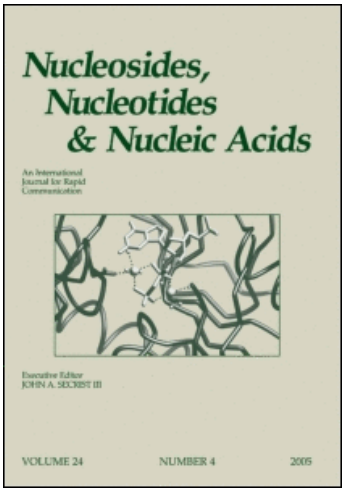

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Synthesis and Incorporation of Pyrrole Carboxamide Nucleoside Triphosphates by DNA Polymerases

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SYNTHESIS AND INCORPORATION OF PYRROLE CARBOXAMIDE NUCLEOSIDE TRIPHOSPHATES BY DNA POLYMERASES.

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Abstract: We have synthesised and examined the enzymatic incorporation properties of the 5'-triphosphates of 2'-deoxyribosyl pyrrole 3-monocarboxamide (dMTP) and 2'-deoxyribosyl pyrrole 3,4-dicarboxamide (dDTP). These analogues we had hoped would behave as ambivalent base analogues in that they can present two alternative hydrogen-bonding faces either by rotation about the carboxamide group or about the glycosidic bond. The two pyrrole derivatives, dMTP and dDTP, exhibit a preference for incorporation with Klenow polymerase. They are preferentially incorporated as either A or C.

Introduction:

It is well known that when hydrogen-bond degenerate nucleoside analogues are present in a DNA template or as their 5' -triphosphate substrates, they constitute mutagenic species.[1,2] It has been demonstrated repeatedly that hydrogen-bonding capability is crucial for enzyme recognition (but see refs. 3,4). In simple hybridisation terms, however, the requirements are much less rigid, and many nucleoside analogues have been prepared which have little or no hydrogen-bond capability, but nevertheless still allow relatively stable duplexes to form when incorporated into oligonucleotides, (ref. 5 and references therein).

Mutagenic analogues have a number of potential uses: they are not only useful as mutagenic reagents[6,7] (random or site-directed), but may also be introduced at ambiguous sites in probes and primers to diminish chain multiplicity.[8] Very few analogues have been prepared which are capable of behaving as more than one of the natural nucleosides/nucleotides in a non-discriminatory manner, when they do, they mostly lead to transitions.[8,9] Of the 12 possible single site mutations in dsDNA, 8 are transversions: there is therefore a need for new derivatives that are capable of (i) being less discriminating and (ii) extending the repertoire of transversion analogues.

Heterocyclic structures incorporated into nucleotides have been designed on the following principle so as to provide a means of producing transversion (as well as transition) mutations during nucleic acid replication *in vitro*. If a heterocyclic base has two hydrogen bonding orientations, either by rotation about a bond in the base or at the glycosidic centre in a nucleoside containing it, the alternative base pairing structures may include those potentially resulting in transversions. An example is 8-oxo-2'-deoxyguanosine **1** (Figure 1), which, in a template or as its 5'-triphosphate, gives at a low frequency, G:C to T:A transversions,[10] because the 8-oxo group drives the base into the *syn* conformation. A number of heterocyclic derivatives have also been designed on this basis. Imidazole carboxamide, **2**, can in principle exist as two amide-bond conformers as shown and thus present either an 'inosine'-face (I-face) or an 'adenosine'-face (A-face),[11] in turn forming base pairs with each of the natural nucleosides. A number of related analogues have been prepared, as their phosphoramidite monomers, and incorporated into oligonucleotides with this concept in mind. These include the pyrrole, **3**, and pyrazole carboxamides **4**, **5**,[12] as well as imidazole and triazole carboxamides.[13]

Some work has previously been reported on the use of azole carboxamide derivatives in oligonucleotides, both in terms of their hybridisation properties, and as template bases for DNA polymerases.[11-13] Little work has yet been reported on the substrate specificity of triphosphate analogues. As part of our work to investigate degenerate nucleoside derivatives, we decided to investigate the 2'-deoxyribosyl 5'-triphosphates of pyrrole 3-monocarboxamide **3**, and pyrrole 3,4-dicarboxamide **6**. The rationale for investigating the latter was that as there are two amide groups they would be held, by an internal hydrogen-bond, to present both an A-face and an I-face within

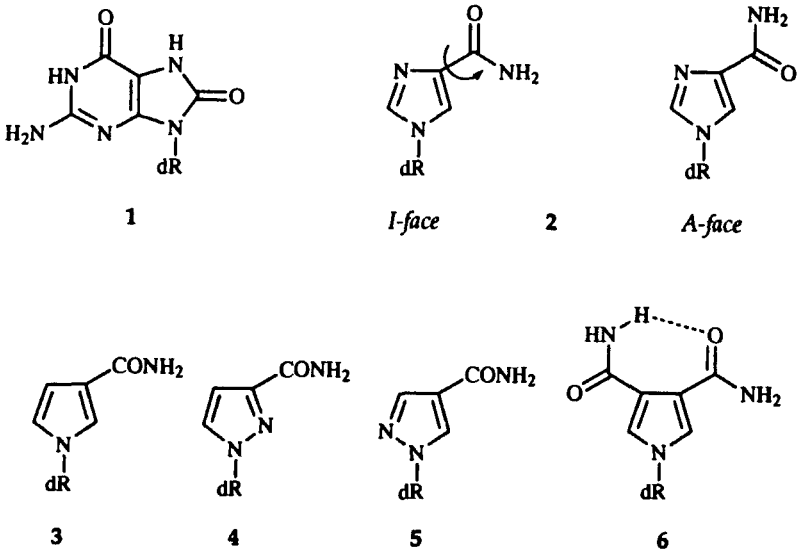


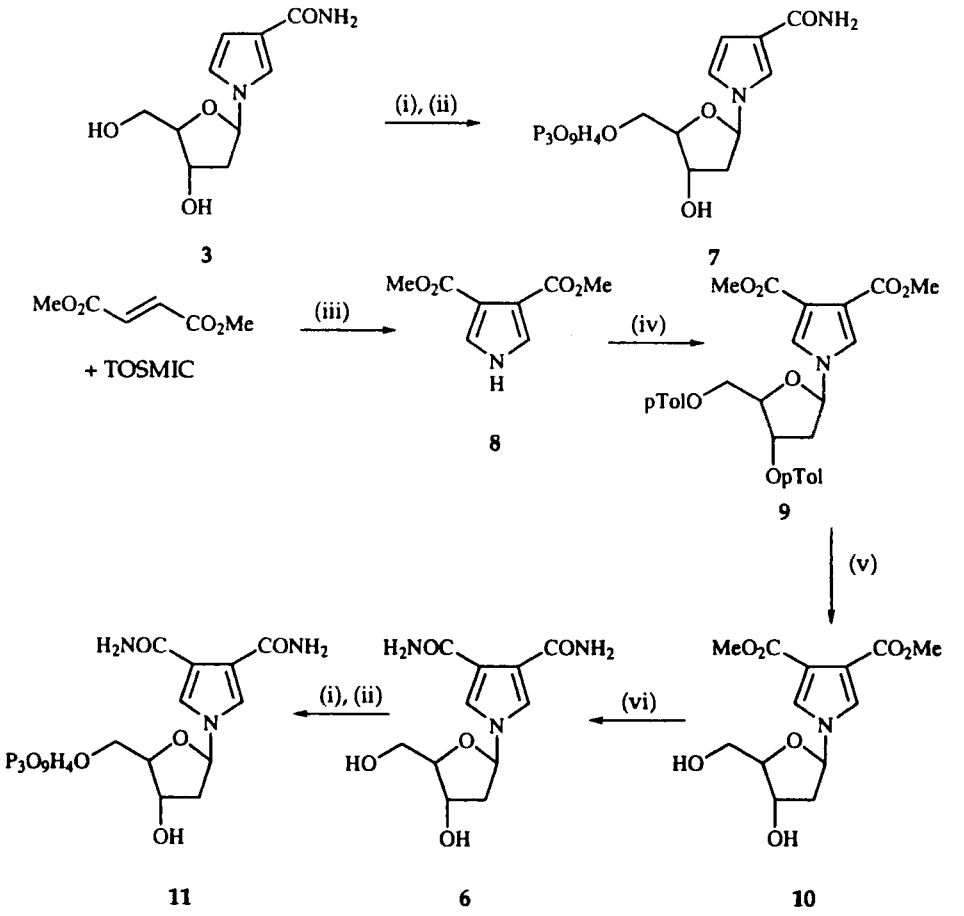
Figure 1

the same molecule. It was reasoned that rotation around the glycosidic bond, at the demand of the system, would allow the molecule to form base pairs with each of the natural nucleosides.

Results

2'-Deoxyribosyl pyrrole-3-monocarboxamide (dM) **3** was synthesised in a similar manner to that described for its ribonucleoside,[14] and then converted to its 5'-triphosphate (dMTP) **7**. Dimethyl-pyrrole-3,4-dicarboxylate **8** was prepared by the reaction of dimethylfumarate with tosylmethyl isocyanide (TOSMIC)[15] and then converted to the 2'-deoxynucleoside **10** as described[16] (Scheme 1). This was converted to the dicarboxamide (dD) **6** by treatment with 0.880 aqueous ammonia at 50°C overnight, and then to its 5'-triphosphate[17] (dDTP) **11**.

The pyrrole dicarboxamide derivative **6** was crystallised and the X-ray crystal structure obtained, Figure 2. As expected there is an internal hydrogen bond between the two carboxamide groups holding them essentially within the plane of the ring.

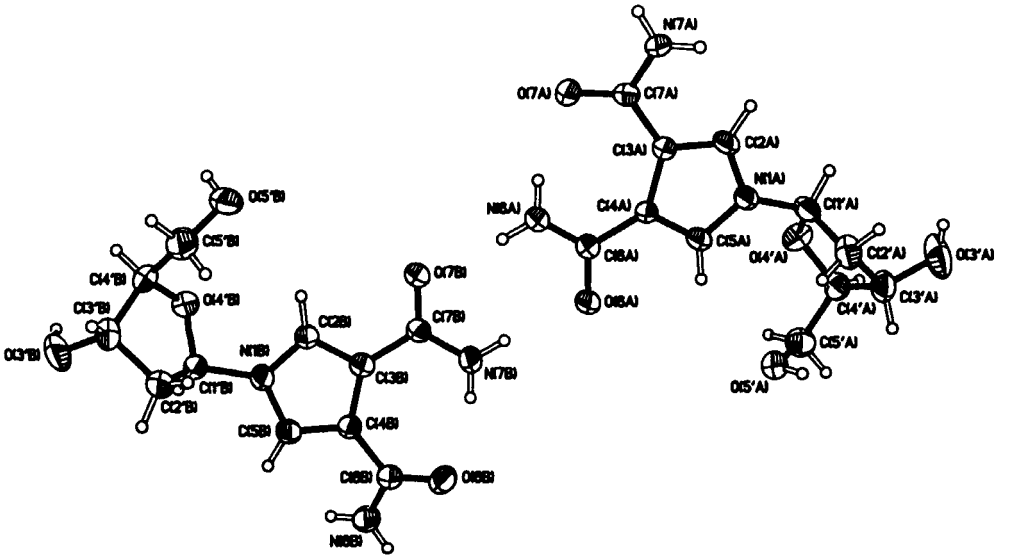


Synthesis of dMTP and dDTP. (i) $POCl_3$. (ii) $P_2O_6Bu_4$. (iii) NaH. (iv) NaH, 1-(3,5-di-O-p-toluoyl-2-deoxyribosyl) chloride. (v) NaOMe. (vi) 0.880 NH_3, 50°C.

Scheme 1.

DNA polymerase incorporation of dDTP and dMTP

The two 2'-deoxyribosyl pyrrole carboxamide 5'-triphosphates (dDTP, dMTP) were studied as substrates for exonuclease free Klenow fragment of *E. coli* DNA polymerase I (EFK). The assays used a ^{33}P 5'-end labelled 15-mer primer hybridised to each of four different 24-mer templates. The sequences of the primer and templates used are given in the experimental.

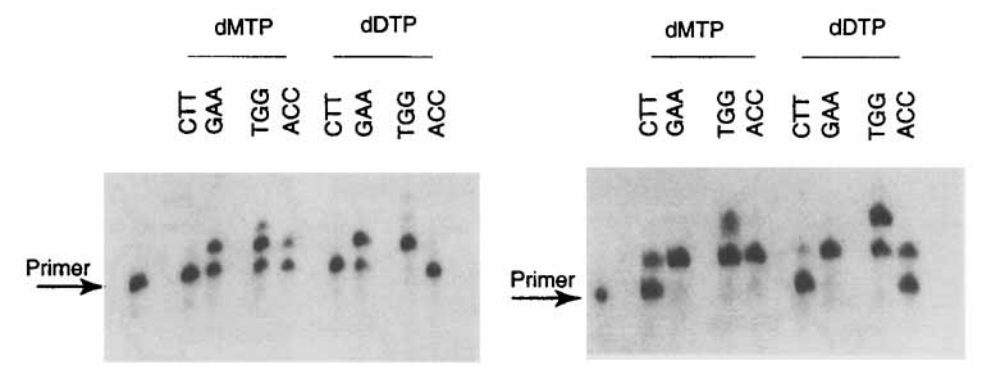


Thermal ellipsoid (50% probability) plot of the pyrrole dicarboxamide nucleoside **6**.

Figure 2

The data that we have obtained concerns both incorporation and extension of the pyrrole carboxamide triphosphates. By incorporation it is meant that a single base addition has occurred; when discussing extension a single (usually) modified base triphosphate has been incorporated and this is extended by the incorporation of further, usually, natural triphosphates.

At high enzyme concentrations, both the pyrrole monocarboxamide and the dicarboxamide 5'-triphosphates were incorporated opposite each of the four natural template bases (Figure 3, right panel), being best incorporated as A, i.e. opposite template T. At lower enzyme concentrations (0.5 units/reaction), it becomes clear that pyrrole monocarboxamide incorporates preferentially as A or C, with a little incorporation as T. Incorporation as G was not observed under these conditions. The dicarboxamide only incorporates as A or C (Figure 3, left panel). However, differences occur when subsequent extension was examined. When incorporated as either A or T, extension products were observed with pyrrole monocarboxamide, with effectively chain termination when incorporated as G or C. Pyrrole dicarboxamide, however, gave



Klenow extension using dMTP and dDTP on the four templates (see experimental). The sequence above each panel shows the next three template bases. The left-hand panel is at low enzyme concentration (0.5 units/reaction), the right hand panel at high concentration (5 units/reaction).

Figure 3

extension products when incorporated as A or C, but not as T or G (data not shown). A summary of the nucleotide analogue incorporation on the various templates is shown in Table 1.

The observed rate differences in incorporation of these two triphosphates were further examined. The assay was designed to look at a single base incorporation of various concentrations of the analogues using Template 3, which requires the addition of the equivalent of ACC. However, due to the ambiguous base pairing of the analogues, especially dDTP, more than a single base incorporation was seen. To each reaction was added a single triphosphate at concentrations between 1 and 2000μM. Autoradiographs were analysed and the data plotted as shown in Figure 4. It is clear from this study that dDTP is a far better substrate than the dMTP when incorporated as an "A" derivative. We are further investigating the kinetics of incorporation of the two pyrrole carboxamide derivatives, and will report on these later.

Terminal deoxynucleotidyl transferase (TdT)

The ability of dDTP and dMTP to act as substrates for terminal deoxynucleotidyl transferase (TdT) were compared with the natural dNTP's. As expected, incubation with any of the natural dNTP's gave 3' tails in the region of 50-150 nucleotides. We found

Table 1 Summary of extension of dMTP and dDTP with exonuclease free Klenow (5U) using the four templates as described in the text. The next three template bases are given for each of the 4 templates.

Nucleotides Added	Template			
	1 (GAA)	2 (ACC)	3 (TGG)	4 (CTT)
dMTP	+1 (C)	+2 (TG)	+1 and +2 (AC)	+1 (G)
dGTPαS, dMTP	nd	nd	nd	+3 (GAA)
dTTPαS, dMTP	nd	+2 (TG)	nd	nd
dATPαS, dMTP	nd	nd	+2 (AC)	nd
dCTPαS, dMTP	+4 (CTTG)	nd	nd	nd
dDTP	+2 (CT)	+1 (T)	+2, +3, +4 (ACCT)	+1 (G)
dGTPαS, dDTP	nd	nd	nd	+4 (GAAC)
dTTPαS, dDTP	nd	+2 (TG)	nd	nd
dATPαS, dDTP	nd	nd	+2, +3, +4 (ACCT)	nd
dCTPαS, dDTP	+2 (CT)	nd	nd	nd

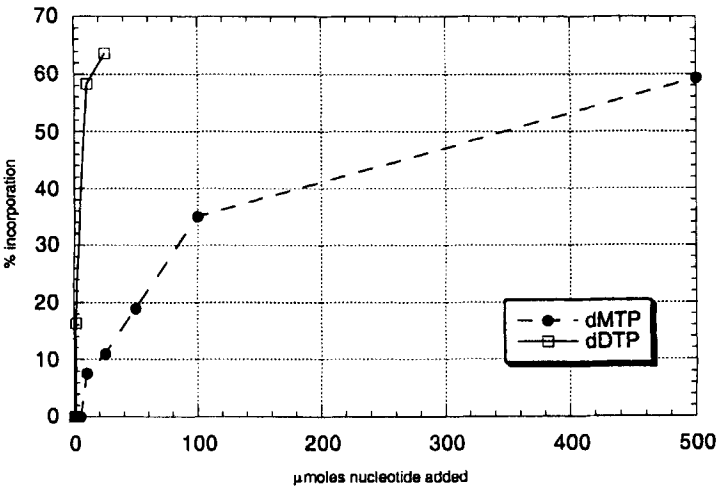


Figure 4

that both dDTP and dMTP are very good substrates for TdT. For dMTP, two major products were obtained. The shorter product was similar in size to that produced by dCTP and the longer had a similar mobility to the dATP and TTP products. The 3'-tail produced by dDTP was longer than those produced using either dATP or TTP.

Discussion

Much of the literature on azole 2'-deoxynucleoside carboxamide analogues has been on the imidazole **2**[18] and it's 5-amino-derivative.[19] The pyrrole monocarboxamide **3** (as its ribo derivative) and the dicarboxamide **6** have been synthesised previously,[14,20] though their incorporation into DNA (RNA) has not been described. The imidazole **2** has been incorporated into DNA as its 5'-triphosphate using Klenow fragment where it was only efficiently incorporated as dATP, and once incorporated it was copied as either A or G leading to a transversion mutations.[18]

More recently, the templating properties of the analogues **2-5** have been examined.[21] The analogues **2**, **3** and **5** behaved in a template as either A or T, whilst the pyrazole **4** behaved as C/T (with *Taq* polymerase). Other than the imidazole derivative **2**, none of the other azole carboxamides have been examined as their 5'-triphosphates. We have examined the incorporation properties of the 5'-triphosphates of two such analogues, pyrrole monocarboxamide **7**, and the dicarboxamide **11**.

There is a difference between the incorporation of the natural triphosphates opposite a modified template base and the incorporation of a modified base, as its 5'-triphosphate, opposite one of the natural template bases. When a modified base is present in the template there is only the expected competition, depending upon the hydrogen bonding pattern of the modified base, for incorporation of the natural triphosphates. When the hydrogen bonding pattern of the modified base is non-standard, or absent, then the polymerase may still force the incorporation of one of the natural triphosphates opposite it, e.g. by its extendase activity.[22] However, as a triphosphate, the modified base must first meet the demand of recognition by the polymerase, and then be recognised by the template base too. Once the modified base has been incorporated, it should potentially form a 3'-terminus for further extension of the growing DNA chain. When the modified base triphosphate is present with the four natural triphosphates, it must additionally compete with the natural bases to enter the polymerase active site.

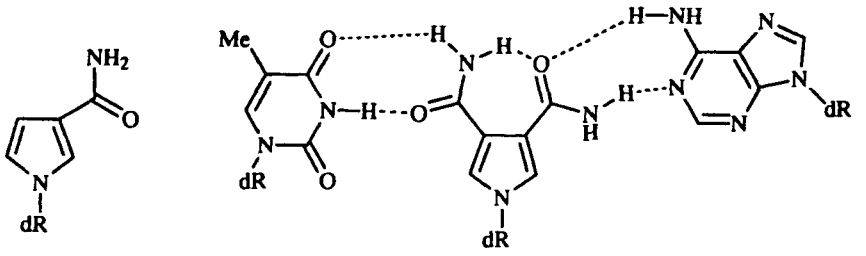
Therefore, the use of modified base triphosphates is a more demanding challenge for incorporation into DNA than their use as modified template bases, though both are important.

The two pyrrole derivatives, dMTP and dDTP, have different hydrogen-bond recognition patterns. With exonuclease free Klenow fragment they can both be incorporated opposite each of the natural DNA nucleotides. However, the incorporation of dMTP as either C or G or dDTP as either T or G is an apparent termination step. In both cases it is unclear whether the observed termination is a defined stop or that the 3' end becomes a much poorer terminus for extension, significantly raising the apparent Km for the insertion of the next nucleotide. Using a limiting concentration of Klenow polymerase the two analogues exhibit a preference for incorporation, in which dMTP is incorporated as either A or C, and dDTP as A or T.

The apparent preference of the monocarboxamide to be incorporated as either A or C can be envisaged as deriving from a single conformation whereby the A-face is preferred (see **3a**, Figure 5). In the dicarboxamide derivative **6**, the two carboxamide groups are held in a fixed orientation by an internal hydrogen bond, as shown by its x-ray crystal structure. We assume there will be a high-energy barrier to rotation about both carboxamide bonds. The fact that dDTP shows a preference for incorporation as A or T is a most easily explained by the nucleotide existing in both glycosyl bond rotamers.

Experimental

General methods: ^{1}H-nmr spectra were obtained on a Bruker DRX 300, and were obtained in d^{6}-DMSO unless otherwise stated. ^{31}P-nmr spectra are externally referenced to phosphoric acid. Mass spectra were recorded on a Kratos MS890. Ultraviolet spectra were recorded on a Perkin-Elmer Lambda 2 spectrophotometer in methanol unless otherwise stated. Tlc was carried out on pre-coated F_{254} silica plates and column chromatography with Merck kieselgel 60. Oligonucleotides were synthesised on an Applied Biosystem ABI 380B synthesiser with the normal synthesis cycle. Cy5 phosphoramidite was purchased from Pharmacia. Purification of oligonucleotides was carried out by polyacrylamide gel electrophoresis using 20% polyacrylamide gel, extraction using 0.5M ammonium acetate, 1mM EDTA buffer followed by



3a 6

Figure 5

concentration and desalting using a Sephadex G25 column (NAP-10, Pharmacia). Unless otherwise stated reactions were worked up as follows: After removal of the solvent, the product was dissolved in chloroform and washed with aqueous sodium bicarbonate solution. The combined organic fractions were dried over sodium sulphate and evaporated.

Synthesis

1-(2'-deoxy-β-D-ribofuranosyl)-pyrrole-3-carboxamide 3. This was prepared following the procedure for the ribonucleoside. 3-Cyanopyrrole[23] (0.77g, 8.4mmol) was dissolved in dry acetonitrile (25ml) and sodium hydride added (60%, 0.38g, 9.5mmol) and the solution stirred at room temperature for 30 minutes. To this was then added 1-(3,5-di-O-p-toluoyl-2'-deoxyribofuranosyl) chloride[24] (3.4g, 10mmol) and the solution stirred at room temperature for 2 hours. The solvent was removed and the product chromatographed ($CHCl_3$) and then recrystallised from methanol to give 1-(3',5'-di-O-p-toluoyl-2'-deoxy-β-D-ribofuranosyl)-3-cyanopyrrole as an off-white solid. Yield 2.06g (second crop yielded a further 1.18g, total yield 87%). δ_H 2.37, 2.39 (6H, 2 x s, 2 x $PhCH_3$), 2.69-2.81 (2H, m, H2', H2"), 4.48-4.59 (3H, m, H5', H5", H3'), 5.62-5.63 (1H, m, H4'), 6.20 (1H, t, J=6.2 Hz, H1'), 6.51 (1H, d, J=2 Hz, H5), 7.17 (1H, d, J=2 Hz, H4), 7.31-7.37, 7.84-7.94 (9H, m, H2, Ph). FAB mass, 445.3 $(M+H)^+$.

The above nucleoside (3.2g, 7.2mmol) was heated at reflux in 10% triethylamine in methanol (25ml) for 12 hours. The solvent was evaporated and the product

chromatographed ($CHCl_3$/5% MeOH) to give 1-(2'-deoxy-β-D-ribofuranosyl)-3-cyanopyrrole as a clear gum. Yield 1.02g (70%). δ_H 2.16-2.34 (2H, m, H2', H2''), 3.40-3.49 (2H, m, H5', H5''), 3.77-3.81 (1H, m, H4'), 4.28 (1H, Br. s, H3'), 4.97 (1H, s, OH), 5.24 (1H, s, OH), 5.95 (1H, t, J=6.4 Hz, H1'), 6.47 (1H, d, J=2 Hz, H5), 7.15 (1H, d, J=2 Hz, H4), 7.82 (1H, s, H2).

The preceding cyanopyrrole (1g, 4.4mmol) was dissolved in methanol (50ml) and dioxan (5ml) and the pH adjusted to pH 9 with ammonia solution. Hydrogen peroxide (30%, 5ml) was then added to the solution and then stirred at room temperature overnight. The solution was evaporated and chromatographed ($CHCl_3$/20% MeOH) to give a white foam. Yield 0.65g, 60%. δ_H 2.12-2.31 (2H, m, H2', H2''), 3.38-3.41 (2H, m, H5', H5''), 3.74-3.78 (1H, m, H4'), 4.24-4.27 (1H, m, H3'), 4.85 (1H, t, 5'-OH), 5.22 (1H, d, 3'-OH), 5.86 (1H, t, J=6.3 Hz, H1'), 6.43 (1H, d, J=2 Hz, H4), 6.70 (1H, NH), 6.94 (1H, d, J=2 Hz, H5), 7.27 (1H, NH), 7.50 (1H, s, H2). uv (H_2O), λmax/nm 229 (ε=8000), ε260 (μM) = 3.4.

1-(2'-deoxy-β-D-ribofuranosyl)-pyrrole-3-carboxamide-5'-triphosphate.

To a stirred solution of the pyrrole-3-carboxamide nucleoside (**3**) (220mg, 1mmol) in a 1:1 solution of triethyl phosphate and trimethyl phosphate (2ml) at 0°C was added phosphoryl chloride (105μl, 1.1mmol) dropwise. After stirring at 0°C for 45 minutes the reaction mixture was treated with bis-tri-n-butyl ammonium pyrophosphate (5.13ml of 0.6M solution in DMF, 2.98mmol) and tri-n-butylamine (713μl, 3mmol). The solution was then stirred at room temperature for 20 minutes and then it was neutralised by the addition of 1.0M TEAB buffer. The crude triphosphate was purified on a DEAE Sephadex A-25 column using a linear gradient from 0 to 1.0M TEAB (pH 7.5). The triphosphate peak fractions were collected (0.7-0.9M), and concentrated. They were then purified by reverse phase HPLC (Waters) on a semi-prep-Delta Pak 15 microns C-18 column (5x30cm), using a gradient of 0-100% buffer A (0.1M TEAB) and buffer B (25% acetonitrile in 0.1M TEAB) at 13ml/min in 40 minutes to afford pure **7** (58mg). δ_P (D_2O/EDTA) -10.31 (d, γ-P), -10.73 (d, α-P), -22.68 (t, β-P).

Dimethyl pyrrole-3,4-dicarboxylate 8. Using an alternative synthesis,[16] TOSMIC (1.5g, 7.7mmol) was added to a solution of dimethyl fumarate (1g, 6.9mmol) and sodium hydride (60%, 0.5g, 12.5mmol) in dry DMF (25ml) at 0°C, and the solution stirred at 0°C for 15 minutes. The reaction was poured onto ice-water and the product

filtered and recrystallised from aqueous ethanol. 0.61g, 48%. δ_H 3.68 (6H, s, 2 x CH_3), 7.39 (2H, s, 2 x CH), 11.81 (1H, s, NH).

Dimethyl-1-(3',5'-di-O-p-toluoyl-2'-deoxy-β-D-ribofuranosyl)-pyrrole-3,4-dicarboxylate 9. The pyrrole **8** (1g, 5.5mmol) was dissolved in acetonitrile (25ml) and to this was added sodium hydride (60%, 0.25g, 6.2mmol) and the solution stirred at room temperature for 30 minutes. To this was then added 1-(3,5-di-O-p-toluoyl-2-deoxyribofuranosyl) chloride (2.5g, 6.4mmol) and the solution stirred for 2 hours. The solvent was evaporated and the product worked up as usual and chromatographed ($CHCl_3$/1% MeOH) to give a yellow foam. Yield 1.75g, 60%. δ_H 2.37, 2.39 (6H, 2 x s, 2 x $ArCH_3$), 2.71-2.75 (2H, m, H2', H2''), 3.66 (6H, s, 2 x OCH_3), 4.45-4.60 (3H, m, H3', H5', H5''), 5.61-5.63 (1H, m, H4'), 6.22 (1H, t, J=6.3 Hz, H1'), 7.30-7.37, 7.70-7.94 (8H, m, Ar-H), 7.67 (2H, s, H2, H5).

Dimethyl-1-(2'-deoxy-β-D-ribofuranosyl)-pyrrole-3,4-dicarboxylate 10.

The di-toluoyl nucleoside above (1g, 1.9mmol) was suspended in methanol (25ml) and sodium methoxide (20mg) added and the solution heated at reflux for 2 hours. The solvent was removed and the product chromatographed ($CHCl_3$/5% MeOH) to give a pale yellow gum. Yield 0.45g, 81%. δ_H 2.16-2.33 (2H, m, H2', H2''), 3.69 (6H, s, 2 x OCH_3), 3.51 (2H, br. s, H5', H5''), 3.79-3.83 (1H, m, H3'), 4.26-4.28 (1H, m, H4'), 4.90 (1H, br. s, OH), 5.21 (1H, br. s, OH), 5.96 (1H, t, J=6.4 Hz, H1'), 7.64 (2H, s, H2, H5).

1-(2'-Deoxy-β-D-ribofuranosyl)-pyrrole-3,4-dicarboxamide 6.

The nucleoside di-ester **10** (0.36g, 1.2mmol) was dissolved in 0.880 ammonia solution (10ml) and heated at 50°C overnight in a sealed bottle. The solution was cooled and evaporated and the product crystallised from ethanol. Yield 0.26g, 80%. Spectra as described.[16]

1-(2'-Deoxy-β-D-ribofuranosyl)-pyrrole-3,4-dicarboxamide-5'-triphosphate 11. The triphosphate **11** was prepared as described for the monocarboxamide **7**. The yield was 80mg from 202mg of the nucleoside **6**. δ_P (D_2O/EDTA) -10.39 (d, γ-P), -10.80 (d, α-P), -22.66 (t, β-P).

X-ray structure determination of 6. Crystals (colourless blocks) were obtained by slow cooling of an ethanolic solution of **6**. A suitable specimen 0.5 x 0.2 x 0.1 mm was mounted on a glass fibre. Data were collected at 293 K using a Rigaku AFC7R four-

circle diffractometer and Mo Kα radiation (λ = 0.701069 Å) from a Rigaku RU200 rotating anode source and graphite monochromator. Of the 2242 reflections measured in the range *h* 0 to 9, *k* -11 to +12, *l* -9 to +9, 2021 were treated as observed (>2σ). They were processed without absorption correction. Crystal data: $C_{22}H_{30}N_6O_{10}$ (two molecules in the asymmetric unit), space group *P*1, *a* = 8.295(5), *b* = 10.118(5), *c* = 8.2090(5) nm, α = 108.923(5)°, β = 110.894(5)°, γ = 71.186(5)°, Z = 1. The structure was solved using SHELXS-86.[26] Refinement (SHELXL-93[27]) converged at *R* = 0.0428 (R_w = 0.1080) for all 2021 data (goodness of fit 1.049). Hydrogen atoms were added during the course of refinement according to standard methods. The coordinates have been deposited with the CCDC (ref. code REGCOV).

DNA polymerase incorporation.

Template dependence of analogue nucleoside triphosphate incorporation into DNA with Klenow exo⁻ (EFK) polymerase.

Primer:	5'	TGCATGTGCTGGAGA 3'	
Template 1:	3'	ACGTACACGACCTCT**GAA**CTAGTC	5'
Template 2:	3'	ACGTACACGACCTCT**ACC**TTGCTA	5'
Template 3:	3'	ACGTACACGACCTCT**TGG**CTAGTC	5'
Template 4:	3'	ACGTACACGACCTCT**CTT**GATCAG	5'

Control reactions using EFK (5U/reaction) and the individual dNTPαS were set up using all four templates. In all cases extension of the primer was only seen in the presence of the correct dNTPαS as expected (se of natural dNTP's may give rise to misincorporation and hence artefact bands, dNTPαS does not). Similarly, chain extension products were seen in the presence of both nucleoside analogue triphosphates. However, there were surprising differences in incorporation of the analogues on the different templates. These results could not be repeated by using the correct dNTPαS and a non-complementary dNTPαS at high concentration. Thus, it showed that the results obtained in the presence of the analogue triphosphate are due to specific incorporation of the analogue, and not misincorporation.

One pmole ^{33}P labelled primer (5' end labelled using T4 polynucleotide kinase (New England Biolabs) and [$\gamma^{33}P$] ATP 3000Ci/mmol (Nycomed Amersham plc)) was

hybridised to 2 pmoles of each template in x2 Klenow buffer (100mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM β-mercaptoethanol). To this was added either 4μM dNTPαS or 40μM pyrrole monocarboxamide-2'-deoxyribose-5'-triphosphate or pyrrole dicarboxamide-2'-deoxyribose-5'-triphosphate or mixtures of 4μM dNTPαS and 40μM analogue 5'-triphosphate. Five units EFK (New England Biolabs) and 2mU inorganic pyrophosphatase (Nycomed Amersham plc) were used per reaction. Primer alone, primer plus template, primer plus template plus enzyme controls were also carried out. The reactions were incubated at 37°C for 30 minutes. Reactions were then stopped by the addition of formamide EDTA stop solution. Samples from each assay were electrophoretically separated on 19% polyacrylamide 7M urea gels. The gels were then fixed and dried before exposure to Kodak Biomax autoradiography film.

Exonuclease free Klenow Fragment "kinetic" Assay.

One pmole ^{33}P labelled primer (see above) (5'-end labelled using T4 polynucleotide kinase and [$\gamma^{33}P$] ATP 3000Ci/mmol) was hybridised to 2pmoles of Template 3 (see above) in 2 x Klenow buffer. To this was added either 1, 2, 5, 10, 25, 50, 100 or 500μM dMTP or dDTP. 0.5 units EFK and 2mU inorganic pyrophosphatase were used per reaction. Primer alone, primer plus template, primer plus template plus enzyme controls were also carried out. The reactions were incubated at 37°C for 30 seconds. Reactions were then stopped by the addition of formamide EDTA stop solution. Samples from each assay were electrophoretically separated on 19% polyacrylamide 7M urea gels. The gels were then fixed and dried before exposure to Kodak Biomax autoradiography film and a phosphor screen. The phosphor screen was analysed on a Molecular Dynamics Storm 860 instrument and quantified using ImageQuant software. The product fragments were sized by comparison with a ^{33}P labelled 8 to 32 base oligonucleotide ladder.

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