminary crystallographic interpretations of data obtained from nuclease complexes of two of these analogues.

2. Experimental

2.1. 1-[2,5,6-Trideoxy-6-[di(2-phenylthioethoxy)]phosphinyl-βD-erythro-hexofuranosyl]thymine ((PTE)$_2$pcdT, 2b)

n-Butyllithium (30 ml of a 1.6 M solution in hexane) was added to a stirred solution of **1a** (3.09 g, 6 mmol) and 2-phenylthioethanol (24 ml) in dimethyl sulfoxide (60 ml) at room temperature. After 20 min, aqueous acetic acid (53 ml of 1 M) was added and the mixture was extracted twice with ethyl acetate (300 ml). The extracts were washed with water (3×100 ml) and the organic phase was evaporated. The residue was chromatographed on a column of silic acid (300 g) using elution with chloroform and then chloroform–methanol (9:1). The latter eluate was evaporated and the residue was stirred with ether to remove some 2-phenylthioethanol. The residue was crystallized from chloroform–hexane to give 1.85 g (52%) of **2b** with m.p. 93–95 °C. λ_{max} (MeOH) 254 nm (ϵ 19 200). $[\alpha]_D^{23}$ 10.8° (c 0.13, MeOH). ORD (MeOH) $[\Phi]_{287}^{pk}$ 1.700°, $[\Phi]_{273}$ 0°, $[\Phi]_{259}^{tr}$ −4200°. ^{1}H NMR ($CDCl_3$, ppm): 1.87 (br s, 3, CH_3), 3.11 (t, 2, J=7 Hz, SCH_2), 4.13 (dt, 2, J(H, H)=7 Hz, J(H, P)=8.5 Hz, OCH_2P), 6.18 (dd, 1, $J_{1',2'}$=5, 6.5 Hz, C_1, H).

Anal. Calc. for $C_{27}H_{33}N_2O_7PS_2$ (592.66): C, 54.71; H, 5.61; N, 4.73. Found: C, 54.64; H, 5.55; N, 4.54%.

[2] The conversion of 3′-*O*-acetylthymidine 5′-aldehyde to $(Ph)_2$pcdTOAc (**1a**) was achieved essentially as described for related uridine and adenosine derivatives [13]. Subsequent platinum catalyzed hydrogenolysis then gave pcdTOAc. These procedures will be described in detail elsewhere.

[3] The isosteric phosphonate analogues are abbreviated using the normal nucleotide convention with the addition of a 'c' to indicate the location of the phosphate methylene group that replaces an ester oxygen in conventional nucleotides. Thus pcdT describes structure **3b** and is correctly named 1-[2,5,6-trideoxy-6-(dihydroxyphosphinyl)-βD-erythro-hexofuranosyl]thymine. The symbol PTE refers to the 2-phenyl-thioethyl esters of phosphates or phosphonates. Thus $(PTE)_2$pcdT is the bis(2-phenylthioethyl) ester of pcdT (**2b**).

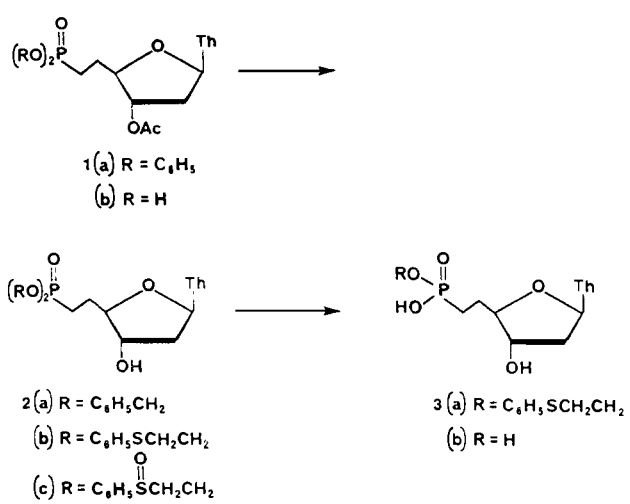
2.2. 1-[2,5,6-Trideoxy-6-(2-phenylthioethoxyhydroxyphosphinyl)-β-D-erythro-hexofuranosyl]thymine (PTEpcdT, 3a)

A solution of $(PTE)_2$pcdT (**2b**, 300 mg, 0.5 mmol) in a mixture of dioxane (10 ml) and 1 N sodium hydroxide (10 ml) was stored at room temperature for 24 h and then neutralized with Dowex 50 (H^+) resin. The filtered solution was applied to a 1.8×15 cm column of DEAE Sephadex (HCO_3^-), and after a thorough water wash, elution was effected with 0.1 M triethylammonium bicarbonate. The major peak was evaporated and the residue passed through a 1.8×7 cm column of Dowex 50 (H^+) resin. Evaporation of the eluates and precipitation of the residue from methanol by addition of ether gave 170 mg (75%) of **3a** as the chromatographically and electrophoretically homogeneous free acid. λ_{max} (MeOH) 256 nm (ϵ 13 400). $[\alpha]_D^{23}$ 16.8° (c 0.4, MeOH). ORD (MeOH) $[\Phi]_{288}^{pk}$ 2400°, $[\Phi]_{274}$ 0°, $[\Phi]_{258}^{tr}$ −4300°.

Anal. Calc. for $C_{19}H_{25}N_2O_7SP$ (456.46): C, 49.99; H, 5.52; N, 6.14; P, 6.79. Found: C, 50.18; H, 5.48; N, 6.05; P. 6.61%.

2.3. 1-[2,5,6-Trideoxy-6-[di-(2-phenylsulfinylethoxy)]phosphinyl-β-D-erythro-hexofuranosyl]thymine (2c)

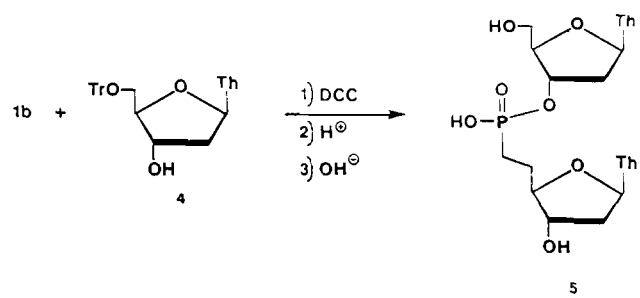
A solution of sodium periodate (171 mg, 0.8 mmol) in water (8 ml) was added to a solution of **2b** (120 mg, 0.2 mmol) in ethanol (8 ml). After 2 h at 40 °C the reaction was complete as judged by TLC and the solvents were evaporated. The residue was partitioned between chloroform and water and the dried organic phase was evaporated leaving 130 mg (98%) of TLC homogeneous **2c** as an amorphous dihydrate. λ_{max} (MeOH) 247 nm (ϵ 11 100), 261 (11 100). $[\alpha]_D^{23}$ 11.3° (c 1.0, $CHCl_3$). ORD (MeOH) $[\Phi]_{289}^{pk}$ 2300°, $[\Phi]_{278}$ 0°, $[\Phi]_{264}^{tr}$ −3300°. ^{1}H NMR ($CDCl_3$, ppm): 7.15 (br s, 1, C_6H), 6.20 (dd, 1, $J_{1',2'}$=6 Hz, C_1-H), 3.12 (m, 4, $ArSOCH_2$).



Anal. Calc. for $C_{27}H_{33}N_2O_9PS_2 \cdot 2H_2O$ (660.69): C, 49.08; H, 5.65; N, 4.24. Found: C, 49.10; H, 5.64; N, 4.11%.

2.4. Preparation of dTpcdT (5)

A solution of pcdTOAc (from 0.1 mmol of sodium salt) and 5′-*O*-tritylthymidine (48 mg, 0.1 mmol) in pyridine (10 ml) was evaporated to dryness and rendered anhydrous by two evaporations with pyridine. The final residue was dissolved in pyridine (1 ml) and dicyclohexylcarbodiimide (103 mg, 0.5 mmol) was added. After 20 h at room temperature, water (3 ml) was added and after 4 h the mixture was evaporated to dryness. The residue was extracted twice with ether and then extracted with 90% ethanol. The extracts were filtered and evaporated leaving a residue that was treated with 80% acetic acid at 100 °C for 20 min. Following evaporation of the solution and co-evaporation of the residue with methanol, the residue was treated for 3 h at room temperature with conc. ammonium hydroxide and evaporated to dryness. A filtered aqueous solution of the residue was applied to a 1.7×40 cm column of DEAE Sephadex (HCO_3^-) and eluted with a linear gradient of 0.005–0.1 M triethylammonium bicarbonate (2 l) giving one major and three minor peaks. The major peak, which contained 971 OD units at 267 nm (51% yield) of dTpcdT, was evaporated to dryness and co-evaporated several times with methanol. An aqueous solution of the resulting triethylammonium salt of dTpcdT was stable upon storage at 0 °C and could be converted to either the free acid or sodium salt by ion exchange techniques. It exhibited a single monoanionic spot upon paper electrophoresis at pH 7.6 and a single spot with R_f 0.49 on paper chromatography using n-propanol–conc. ammonium hydroxide–water (6:3:1). Upon incubation with crude *Crotalus adamanteus* venom at pH 8 for 2 h it was quantitatively cleaved giving thymidine and pcdT in a ratio of 1:1.07 as measured by paper chromatography.



2.5. Preparation of PTEpcdTpcdT (7a)

An aqueous solution of the sodium salt of pcdTOAc (**1b**, 406 mg, 1 mmol) was passed through a 1.6×13

cm column of Dowex 50 (pyridinium) resin and the column was washed with four bed volumes of pyridine–water (1:9). After addition of pyridine (100 ml) the solution was evaporated to dryness (bath below 35 °C). Following addition of $(PTE)_2$pcdT (**2b**, 593 mg, 1 mmol) the mixture was evaporated three times with 10 ml portions of anhydrous pyridine. Dicyclohexylcarbodiimide (1.03 g, 5 mmol) was added to a solution of the residue in pyridine (5 ml) and then stored at room temperature for 64 h. Further portions of $(PTE)_2$pcdT (300 mg, 0.5 mmol) and dicyclohexylcarbodiimide (500 mg, 2.5 mmol) were then added and after 32 h the mixture was quenched with water (5 ml) and stored overnight. Pyridine (10 ml) and 1 N sodium hydroxide (25 ml) were added and the mixture was stirred at room temperature for 2 days before being adjusted to pH 7–8 by addition of Dowex 50 (H^+) resin. The mixture was filtered and the residue was well washed with water. The filtrates (~500 ml) were absorbed on a 3×40 cm column of DEAE Sephadex (HCO_3^-) and the column was thoroughly washed with water. Elution was effected with a linear gradient of 0.005 to 0.25 M triethylammonium bicarbonate (6 l) giving two well separated peaks. The first peak contained 7000 OD units (265 nm) of PTEpcdT identical to that above, while the second peak contained 18 200 OD units (265 nm) of the desired product. This peak was evaporated to dryness and co-evaporated several times with methanol. An aqueous solution of the final residue was passed through a 1×6 cm column of Dowex 50 (H^+) resin and the effluent was adjusted to pH 3.6 with sodium hydroxide and evaporated to dryness. A solution of the dry residue in methanol (25 ml) was added to ether (500 ml) with stirring and the precipitate was collected, washed with ether and dried in vacuo giving 626 mg (72%) of the disodium salt of PTEpcdTpcdT (**7a**) as the tetrahydrate. λ_{max} (MeOH) 261 nm (ϵ 20 400).

Anal. Calc. for $C_{30}H_{38}N_4O_{13}P_2SNa_2 \cdot 4H_2O$ (874.70): C, 41.19; H, 5.31; N, 6.41. Found: C, 41.45; H, 4.76; N, 6.73%.

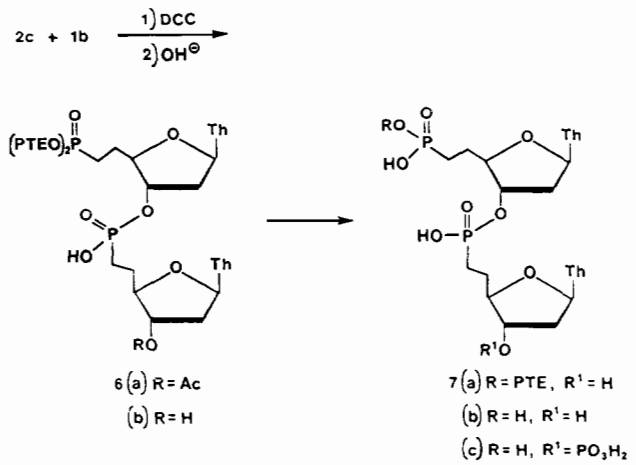
2.6. Hydrolysis of PTEpcdTpcdT to pcdTpcdT (7b)

A solution of PTEpcdTpcdT (**7a**, 430 mg, 0.49 mmol) in aqueous 0.1 N sodium periodate (10 ml) was stored at 40 °C for 2.5 h and then evaporated to dryness. The residue was dissolved in 1 N sodium hydroxide (10 ml) and stored at room temperature for 3 h. The solution was adjusted to pH 8 with 1 N hydrochloric acid, diluted to 300 ml, and applied to a 2.2×48 cm column of DEAE Sephadex (HCO_3^-). The column was eluted with a linear gradient of 0.1 to 0.3 M triethylammonium bicarbonate (4 l) giving a small peak (1400 OD units at 264 nm) of what is believed to be the unhydrolyzed sulfoxide of PTEpcdTpcdT followed by a major peak containing 8200 OD units (267 nm) of pcdTpcdT. The latter peak was evaporated, and after several co-evaporations with methanol a solution of the residue was passed through a 1×9 cm column of Dowex 50 (H^+) resin. The effluent was adjusted to pH 7 with sodium hydroxide and evaporated to dryness. A solution of the dried residue in methanol (10 ml) was added with stirring to ether (200 ml). The resulting precipitate was collected, washed with ether and dried in vacuo giving 300 mg (83%) of the trisodium salt of pcdTpcdT as the trihydrate. λ_{max} (0.01 N HCl) 266 nm (ϵ 18 200). $[\alpha]_D^{23}$ 0.6° (c 0.7, H_2O). ORD (H_2O) $[\Phi]_{295}^{pk}$ 730°, $[\Phi]_{282}$ 0°, $[\Phi]_{265}^{tr}$ −1400°, $[\Phi]_{247}$ 0°.

2.7. Preparation of pcdTpcdTp (7c)

A solution of the free acid form of PTEpcdTpcdT (**7b**, 0.17 mmol) and mono-2-cyanoethyl phosphate (from 1 mmol of the barium salt) in aqueous pyridine was evaporated to dryness and the residue was co-evaporated three times with anhydrous pyridine (10 ml). Dicyclohexylcarbodiimide (1.0 g, 4.8 mmol) was added to a solution of the final residue in pyridine (3 ml) and the mixture was stored at room temperature for 2 days. After addition of water (1 ml) the mixture was filtered and the residue washed with aqueous pyridine. The filtrates were evaporated, treated with 0.1 N sodium hydroxide at 100 °C for 1 h, passed through a column of Dowex 50 (H^+) resin, neutralized with ammonia and evaporated to dryness. The residue was treated overnight with 10 ml of 0.1 M sodium periodate, evaporated, and then reacted for 3 h at room temperature with 5 ml of 1 N sodium hydroxide. The solution was neutralized with Dowex 50 (H^+) resin and applied to a 1.8×37 cm column of DEAE Sephadex (HCO_3^-) which was eluted with a linear gradient of water to 0.5 M triethylammonium bicarbonate (4 l) giving one major and two minor peaks. The major peak contained 1350 OD units at 267 nm (54%) of product containing a single UV-absorbing material by paper chromatography (R_f 0.05 in n-propanol–conc. ammonia–water, 6:3:1). It was, however, contaminated with a trace of a non-nucleotidic, phosphate-containing material that remained on the origin. Accordingly, a portion was converted to the free acid with Dowex 50 (H^+) resin and adsorbed on 1.3 g of Shirasagi charcoal (Takeda Chemical Industries, Osaka, Japan). Following careful washing with water, the product was eluted with 50% ethanol containing 5% conc. ammonium hydroxide. The eluate was evaporated and chromatographed on DEAE Sephadex (HCO_3^-) as above. The single peak was evaporated to dryness and the residue repeatedly co-evaporated with methanol. The final residue was passed through a 1×5 cm column of Dowex 50 (H^+) resin and the eluate was adjusted to pH 7.5 with sodium

hydroxide and evaporated. A solution of the residue in water (0.5 ml) was precipitated by addition of methanol (4 ml) and ether (10 ml) giving 40 mg of the sodium salt of chromatographically homogeneous pcdTpcdTp. The ratio of total phosphorus:thymine was 1.54:1.00 assuming $\epsilon = 18\,400$ as shown for pdTpdT [15]. Treatment of this material with bacterial alkaline phosphatase led to complete conversion to pcdTpcdT as shown by paper chromatography using n-propanol–conc. ammonium hydroxide–water (6:3:1).



2.8. Nuclease hydrolyses

Staphylococcal nuclease was purified as previously described [16]. Each of the three dinucleoside phosphonates (**7b**, **5** and **7c**) was dissolved at a concentration of $\sim 1\times10^{-5}$ M in 1.0 ml of 0.05 M Tris–HCl buffer, pH 8.8, containing 0.01 M $CaCl_2$ and incubated with an excess (0.15 mg/ml) of nuclease, $E_{1\%}$ 1 cm, 180 nm = 9.70 [17], at 30 °C. Aliquots (about 15 OD units) were removed at fixed-time intervals and examined by electrophoresis, as described above, for possible hydrolysis products. Similar degradation experiments were carried out in the presence of 40% (wt./wt.) 2-methyl-2,4-pentanediol (MPD) to assess potential hydrolysis under the crystal growing conditions (see below). All mobilities were compared to those obtained for the appropriate controls.

2.9. Kinetic measurements with nitrophenyl-pdT

The spectrophotometric assay used was essentially the same as that described by Cuatrecasas et al. [18]. The nuclease catalyzed formations of *p*-nitrophenyl phosphate can be quantitated from the change in absorbancy at 340 nm (ΔA_{340}), knowing the difference in the molar extinction coefficients of the ionized and the unionized forms of *p*-nitrophenyl phosphate. Since the *Em* has been reported as 3580 [18], the rate of change per min can be calculated by:

$$v/\text{min} = \Delta C/\text{min} = \frac{\Delta A_{340} \times \text{S.F.}}{3580}$$

where v = initial velocity, C = concentration in moles, S.F. = an appropriate instrument scale factor.

Several preliminary kinetic experiments were carried out. The pH was monitored for 30 min after the addition of 5–25 μl samples of nuclease (0.15 mg/ml) to 1.0 ml nitrophenyl-pdTp in 0.05 M Tris–HCl buffer, pH 8.8. Also the change in absorbance at 340 nm was followed for at least 10 min in the absence and in the presence of dinucleoside phosphonate inhibitors (1×10^{-5} M) on a Unicam SP 1800 recording spectrophotometer, equipped with an automatic cell changer. The cell temperature was maintained at 25 °C to prevent non-enzymatic substrate hydrolysis.

The inhibition of nuclease activity by the dinucleoside phosphates was then examined by steady state kinetic analysis. The Michaelis constant, K_{mapp}, and the catalytic rate constant, K_{cat}, for the hydrolysis of nitrophenyl-pdT were obtained from a Lineweaver–Burke plot [19]. The reaction mixture consisted of 0.9 ml of 0.05 M Tris–HCl buffer, pH 8.8, containing 0.01 M $CaCl_2$ and 0.01 ml of the substrate at concentrations varying from 0.1 to 2.0 mM. The reaction was initiated with the addition of 10 μl of nuclease (0.15 mg/ml). Dissociation constants (K_i) were determined from Dixon plots [20] at two substrate concentrations (0.1 and 0.2 mM) and four inhibitor concentrations under otherwise identical reaction conditions as above. The graphically interpreted data, fitted by the method of least-squares, were determined with an estimated accuracy of ±10%.

2.10. Binding studies by gel filtration

Direct measurements of dissociation constants were made in order to provide an independent, non-kinetic estimate of the nuclease dinucleoside phosphonate interaction under the conditions of optimal enzymatic activity. We chose the gel permeation technique [21] since it has been shown to be faster, more reproducible, and less susceptible to many of the problems incurred in dialysis equilibrium [22]. The dissociation constant is calculated from the equation

$$K_i = \frac{[P][I]}{[PI]}$$

where [P] and [I] represent the concentration of protein and inhibitor. The area of the trough corresponds to bound inhibitor and the area of the peak corresponds to (a) bound inhibitor, (b) bound protein, calculated by multiplying the area of bound inhibitor (trough) by the ratio of the *Em* values of protein to inhibitor, and (c) free protein, equivalent to the total area minus the sum of a and b. Since the base-line absorbance equals the free inhibitor concentration, then

$$K_i = [\text{free I}] \times \frac{\text{area free P}}{\text{area bound P}}$$

By varying the inhibitor concentration, the protein:nucleoside molar binding ratio can also be determined.

A 1.6×40 cm (Pharmacia, type K16) column thermojacketed at 25 °C was packed with about 20 g of Sephadex G-25 (fine grade) and equilibrated in 0.05 M Tris–HCl buffer, pH 8.8, plus 0.01 M $CaCl_2$ containing the following concentrations of the dinucleoside phosphonates: dTpcdT, 8.45×10^{-4} M; or pcdTpcdT, 2.11×10^{-4} M; or pcdTpcdTp, 5.70×10^{-5} M. The nuclease sample (1 mg) was dissolved in about 0.5 ml of the above solution, containing the appropriate inhibitor at the same concentration as that in the column, and applied in a descending fashion. The elution profile at 273 nm was determined by graphical plotting of the absorbance of the collected fractions versus elution volume. The selection of 273 nm to monitor the binding reaction was based on the requirement of additivity [21] of the nucleoside and enzyme absorbances. The areas of the peak and trough were calculated by two methods. In the first, a summation was carried out for all fractions (within the trough and peak regions, respectively), measuring the difference between the absorbance of the fraction and that of the baseline absorbance. In the second, the graphically plotted areas for the peak and trough were individually cut-out and accurately weighed on a Mettler balance. The peak to trough area ratios obtained from these two different techniques differed by less than 8%.

2.11. Tryptic digestion in the presence of the dinucleoside phosphonates and Ca^{2+}

The effect of ligand binding in the presence of Ca^{2+} in protecting against tryptic inactivation of nuclease [23] was studied for each of the phosphonate analogues. A digestion mixture containing, in 2.0 ml of 0.05 M Tris–HCl buffer, pH 8.0, 170 μg of nuclease, 10 μg of trypsin, 1.0×10^{-1} μM of dinucleoside phosphonate (a slight molar excess of nuclease), and 0.01 M $CaCl_2$ was incubated at 25 °C. Timed aliquots (0.1 ml each) were removed from the mixture and immediately frozen in liquid nitrogen to stop proteolysis. DNAse assays [5] were performed in duplicate on the appropriately diluted aliquots. Control digestions were carried out with nuclease in the presence of no ligands and in the presence of pdTp and Ca^{2+}.

2.12. Crystal growing

Crystallization of the nuclease–dinucleoside phosphonate-Ca^{2+} complex was performed according to the methods of Cotton and co-workers [24,25] used for the crystallization of the native (Type I) and pdTp inhibited (Type II) nuclease. In initial trials, nuclease was dissolved at a concentration of about 1.7 mg/ml in a solution containing 0.015 M potassium phosphate, pH 8.60, at 4 °C. Redistilled MPD was added dropwise to the solution at 4 °C to final concentrations ranging from 15 to 35% (wt./wt.). To this solution, 25 μl of an aqueous solution of the appropriate dinucleoside phosphonate at 2.5×10^{-5} mM and 5 μl of a solution of 0.01 M $CaCl_2$ in 0.02 M potassium citrate buffer were added. A nuclease:inhibitor:Ca^{2+} molar ratio of 1:1:2 was thereby achieved. Small crystals of the nuclease–pcdTpcdT-Ca^{2+} (Type III) and nuclease–pcdTpcdTp-Ca^{2+} (Type IV) complexes grew from the solutions after about a month, at MPD concentrations of approximately 28 and 25%, respectively. No crystals of dTpcdT were obtained. Having determined the general requirements for crystallization, methods were employed to optimize the size and morphology of crystals. About 0.5 ml of the nuclease–inhibitor-Ca^{2+} solutions, having an MPD concentration 1/2 to 1/3 of that to be reached at equilibrium, were placed in a cut-down one dram vial. To another vial, the same volume of buffer alone, containing 1/2 to 1/3 higher concentration of MPD than the equilibrium value, was added. These vials were then sealed inside a clear plastic box which could be monitored under a microscope without disturbing the incubation. Within 6 weeks, Type III crystals had grown to usable dimensions, about 0.7×0.5×0.3 mm, at a final MPD concentration of 29.9%. Type IV crystals reached a maximum size in about 10 weeks of 0.5×0.4×0.3 mm at 25.2% MPD.

Before being used for data collection, the crystals were soaked for several days in 0.032 M Tris–HCl buffer pH 8.20, containing 40% (wt./wt.) MPD. This procedure is standard for all nuclease crystal types because it empirically yields better reproducibility in unit cell constants.

2.13. X-ray data collection

All data collection procedures were carried out in a cold room at 4 °C on a computer controlled Syntex P1 diffractometer equipped with a graphite monochromator mounted in the parallel mode as previously described [9].

The cell constants for the crystals of the nuclease–pcdTpcdT-Ca^{2+} complex (Type III) were $a = 48.20, b = 48.19$ and $c = 63.19$ Å. Of the approximately 24 000 data in the 1.5 Å sphere, 22 905 were observed above background and were collected with about 276 h of total radiation exposure. The overall change in cell volume was 9.46%. For the subsequent reduction procedures, the observed reflections in the 2.0 Å sphere (9986) were used.

The nuclease–pcdTpcdTp-Ca^{2+} crystals (Type IV) ($a=48.03$, $b=47.96$, $c=64.22$ Å) gave very weak diffraction and were not amenable to even low resolution data collection.

2.14. X-ray data reduction and phasing

The recorded intensities were corrected for degradation, absorption, backgrounds, and Lorentz–polarization as previously described [9,24,25]. The Type III data were then level scaled to that of the 1.5 Å Type II native set [9, M. Brice et al., unpublished] by adjusting the value of ΣF^2. Using the Type II native phases refined by the real space method of Collins [26], an electron density map was calculated and prospectives of the map were contoured in appropriate sections. Maps of the active site region were prepared in sections perpendicular to the z-axis. The spacings between sections were ~0.5 Å. Difference maps of the type ($F_{\text{Type III}} - F_{\text{Type II}}$) were computed and plotted in a similar fashion.

3. Results

3.1. Syntheses of dinucleoside phosphonates

The synthesis of pcdTpcdT (**7b**) was based upon the condensation of the phosphonate moiety of pcdTOAc (**1b**) with the 3′-hydroxyl group of a suitable diester of the phosphonic acid of pcdT. A suitable compound of the latter type was prepared by transesterification of $(Ph)_2$pcdTOAc (**1a**) with an excess of the lithium salt of 2-phenylthioethanol in dimethyl sulfoxide which readily gave crystalline $(PTE)_2$pcdT (**2b**) in 52% yield. The monophenylthioethyl group has previously been used as a phosphonate blocking group during oligonucleotide synthesis via the diester approach [27,28].

Removal of the phenylthioethyl (PTE) groups could be achieved in several ways. Direct alkaline hydrolysis of $(PTE)_2$pcdT (**2b**) with 0.5 N sodium hydroxide in aqueous dioxane led, as expected, to selective hydrolysis of one ester group giving PTEpcdT (**3a**) which was isolated in analytically pure form by ion exchange chromatography in 75% yield. Alternatively, direct oxidation of the sulfur function of **2b** to the bis sulfoxide **2c** could be achieved by treatment with an excess of sodium periodate in aqueous ethanol. Since the product was isolated as a dihydrate, it was not possible to directly assign the sulfoxide structure rather than the related sulfone by combustion analysis. The ^{1}H NMR spectrum was also not diagnostic, the $SOCH_2$ groups appearing as a 4-proton multiplet at 3.12 ppm. General broadening of the signals for other discrete protons was, however, apparent and suggested the presence of diastereomeric sulfoxide groups. Further confirmation came from the UV spectrum, which showed maxima at 247 and 261 nm, the first peak indicating the contribution of the phenylsulfinyl groups (λ_{max} 250; ϵ 4100) as opposed to phenylsulfones (λ_{max} 266, 273; ϵ 800) [29]. Treatment of the bis sulfoxide with 1 N sodium hydroxide at room temperature led to very rapid cleavage of one ester group in less than 1 min as shown by TLC and paper electrophoresis. Elimination of the second group was considerably slower, the half-time being approximately 40 min. The product of this reaction was pcdT (**3b**).

As a model for the preparation of the dinucleotide analogues, pcdTOAc (**1b**) was condensed with 5′-*O*-trithylthymidine (**4**) in the presence of dicyclohexylcarbodiimide. Without purification the crude product was treated sequentially with 80% acetic acid and conc. ammonium hydroxide in order to remove the trityl and acetyl protecting groups. Ion exchange chromatography then permitted the isolation of dTpcdT (**5**) in an overall yield of 51%. This chromatographically and electrophoretically homogeneous product has mobilities very similar to those of authentic dTpdT [30]. It was, as expected, quantitatively degraded to equimolar amounts of thymidine and pcP upon digestion with crude *Crotalus adamanteus* venom at pH 8.

A direct extension to the synthesis of the dinucleotide analogue pcdTpcdT (**7b**) involved the dicyclohexylcarbodiimide catalyzed condensation of $(PTE)_2$pcdT (**2b**) with pcdTOAc (**1b**). Without purification the resulting $(PTE)_2$pcTpcTOAc was treated with sodium hydroxide in aqueous pyridine in order to hydrolyze one PTE group and the acetyl function. The resulting PTEpcdTpcdT (**7a**) was isolated in an overall yield of 72% by ion exchange chromatography as its sodium salt. As would be expected, **7a** showed λ_{max} at 261 nm due to a superimposition of the maxima of the thymidine (λ_{max} 267 nm) and phenylthioalkyl (λ_{max} 255 nm) absorptions. Oxidation of **7a** to the related sulfoxide was readily achieved with periodate and subsequent treatment with 1 N sodium hydroxide at room temperature for 3 h led to almost complete elimination of the ester group. Ion exchange chromatography led to the isolation of the sodium salt of pcdTpcdT (**7b**) in a yield of 83% from **7a**. The latter compound showed the expected UV spectrum typical of thymidine (λ_{max} 267 nm) and behaved very similarly to pdTpdT on paper chromatography and paper chromatography and paper electrophoresis. Hydrolysis with crude *Crotalus adamanteus* venom led exclusively to pcdT.

Finally, we undertook the synthesis of pcdTpcdTp (**7c**), the 3′-phosphate ester of **7b**. This was achieved via phosphorylation of PTEpcdTpcdT (**7a**) using the cyanoethyl phosphate-dicyclohexylcarbodiimide method [31]. Following elimination of the resulting cyanoethyl ester by treatment with 0.1 N sodium hydroxide at 100 °C, the PTE group was cleaved by sequential reaction

with sodium periodate and sodium hydroxide as above. The desired **7c** was obtained in 54% yield by ion exchange chromatography but was found to be contaminated with a trace of a non-UV absorbing phosphorus-containing product that remained on the origin on paper chromatograms. This impurity was removed by adsorption of the nucleotide on charcoal followed by elution and ion exchange isolation giving homogeneous pcdTpcdTp (**7c**) as the sodium salt. This compound showed the expected ratio of phosphorus to thymine and was quantitatively degraded to pcdTpcdT by bacterial alkaline phosphatase.

3.2. Hydrolyses and preliminary kinetics

Extended incubations of the dinucleoside phosphonates with molar excess quantities of nuclease produced no detectable hydrolysis. In Table 1, the R_f values as a function of incubation time and incubation condition (with or without MPD) are listed for the three dinucleoside phosphonates in the presence and absence of enzyme. The values for the potential hydrolysis products, based on the known specificity requirements of the nuclease are also included. Migration was as expected for the monoanionic (dTpcdT) and trianionic (pcdTpcdT) species relative to pdTp (4 formal negative charges at pH 7). However, pcdTpcdTp, with 5 negative charges, migrated with the same approximate R_f as pdTp, both in the presence and absence of enzyme. Furthermore, a small additional spot, appearing at the origin ($R_f = 0.01$) in the pcdTpcdTp pattern, was evident under all control and experimental conditions. Nuclease hydrolysis of this species would yield pcdTp ($R_f \simeq 1.0$) and dTp ($R_f \simeq 0.4$), neither present in the migration profile. Since this apparently uncharged component was not detected in the purity examination, it probably was caused by subsequent non-enzymatic breakdown or exogenous contamination.

Preliminary kinetic experiments were conducted to further validate the steady-state studies and to establish suitable concentrations, etc. The pH was found to remain constant for at least 30 min following addition of nuclease. The change in absorbance was linear in the presence or absence of inhibitor (data not shown) up to 10 min after initiation of the reaction with enzyme at the concentrations employed. Thus, no detectable product (or substrate) inhibition had occurred under these conditions.

3.3. Competitive inhibition by dinucleoside phosphonates

The results of the steady-state analyses are summarized in Table 2. The K_{Mapp} of 6.0×10^{-3} M for the nuclease hydrolysis of nitrophenyl-pdT (Fig. 2) is in good agreement with published results [6]. The graphic interpretation of the effects of dTpcdT, pcdTpcT and pcdTpcdTp on nuclease activity at two different nitrophenyl-pdT concentrations are shown in Fig. 3(a)–(c), respectively. A model of competitive binding is supported by the linearity of the data at each nitrophenyl-pdT concentration and the common intercept of both sets of data.

3.4. Binding of dinucleoside phosphonates

The elution profiles of the 273 nm absorbance accompanying the passage of nuclease through an inhibitor-equilibrated G-25 column are given in Fig. 4. The dissociation constants of the three dinucleoside phosphonate–nuclease complexes, compared to those

Table 1
Electrophoretic mobilities at pH 7.5 of the dinucleoside phosphonates following timed incubations with nuclease at 30 °C [a]

Incubation time [b] (h)	Electrophoretic R_F [c]				
	dTpcdT	pcdTpcdT	pcdTpcdTp	pdTp	dTp
None (controls)	0.34	0.55	0.83	1.00	0.48
None + MPD (controls)	0.35	0.51	0.82	1.00	0.45
0.5	0.34	0.57	0.88	1.00	
1.0	0.37	0.56	0.81	1.00	
4.0	0.36	0.56	0.81	1.00	
8.0	0.35	0.55	0.86	1.00	
12.0	0.35	0.57	0.86	1.00	0.39
0.5 + MPD	0.32	0.53	0.81	1.00	
1.0 + MPD	0.32	0.55	0.83	1.00	
8.0 + MPD	0.34	0.53	0.83	1.00	
12.0 + MPD	0.34	0.51	0.87	1.00	0.43

[a] Conditions given in Section 2.
[b] Reactions were stopped at the indicated times by immediate freezing of aliquots in liquid nitrogen following removal from incubation.
[c] Relative to pdTp.

Table 2
Comparison of dissociation constants of the dinucleoside phosphonate–nuclease-Ca^{2+} complexes obtained from kinetic and gel filtration studies [a]

Nucleotide	K_i (m)	
	Kinetics	Gel filtration
dTpcdT	5.5×10^{-5}	4.0×10^{-5}
pcdTpcdT	2.3×10^{-6}	9.1×10^{-7}
pcdTpcdTp	6.5×10^{-7}	8.2×10^{-8}
pdTp	1.6×10^{-7}	

[a] Conditions given in Section 2.

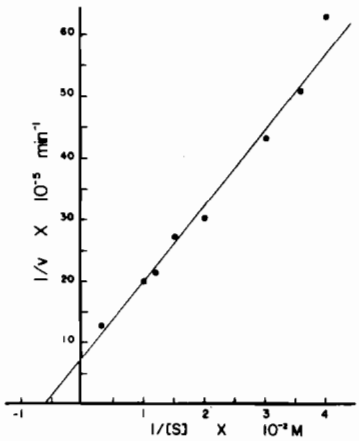


Fig. 2. Lineweaver–Burke plot for the nuclease-catalyzed hydrolysis of nitrophenyl-pdT. Assay conditions are described in Section 2.

derived from kinetic data, are shown in Table 2. The K_i values from both experiments correlate reasonably well for two of the substrate analogues although the estimates from gel filtration suggest stronger affinity. This is especially true in the case of pcdTpcdTp where the difference is close to an order of magnitude. An initial attempt was made to explain this discrepancy in K_i values by pressure effects, inherent in the gel filtration study. For example, expansion of the solvent–small molecule (inhibitor) system as the protein is introduced in the presence of its water of hydration (partial specific volume, $\bar{v}$) will lower the concentration of the inhibitor [32]. Thus, a contribution to the trough not resulting from binding will occur. However, a trial calculation [33] based on a $\bar{v}=0.74$ ml/g for nuclease gave a corrected K_i less than 5% that of the uncorrected value. Although it is probable that these differences are methodological ones, the existence of non-inhibitor, secondary binding sites for phosphonates cannot be discounted under the conditions of the gel filtration experiment. Multiple substrate binding has been observed before in kinetic studies of nuclease hydrolysis of nitrophenyl–pdTp [34].

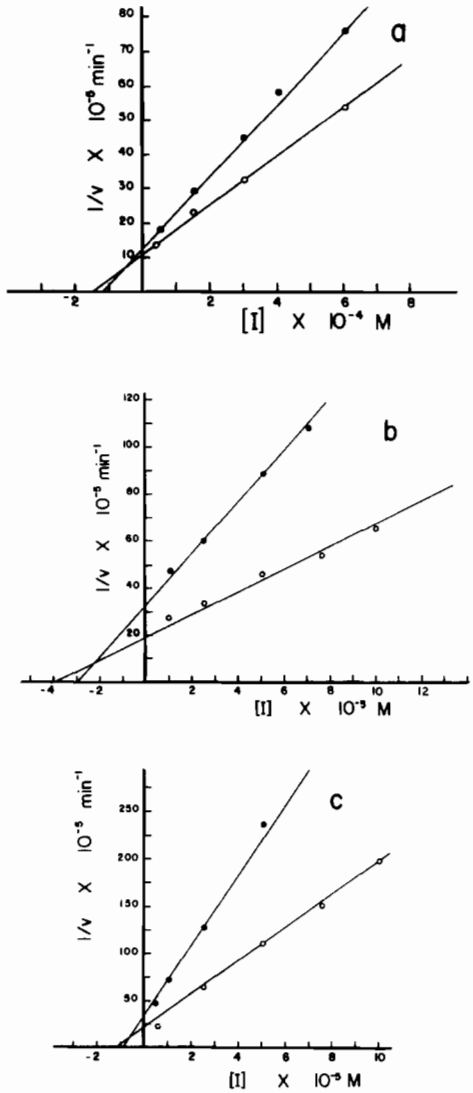


Fig. 3. Kinetic analysis of the inhibition of nuclease by phosphonate analogues. Nuclease (5–15 μl at 0.15 mg/ml) was added to a solution containing 0.025 M Tris–HCl (pH 8.8), 0.01 M $CaCl_2$, 10 μM (●–●) or 20 μM (○–○) nitrophenyl pdT and the amounts of inhibitor indicated. (a) pdTpcdT; (b) pcdTpcdT; (c) pcdTpcdTp.

3.5. Resistance to proteolysis

In the absence of ligands, tryptic digestion rapidly destroyed all enzymatic activity (Table 3). However, the protective effect of the dinucleoside phosphonates and Ca^{2+} was similar to that rendered by pdTp and Ca^{2+}. Activity was more reduced in the presence of dTpcdT than the other two inhibitors, dropping to zero after 6 h incubation.

3.6. Crystallographic studies

The F_{obs} electron density maps constructed (data not shown) bisect the binding site of the nuclease–pcdTpcdT-Ca^{2+} complex at 2.0 Å resolution. On initial

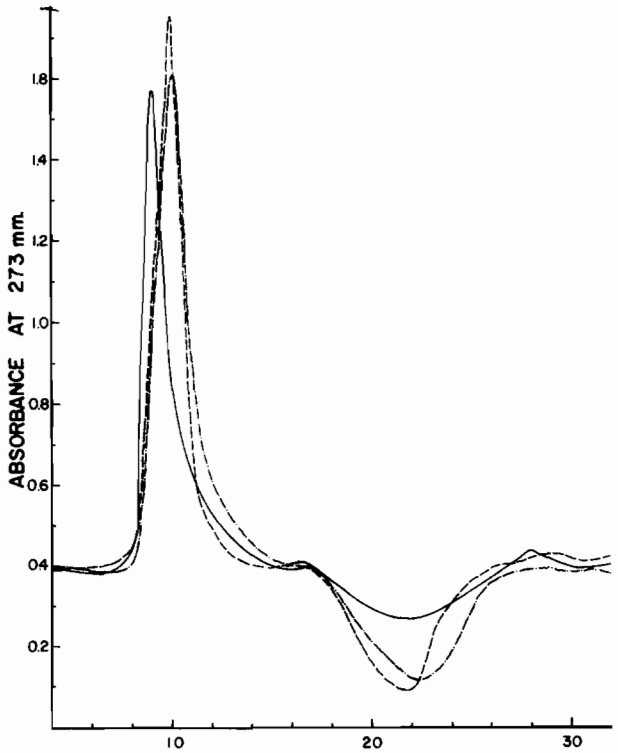


Fig. 4. Gel filtration analysis of the binding of phosphonate analogues to nuclease in the presence of Ca^{2+}. The 273 nm elution profiles are designated as (——) dTpcdT, (–·–) pcdTpcdT and (– – –) pcdTpcdTp. See Section 2 for details.

inspection, these maps appeared indistinguishable from the same sections of the nuclease–pdTp-Ca^{2+} active site. The phosphate positions have moved less than 0.1 Å from those in the Type II map. Likewise, only one nucleoside moiety can be located and its position corresponds almost exactly with that in the pdTp inhibited nuclease crystals. Difference maps between F_{obs} (Type III) and F_{obs} (Type II) were essentially flat in the region of the active site. However, the calculated least-squares R factor of 17% between the shell standard 22 reflection of Types III and IV suggested a considerable difference in electron density between the two types of crystals. We offer three interpretations of these results.

(i) pdTp, present as contamination, has preferentially bound and crystallized with nuclease. This seems very improbable since (a) no traces of this impurity were detected via sensitive chromatographic techniques; (b) pdTp is not a potential hydrolysis product of pcdTpcdT; (c) the binding constant of pdTp (even if pdT is present in undetectable concentration) is lower than that of pcdTpcdT and, thus, pdTp should not preferentially bind; (d) Type III crystals formed at 28% MPD concentration, whereas Type II crystals always form at 24% MPD.

(ii) pcdTp, present as a contaminant or as one of the potential nuclease hydrolysis products of pcdTpcdT, has preferentially bound and crystallized with nuclease. The lack of detectable hydrolysis of pcdTpcdT at more favorable conditions (Table 1) than those employed in the crystallization experiments and the well-documented stability of P–C bonds to non-enzymatic hydrolysis [35–37] renders this explanation questionable. However, even such a slight modification as replacement of a 5′-oxygen atom with a methylene group could account for the observed changes in cell parameters and crystallization conditions for Type III and Type II.

(iii) pcdTpcdT has bound and crystallized with nuclease, but its β-nucleoside moiety cannot be located. This explanation, illustrated in Fig. 5, is the only one that is consistent with all solution data and can be justified crystallographically. The similarity of the Type II and Type III F_{obs} maps demands that the dinucleotide analogue must bind with the 5′-phosphate of pdTp and its 3′→5′ phosphonate ester linkage at the position of the 3′-phosphate of pdTp. Inspection of the 2.8 Å Kendrew model of the nuclease–pdTp-Ca^{2+} complex reveals that a second or β-thymidyl moiety, if bound in the above fashion, would extend into a rather large groove where very few side-chain contacts of hydrogen-bonding interactions are available. Thus, structural elements in this region of low density would probably show a high degree of disorder and would as a result be difficult to locate. Furthermore, the use of Type II refined phases in creating the Type III F_{obs} maps infers a structural 'bias' to the Type III amplitudes, resulting

Table 3
Effect of the presence and absence of the dinucleoside phosphonates and Ca^{2+} on the tryptic [a] digestion of nuclease

	% Activity [b] remaining after				
	30 min	60 min	120 min	180 min	240 min
Nase, Ca^{2+}	0	0	0	0	0
Nase + pdTp, Ca^{2+}	47	17	11	8	12
Nase + dTpcdT, Ca^{2+}	31	12	5	3	0
Nase + pcdTpcdT, Ca^{2+}	40	14	10	10	11
Nase + pcdTpcdTp, Ca^{2+}	45	18	9	7	12

[a] 10% wt./wt. ratio of trypsin to nuclease in the presence (or absence) of one molar equivalent of nucleotide inhibitor.
[b] The average of duplicate assays performed according to Cuatrecasas et al. [23].

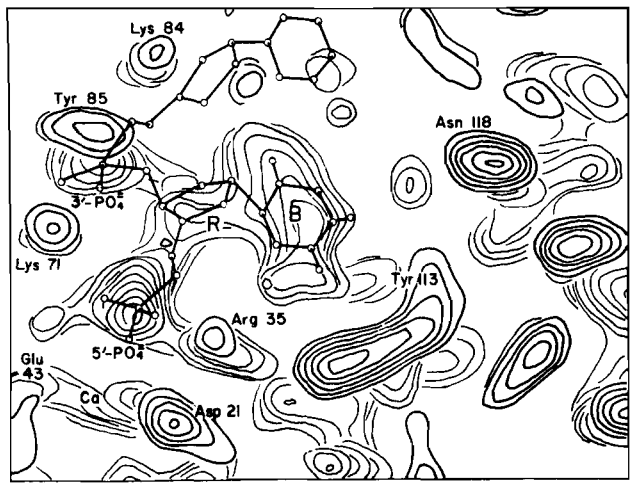


Fig. 5. The proposed position of pcdTpcdT in the nuclease active site. The model of pcdTpcdT is imposed upon the high resolution Type III electron density map, viewed from the side (parallel to the x–y plane).The lowest contour was drawn at 0.4 e $Å^{-3}$ with an increment of 0.25 e $Å^{-3}$ between adjacent contours. Sections with z-coordinates from 0.220 to 0.490 are shown. The symbols R and B refer to the deoxyribose and α-thymine rings, respectively. The location of the β-thymidyl moiety is achieved by the addition of a second nucleoside unit of identical conformation features. Active site residues that interact with the α-moiety are indicated.

in a considerable down-weighting of electron density not present in the Type II structure.

4. Discussion

In this report we have presented a novel synthetic route for three isosteric phosphonate analogues of dinucleotides. We have shown that they bind competitively at the active site of the Staphylococcal nuclease, stabilizing its native, enzymatically active conformation as judged by resistance to tryptic attack in the presence of Ca^{2+}. The X-ray crystallographic data, however, suggest that their mode of binding ala, the 'sub-site' model [6] of Cuatrecasas and co-workers, may be different.

The phosphonate ester, like the native phosphodiester substrate, is monoanionic but differs significantly from the oxygen allostere in charge distribution. When a phosphate is surrounded by four oxygens, the charge is distributed, to a first approximation, symmetrically and, to a second approximation, with some preference toward the unbounded oxygen (Jardetsky in discussion following [38]). Replacement of an oxygen by a methylene group completely distorts this charge distribution even if the net charge is still -1. Thus, the monoanionic analogue, dTpcdT, represents the first case of oligonucleotide inhibition in the absence of a 5′-phosphoryl group. There are no X-ray data for the dTpcdT–nuclease-Ca^{2+} complex, but the presence of only one phosphonate ester group (analogous to the 3′5′ phosphodiester linkage) requires the binding of the α- and the β-nucleosides to subsites (P1 and P2 in Fig. 1), on each side of the scissile phosphorus–oxygen bond. Although this type of binding is productive for hydrolysis of P–O esters, cleavage of the more resistant [35–37] P–C bond is not catalyzed by the nuclease for a number of reasons. Differences in R–O–R versus R–C–R bond angles ($\sim$103 versus 109° [39] and P–O versus P–C bond lengths ($\sim$1.6 versus 1.8 Å) [39–41] could result in significant distortion of the geometry and position of the phosphonate ester. Models of dTpcdT show that the thymine ring of the β-nucleoside moiety, which is the more important in the substrate recognition process [42], is sterically hindered by the extra bulk of the 5′-methylene group. Furthermore, the lower electronegativity and greater van der Waal radius of the methylene group [39] relative to a 5′-oxygen atom would make it energetically more difficult to get into an axial leaving position if, in fact, hydrolysis of dTpcdT proceeds through a pentacoordinate phosphorus reaction mechanism (reviewed in Refs. [2,3]). Available evidence [43,44] suggests that non-enzymatic cleavage of mono- and triphosphonyl esters occurs via a metaphosphate intermediate. Thus, even if the unfavorable steric and conformational factors of the analogue were minimized, the unique geometry of the nuclease active site might prevent the lowest energy route (metaphosphate formation) for phosphonate hydrolysis.

The pcdTpcdT in solution binds to nuclease about fifty times stronger than dTpcdT and is more effective in protecting against trypsin hydrolysis. Therefore we were encouraged that this analogue might provide a 'productive' binding mode (Fig. 1(a)) with its phosphonate ester linkage occupying the hydrolytic site (A). However, precluding the possibility of hydrolysis to pcdTp and dT under the conditions of crystallization, the preliminary X-ray diffraction interpretation (Fig. 5) is consistent only with a mode of 'unproductive' binding (Fig. 1(b)); i.e. the 5′-phosphonate dianionic terminus occupying the hydrolytic site with the α- and β-thymidyl moieties to the 'right' of subsites P2 and P3, respectively. Apparently the binding affinity of pcdTpcdT depends predominantly on the presence of a free 5′-phosphonic acid group with only secondary effects assignable to the analogue 3′→5′-phosphonate ester linkage. The similarity in the K_i values for pcdTpcdT (9.1×10^{-7}) and pdTpdT (6.3×10^{-7}) is consistent with this explanation.

As in the phosphodiester series [5,7], an additional order of magnitude in binding strength is demonstrated by the 3′-phosphate analogue, pcdTpcdTp. The presence of three phosphate (or phosphate analogue) groups allows this derivative either of the schematic binding patterns (Fig. 1). However, the markedly altered diffraction properties (cell constants and intensities) of the Type IV crystals combined with the discrepancy in binding constant measurements in solution (Table 1) suggest possible non-active site binding for pcd-

TpcdTp. Bier [9] has rationalized that the affinity labeling of Trp 140 with the diazonium derivative of dTp–phenylamine, which yields an enzymatically inactive product [45], as binding of the relatively nonspecific dTp in a subsite other than that observed crystallographically for pdTp [9] or chemically for oligonucleotides [7]. Nuclease catalyzed hydrolysis of pcdTpcdT gives pcdTp and dTp. From the present data we cannot say more.

The results presented here can well be accommodated by the binding subsite model of Cuatrecasas et al., but at present add no additional structural information to that provided by the nuclease–pdTp-Ca^{2+} complex. As in the case of pdTp in the Type II structure, pcdTpcdT is apparently inhibiting due to the interaction of its dianionic 5′-phosphonic acid group with the guanidinium groups of arginine residues 35 and 87. However, based on the same arguments offered above for the monoanionic phosphonate, the replacement of the 5′-oxygen ester atom with a methylene group at the 5′-terminus should promote subtle but crystallographically observable changes in the orientation of the neighboring side chains of active site residues Arg 35, Arg 87 and possibly Tyr 113 (Fig. 5). The elucidation of these effects requires further investigation.

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