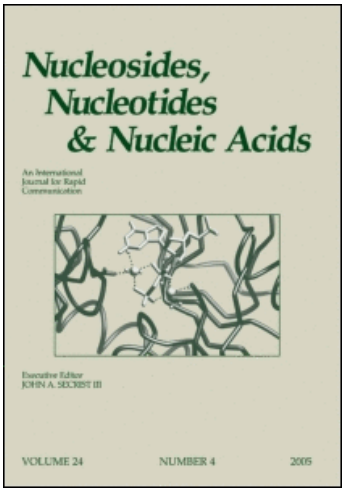

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DNA POLYMERASE INCORPORATION OF UNIVERSAL BASE TRIPHOSPHATES. †

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ABSTRACT: The 5'-triphosphates of various universal deoxynucleosides have been synthesised, and their ability to act as template directed substrates for various polymerases has been investigated.

Nucleoside analogues which do not discriminate between the natural bases when opposite them in a duplex may be described as universal, at least in hybridisation terms. As such they can be used to help overcome sequence ambiguities arising from the degeneracy of the genetic code, and therefore have applications in DNA manipulations. The most commonly used 'universal' base is hypoxanthine, but enzymatically it behaves almost exclusively as guanine, and its triphosphate *in vitro* is only weakly mutagenic.[1,2] One approach has been to develop analogues in which base stacking is probably the dominant duplex stabilising feature. One class of such compounds have been designed to mimic the electronic charge distribution of the natural nucleobases. Their hydrogen bonding potential has been essentially removed in the case of azole nucleoside analogues[3-7] such as 5-nitroindole[3] (**1**) and 3-nitropyrrole[4] (**2**), and they are hydrophobic in nature. Within this category of compounds may be included some hydrophobic aromatic isosteres of purine and pyrimidine bases;[8] these compounds contain

† This paper is dedicated with great regard to the late Professor Tsujiaki Hata

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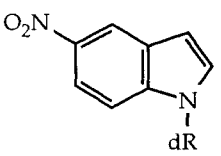
fluorine atoms replacing the carbonyl groups of the natural bases, e.g. replacement of thymine by difluorotoluene, but the evidence would suggest that this still has hydrogen bonding capability.[9] Another category of compounds are more hydrophilic in nature, and include carboxamide derivatives of pyrrole[10] and imidazole.[11,12]

Some of these analogues have been investigated in primers for PCR and sequencing[4,13] and for polymerase recognition when present as a template base.[14] However, to date the triphosphates of hydrophobic base analogues, and their recognition by polymerases has not been reported. A desirable function of an ideal universal base would be that the triphosphate should act as a good substrate for DNA polymerases, extending the 3'-end of a primer. Here we report the synthesis of the nucleoside triphosphates of 3-nitropyrrole (3-NP) and 5-nitroindole (5-NI), and some related indoles, and their recognition by a number of DNA polymerases.



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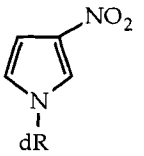
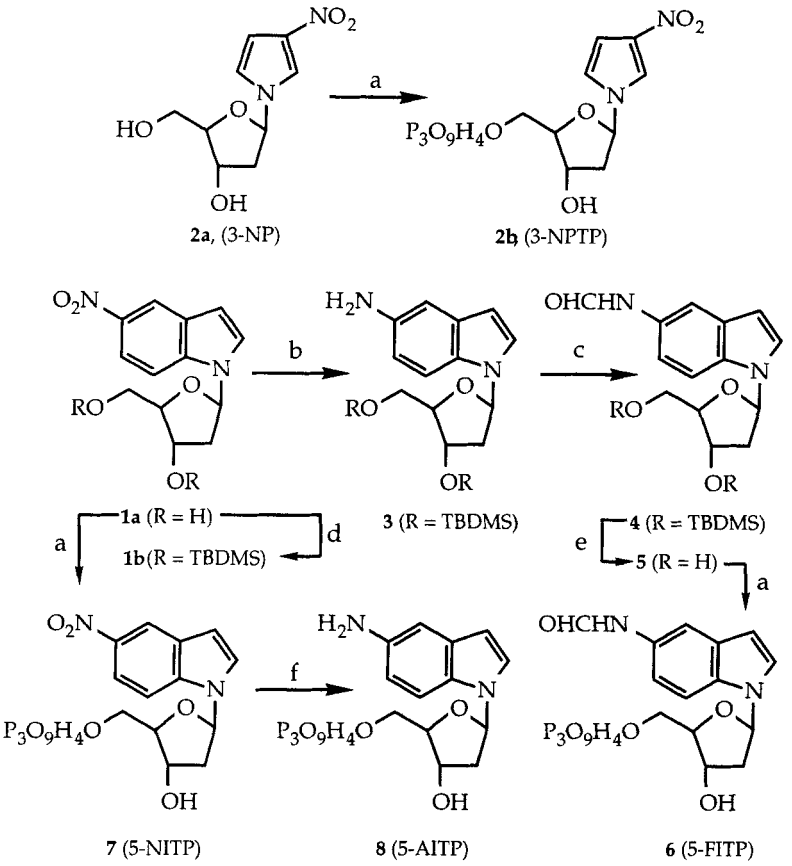


2

Results and Discussion

Synthesis.

The 5'-triphosphates of **1** and **2** were synthesised by reaction of the nucleoside with phosphoryl chloride followed by reaction with pyrophosphate. In addition, the ^{t}-butyldimethylsilylated (TBDMS) derivative of 2-deoxyribosyl-5-nitroindole (**1b**) was reduced with Raney-nickel under an hydrogen atmosphere to the 5-aminoindole (5-AI) nucleoside (**3**). This in turn was converted to its formamido (5-FI) derivative (**4**) by refluxing with ethyl formate in ethanol. Deprotection of **4** with ammonium fluoride provided **5**, so that we could compare non-hydrogen bonding bases with those that have hydrogen bonding functionalities (**SCHEME 1**). Compounds **3** and **5** were then converted to their 5'-triphosphates. During the phosphorylation of the formamidoindole (**5**) two triphosphates were formed, the major triphosphate being identified as the desired 5-formamidoindole 5'-triphosphate (FITP, **6**). The other triphosphate, initially isolated as a pure compound and identified by ^{31}P-nmr decomposed into three unidentified compounds. In order to obtain the 5-aminoindole 5'-triphosphate (5-AITP, **8**), the nitro group in **7** was reduced using hydrogen and 10% Pd/C.



Reaction conditions: a) $POCl_3$, 0.5M Bis-tri-n-butylammonium pyrophosphate, tri-n-butylamine, 0-5°C. (b) Raney-Ni, MeOH, H_2, RT. (c) Ethyl formate, reflux. (d) TBDMSCl, pyridine. (e) Ammonium Fluoride, EtOH, reflux. (f) 10% Pd/C, H_2, H_2O, 30 psi.

SCHEME 1

DNA Polymerase studies:

Exonuclease-free Klenow fragment of DNA polymerase I.

Using these four triphosphates we studied their incorporation into DNA by various polymerases. The assay used a ^{33}P 5'-end labelled 15-mer primer hybridised to each of four different 24-mer templates. The sequences of the templates were designed to give maximum information on the incorporation behaviour of the analogues. The analogues were also competed against the natural dNTPs in assays using both fixed and variable dNTP and analogue

concentrations. The sequences of the primer and templates used are:

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

Using exonuclease-free Klenow fragment of *E. coli* DNA polymerase I, single base extensions were seen as expected with the triphosphates of native bases, depending on template sequence and the identity of the base. Addition of the triphosphates of two different native bases produced the expected increase in product size and full extension could be obtained in the presence of all four natural triphosphates. Similarly, chain extension was seen with all of the universal 2'-deoxynucleoside 5'-triphosphate analogues. However, once incorporated not all were able to support further extension, either alone or in the presence of a natural triphosphate. After a single addition, 5-NITP, 3-NPTP and 5-FITP were unable to produce further extension products on any of the four templates (FIG. 1). 5-AITP showed greater variability in its initial incorporation, being best incorporated opposite adenine, and worst against thymine (data not shown). However, whilst it allowed the extension of the primer by a further base addition when incorporated opposite A or C, thereafter it behaved as a chain terminator as did the other universal base triphosphates. Clearly the ability of hydrogen bond formation for this compound does not greatly enhance its polymerase incorporation. A summary of how these nucleotides were incorporated and extended by Klenow (exo$^-$) is shown in TABLE 1.

the result was full length 24 and 25-mers, (the 25-mers deriving from the polymerase's intrinsic ability to add an extra non-template directed base[15]). This demonstrates that the natural triphosphates can out-compete 3-NPTP, even at 5 mM concentration. With 5-NITP at concentrations from 0.1 to 5 mM, a ladder of products was observed. This clearly shows that 5-NITP is incorporated, but it then causes chain termination, and the intensity of the shorter products is proportional to the concentration of 5-NITP.

In the assay using a fixed analogue triphosphate concentration, when the [dNTP] is above the Km for the enzyme (2 μM)[16] then full length product is observed. As the [dNTP] decreases below the Km then the degree of laddering increases as the extension products decrease in size due to the incorporation of

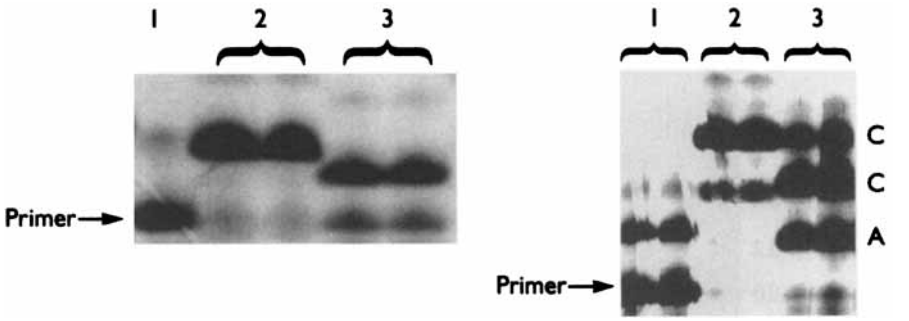


FIG. 1. Klenow (exo-) extension using the triphosphates of universal base nucleoside analogues: Left hand panel; 5-nitroindole (Lane 2) and 3-nitropyrrole (Lane 3). Note that 5-nitroindole product migrates slower than the corresponding product with 3-nitropyrrole. Right hand panel; 5-aminoindole (Lane 1); dATP + dCTP (Lane 2); 5-aminoindole + dATP (Lane 3).

TABLE 1

Base analogue	Incorporates as:	Extends when incorporated as:
5-nitroindole	A/C/G/T	Terminator
3-nitropyrrole	A/C/G/T	Terminator
5-aminoindole	T>G/C>A	T/G
5-formamidoindole	A/C/G/T	Terminator

When 3-NPTP was assayed against fixed concentrations of dNTPs (10 μM)

the analogues and consequent chain termination (FIG. 2). The laddering of products shows chain termination at template purine sites only. This implies that 5-NITP is only incorporated, in a competition assay, as a pyrimidine analogue. This is rather surprising considering the size of the indole nucleosides which are larger than purines. Once incorporated as a pyrimidine, extension of the 3'-terminus is then terminated.

There are two possible explanations why these analogues cause chain termination. Firstly, the lack of hydrogen bonding capability could mean that subsequent to incorporation the 3' end effectively remains as a single strand causing the polymerase to dissociate from the duplex. A second possibility is that after incorporation, and in the absence of 3'-5' exonuclease activity, the 3' end of

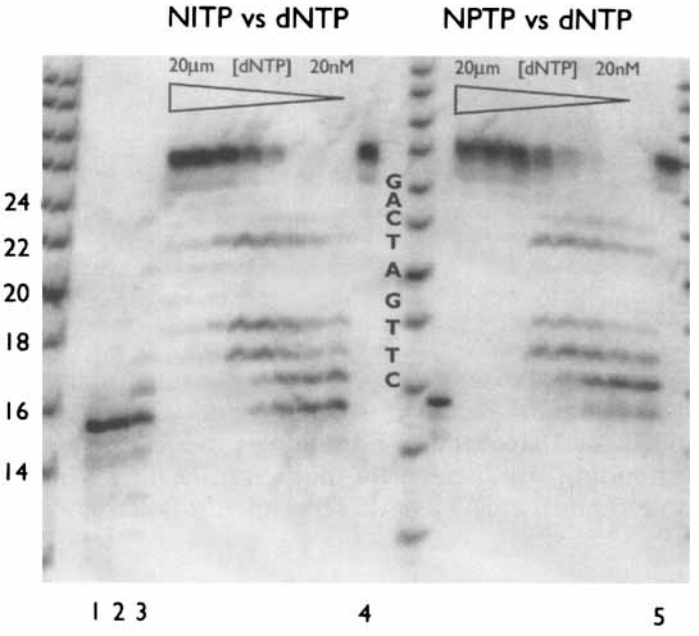


FIG. 2. Klenow (exo-) extension on template 4 using fixed (20 µM) NITP (left hand panel) and NPTP (right hand panel) versus variable (20 µM - 20nm) dNTP concentration. As the dNTP concentration decreases a ladder of products is observed terminating after incorporation of the universal base triphosphate as pyrimidines (sequence shows added nucleotides). Lane 1 primer; Lane 2: primer + template; Lane 3: primer + template + enzyme; Lane 4: no NITP; Lane 5; no NPTP.

the primer creates a poor terminus for extension raising the Km for the addition of the next nucleotide by several orders of magnitude, or a combination of the two.

Similar results were obtained using Sequenase™ and Thermosequenase™ DNA polymerases and AMV reverse transcriptase. The only exception to this was 3-NPTP, which gave no single base extension with AMV-RT (data not shown).

PCR with *Taq* polymerase.

To determine whether incorporation of the analogue triphosphates using *Taq* polymerase in PCRs could produce mutations, two 20-mer primers were used to amplify a 237bp fragment encoding supF, a mutant tRNA gene fragment which inserts tyrosine at amber stop codons in *E. coli*. Cycling between the annealing temperature of 50°C and the denaturing temperature of 96°C is sufficiently slow to allow amplification of the entire fragment without pausing at 72°C for

polymerase extension. Addition of 5-NITP in increasing concentrations (50 μM - 2 mM) to the reactions results in decreasing amount of PCR product such that, at 1 mM [5-NITP] there was no detectable product. However, when an extension time at 72°C of 1 minute was added to each cycle, a small increase in the yield of PCR product was obtained (FIG. 3).

To determine whether 5-NITP had been incorporated into, and produced mutations in the PCR product when 1 mM 5-NITP had been used, the product was re-amplified in the presence of only natural dNTPs both to wash out any analogue residues that may have been incorporated, and to increase the amount of product for cloning. This re-amplified PCR product was then cloned. 25 Clones obtained in this way were tested for supF activity *in vivo* and sequenced. None of the clones had decreased supF activity, and all the clones were unchanged in sequence clearly indicating that the analogue had not been incorporated.

Similar experiments were carried out using 3-NPTP, but in this case larger amounts of the analogue triphosphate were required to decrease the yield of the PCR product. At 1.5 mM [3-NPTP] some PCR product was visible on ethidium bromide stained agarose gels, albeit greatly reduced in yield. Once again, increasing the extension time, to two minutes, resulted in an increased yield of PCR product.

At concentrations above 500 μM of the 5-AITP triphosphate analogue no PCR product was observed even when the extension time was increased to 2 minutes. The 5-FITP analogue though had very little effect on the PCR reaction, with full length product being observed, albeit in reduced yield even at 2 mM [triphosphate] (data not shown). The reason for this loss of inhibitory effect is unclear, but in view of the results for 5-NITP, cloning and sequencing of the PCR products was not carried out. This suggests that 5-aminoindole is an even better inhibitor of *Taq* polymerase than 5-formamidoindole and 3-nitropyrrole: enzyme recognition may be improved by its ability to form one hydrogen bond. However, once in the active site of the polymerase either chain extension occurs leading to termination, or it remains in the active site preventing other dNTPs from entering it, again leading to termination of polymerase activity.

In view of the difficulty in extending a 3'-terminus comprising an incorporated 5-NI residue, it is not clear what is occurring during PCR. That inhibition occurs is understandable, but why increasing the extension time should lead to an increase in product yield is not. We think it likely that the longer extension time simply allows a larger proportion of each primer to complete chain

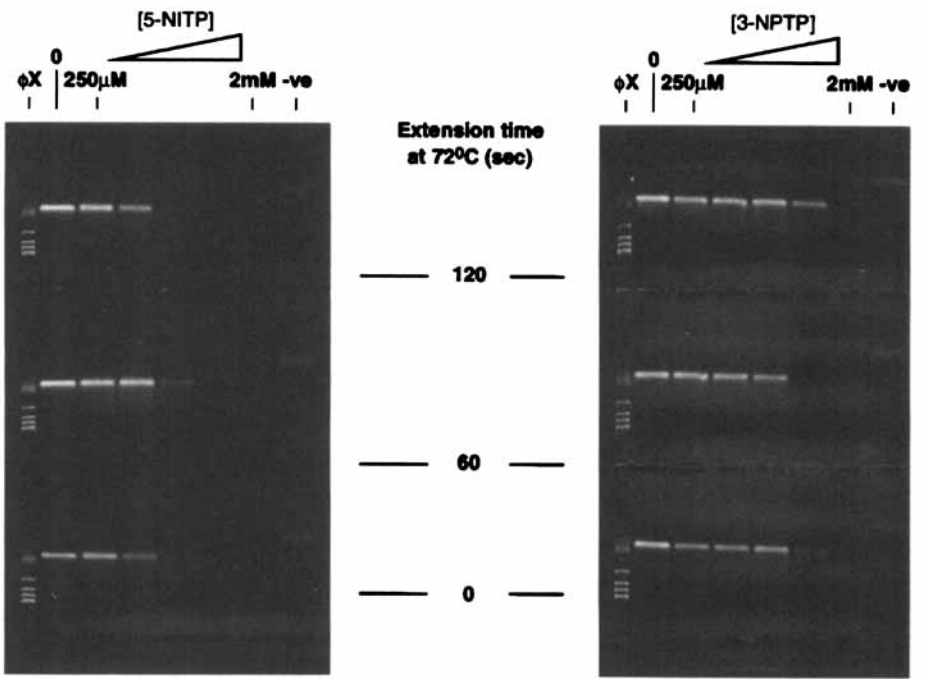


FIG. 3. PCR products using fixed (50 μM) dNTPs and variable (0, 250 μM, 500 μM, 1 mM, 1.5 mM and 2 mM) 5-NITP (left hand panel) and 3-NPTP (right hand panel) triphosphate concentrations, with 0, 60 or 120 second extension time at 72°C. First lane in each panel is φX174 Hae III marker, last lane is a negative control (no template).

extension, without incorporation of the analogue in each cycle. Alternatively, there may be residual 3'-exonuclease activity which removes the inhibiting residues.

In conclusion, the triphosphates of the universal base analogues described have been shown to be substrates for DNA polymerases, though the degree of incorporation appears to be limited to one base. The mechanism of chain termination is unknown at present. However, it is clear that the analogue triphosphates are capable of entering the polymerase active site, and this may be due to them having similar electron charge distribution to the natural dNTPs[17] although they may compete poorly with the natural triphosphates. Once incorporated into the growing DNA chain, they cause termination perhaps because they lack the hydrogen bonding recognition pattern required by the

polymerase. The triphosphates of 5-NI and 3-NP lack any significant hydrogen bonding ability and therefore endorse the currently held view that some base hydrogen bonding is required for efficient chain extension catalysed by DNA polymerases. Even when some hydrogen bonding capability exists, although not optimally aligned, as in the case of 5-aminoindole, chain extension is very poor, and this analogue is actually a very good polymerase inhibitor.

It is clear that the physical properties required of universal base analogues as either components of nucleic acids for hybridisation purposes or DNA polymerase substrates are different. To date, there has not been a universal base analogue that is capable of fulfilling both of these functions. We have demonstrated that the indoles and 3-nitropyrrole nucleoside triphosphate analogues described in this paper are poor substrates for DNA polymerases. However, 5-nitroindole and 3-nitropyrrole have been shown to be very useful when incorporated into short oligomers for use in hybridisation studies[3,7] as probes or primers.[4,12] These applications rely on the analogues ability to stabilise DNA duplex structures by stacking interactions, and base recognition is not required. There are examples of analogues that are good substrates for DNA polymerases, but these tend to be either base specific in their recognition, such as 2'-deoxyinosine, or are recognised as only two of the natural bases.[10,18] Although the indole and 3-nitropyrrole analogues are poor substrates for polymerases they have other potential applications.[19,20] It may be more productive in future to design universal base analogues which concentrate on achieving only one function, either as an enzyme substrate or a component of a nucleic acid for hybridisation.

MATERIALS AND METHODS

General methods

^{1}H-n.m.r. spectra were obtained on Bruker DRX-300 in d^{6}-DMSO and ^{31}P-n.m.r. in D_2O. ^{31}P-n.m.r. are referenced to phosphoric acid. Ultraviolet spectra were recorded on a Perkin Elmer Lambda 2 spectrometer in water unless otherwise stated. TLC was carried out on pre-coated F_{254} silica plates and column chromatography with Merck Kieselgel 60. The phosphoramidite monomer of 5-nitroindole was purchased from Glen research. Oligonucleotides were synthesised on an Applied Biosystem ABI 380B synthesiser with the normal synthesis cycle. Purification was carried out by polyacrylamide gel electrophoresis using 20% acrylamide gel, extraction using 0.5M ammonium

acetate, 1mM EDTA buffer followed by concentration and desalting using a Sephadex G25 column (NAP-10, Pharmacia).

Synthesis

1-(2-Deoxy-β-D-ribofuranosyl)-5-nitroindole 5'-triphosphate (5-NITP, 7). To a stirred solution of 5-nitroindole-2'-deoxynucleoside (**1a**)[3] (400 mg, 1.5 mmol) in a mixture of triethyl phosphate (TEP) and trimethyl phosphate (TMP) (1:1, 10 ml) was added phosphorus oxychloride (0.6 ml, 6 mmol) dropwise at 0-4°C. The mixture was then stirred for 15 h at 0-4°C. The reaction mixture was then treated with Bis-tri-n-butyl ammonium pyrophosphate (7.5 mmol) in anhydrous DMF (20 ml of 0.5 M solution) with simultaneous addition of tri-n-butylamine (7.5 mmol, 1.8 ml). After 10 min the reaction was quenched with 1 M triethylammonium bicarbonate solution (TEAB, 100 ml, pH 7.5) and stirred at room temperature for 2 hours. The crude mixture was purified on a Sephadex A-25 column using a linear gradient from 0 to 1 M TEAB (pH 7.5). The triphosphate peak fractions were collected (0.7-0.9 M), concentrated *in vacuo* and finally purified by reverse phase HPLC, (Waters semi-prep-HPLC delta Pak 15 microns C-18 column (1.9 cm x 30 cm) under the gradient conditions of 0 - 100% buffer A (0.1 M TEAB) and buffer B (25% CH_3CN in 0.1 M TEAB) at 12 ml/min in 30 min). Yield 110 mg. ^{31}P-n.m.r. ∂ (ppm) (D_2O/EDTA) γ-P -9.95, (d); α-P -11.20, (d); β-P -23.02, (t).

1-(2-Deoxy-β-D-ribofuranosyl)-3-nitropyrrole-5'-triphosphate (3-NPTP, 2b).

3-Nitropyrrole-2'-deoxynucleoside-5'-triphosphate was made in a similar manner to that described for 5-nitroindole. Yield: 37 mg from 170 mg of the corresponding nucleoside. ^{31}P-n.m.r. ∂ (ppm) (D_2O/EDTA) γ-P -10.78, (d); α-P -11.41, (d); β-P -23.36, (t).

1-(3,5-Di-O-tert-butyldimethylsilyl-2-deoxy-β-D-ribofuranosyl)-5-nitroindole (1b). To the 5-nitroindole nucleoside **1a** (0.5g, 1.9mmol) in pyridine (25ml) was added tert-butyldimethylsilyl chloride (0.63g, 4.2mmol) and the solution stirred at room temperature overnight. The solution was evaporated, extracted ($CHCl_3$/aqueous $NaHCO_3$), dried and chromatographed ($CHCl_3$) to give **1b**, yield 0.87g, 93% as a pale yellow gum. ^{1}H-n.m.r. δ (ppm) -0.01, 0.09 (12H, 2 x s, Si-Me), 0.85, 0.88 (18H, 2 x s, ^{t}-butyl), 2.27-2.35, 2.47-2.59 (2H, m, H2', H2''), 3.64-3.76 (2H, m, H5', H5''), 3.84-3.88 (1H, m, H4'), 4.51-4.56 (1H, m, H3'), 6.43 (1H, t, J=6.6 Hz, H1'), 6.78 (1H, d, J= 3.4 Hz, H3), 7.75-7.78 (2H, m, H7, H2), 8.0 (1H, dd, J_1=2.3 Hz, J_2=9.1 Hz, H6) 8.53 (1H, d, J=2.3 Hz, H4).

1-(3,5-Di-O-tert-butyldimethylsilyl-2-deoxy-β-D-ribofuranosyl)-5-aminoindole (3).

To the nitroindole **1b** (0.9g, 1.8mmol) in methanol (40ml) was added Raney nickel (300mg) in methanol (10ml) and the solution stirred under a hydrogen atmosphere for 2 hours. The solution was filtered through celite, evaporated and chromatographed ($CHCl_3$) to give **3** (0.80g, 92%) as an off-white foam/gum. ^{1}H-n.m.r. δ (ppm) 0.03, 0.04 (12H, 2 x s, Si-Me), 0.88, 0.90 (18H, 2 x s, ^{t}-butyl), 2.13-2.19 2.46-2.53 (2H, m, H2', H2''), 3.65-3.68 (2H, m, H5', H5''), 3.77-3.8 (1H, m, H4'), 4.48-4.51 (3H, m, H3', NH_2), 6.16-6.21 (2H, m, H1', H3), 6.51 (1H, dd, J_1=2.1 Hz, J_2=8.7 Hz, H6), 6.66 (1H, d, J=1.9 Hz, H4), 7.21 (1H, d, J=8.7 Hz, H7), 7.29 (1H, d, J=3.3 Hz, H2).

1-(3,5-Di-O-tert-butyldimethylsilyl-2-deoxy-β-D-ribofuranosyl)-formamidoindole (4). The aminoindole **3** (0.79g, 1.66mmol) was heated at reflux with ethyl formate (25ml) for 5 hours. The solvent was removed and the product chromatographed ($CHCl_3$/1% MeOH) to give **4** (0.72g, 86%) as an orange gum/foam. ^{1}H-n.m.r. δ (ppm) 0.001, 0.07 (12H, 2 x s, Si-Me), 0.84, 0.87 (18H, 2 x s, ^{t}-butyl), 2.19-2.21, 2.46-2.54 (2H, m, H2', H2''), 3.63-3.67 (2H, m, H5', H5''), 3.77-3.79 (1H, m, H4'), 4.47-4.96 (1H, m, H3'), 6.42 (1H, d, J=3.2 Hz, H3), 7.23 (1H, d, J=8.7 Hz, H7), 7.46-7.50 (2H, m, H6, H2), 7.86 (1H, s, CHO), 8.19 (1H, s, H4), 9.97 (1H, s, NH).

1-(2-Deoxy-β-D-ribofuranosyl)-5-formamidoindole (5). The silylated nucleoside **4** (0.7g, 1.4mmol) in ethanol (25ml) and ammonium fluoride (0.5g, 14mmol) was heated at reflux overnight. The solvent was removed and the product chromatographed ($CHCl_3$/10% MeOH) to give **5** (200mg, 52%) as an off-white powder. ^{1}H-n.m.r. δ (ppm) 2.16-2.22 and 2.43-2.45 (2H, m, H2', H2''), 3.43-3.56 (2H, m, H5', H5''), 3.79-3.80 (1H, m, H4'), 4.32-4.33 (1H, m, H3'), 4.84 (1H, t, 5'-OH), 5.24 (1H, d, 3'-OH), 6.31 (1H, t, J=6.6 Hz, H1'), 6.44 (1H, d, J=3.2 Hz, H3), 7.24 (1H, d, J=8.7 Hz, H7), 7.50 (1H, d, J=8.9 Hz, H6), 7.55 (1H, d, J=2.8 Hz, H2), 7.88 (1H, s, CHO), 8.23 (1H, Br. s, H4), 9.96 (1H, s, NH).

5-Formamidoindole-2'-deoxynucleoside-5'-triphosphate (5-FITP, 6). To a stirred and cooled (0-5°C) solution of 5-formamidoindole-2'-deoxynucleoside **5** (165mg, 0.59 mmol) in trimethylphosphate (2 ml) was added phosphoryl chloride (83μl, 0.89 mmol). After one hour, the reaction mixture was simultaneously treated with 0.5 M DMF solution of tri-n-butyl ammonium pyrophosphate (5.46 ml, 2.98 mmol) and tributylamine (0.71ml, 2.98 mmol). After stirring the reaction mixture at room temperature for 10 min, it was neutralised with 1.0 M TEAB and stirred at room temperature for 3 hours, evaporated under reduced pressure and

the residue obtained was dissolved in SQ water (30 ml). Then the crude triphosphate mixture was loaded onto a Sephadex column (500 ml) and a mixture of two triphosphates isolated using the gradient 0.05 (1L) to 1.0 M TEAB (1L, pH = 7) at 1.3 ml/min flow rate. The triphosphates mixture was further fractionated into individual triphosphates by reverse phase HPLC as before. The desired triphosphate **6** (22mg) with 22 min retention time showed ^{31}P-n.m.r. ∂ (ppm) (D_2O/EDTA) γ-P -10.06, (d); α-P -10.82, (d); β-P -22.61, (t), and remained stable. The triphosphate (30mg, uncharacterised) with 22.5 min retention time isolated initially as the pure triphosphate, decomposed into three compounds.

1-(2-Deoxy-β-D-ribofuranosyl)-5-aminoindole 5'-triphosphate (5-AITP, 8). To an aqueous solution (3ml) of 1-(2-deoxy-β-D-ribofuranosyl)-5-nitroindole 5'-triphosphate **7** (10mg) in a glass Parr hydrogenation pressure vessel was added 10% Pd/C (5mg) and the solution hydrogenated for 2 hours at 30psi hydrogen. The reaction mixture was filtered through celite and the filtrate purified on a delta Pak C-18 column (1.9x30cm, 15 microns), using a gradient of 0-100% buffer A (0.1M TEAB, pH 7.1) and buffer B (25% acetonitrile in 0.1M TEAB) at 12ml/min over 30 minutes to yield the aminoindole triphosphate **8** (6mg, 64%). u.v. λmax (H_2O) 269nm. ^{31}P-n.m.r. ∂ (ppm) (D_2O/EDTA) -6.51 (d, γ-P), -10.46 (d, α-P), -21.99 (t, β-P).

Klenow Fragment - Standard Assay. 1 picomole of ^{33}P labelled primer (5'-end labelled using T4 polynucleotide kinase and [γ^{33}P] ATP 3000Ci/mmol, Amersham International plc) was hybridised to 2 picomoles of template in 2x Klenow buffer (100mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol). To this was added either 4 μM dNTPαS or 40 μM universal base triphosphate analogue or both. Five units of 3'-5' exonuclease free Klenow fragment of polymerase I were used per reaction. The solutions were incubated at 37°C for 30 minute. Primer alone, primer plus template and primer plus template plus enzyme controls were also carried out.

Klenow Fragment - Competition Assay. 1 picomole of ^{33}P labelled primer was hybridised to 2 picomoles of template 4 in 2x Klenow buffer. To this was added either 10 μM dNTP's with 0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 mM universal base triphosphate, or 20μM universal base triphosphate with 0, 0.04, 0.1, 0.2, 0.4, 1, 2, 4, 20 μM dNTP's. Five units of exonuclease free Klenow were used per reaction, and the reactions were incubated at 37°C for 30 minute. Primer alone, primer plus template and primer plus template plus enzyme controls were also carried out.

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 or a phosphor screen. The phosphor screen was analysed on a Molecular Dynamics Storm 860 imager.

Polymerase Chain Reactions (*Taq*). The 5'-triphosphates of four base analogues, 5-nitroindole, 3-nitropyrrole, 5-aminoindole and 5-formamidoindole were examined in a series of PCR reactions. The target for amplification was the amber suppressor gene, supF, present on the plasmid pCDM8 (Invitrogen). The sequences of the primers used were 5'-CAGCTGGATTACCGCGGTGT and 5'-CACACACAAGCAGGGAGCAG. The template was linearised at its unique Hind III site. Each 50 µl reaction contained: 50 µM of each natural dNTP, 0 µM, 250 µM, 500 µM, 1mM, 1.5mM or 2mM of the universal base triphosphate, 2.5 units *Taq* polymerase, 5 µl of 10xPCR buffer, 50 pmoles of primers and 5ng of the template. The reactions were overlaid with 35 µl of mineral oil. PCR reactions were carried out on a Techne PHC-3 apparatus, and cycling conditions were: denaturation at 96°C for 2 minutes (during which time *Taq* polymerase was added), followed by 30 cycles of: denaturing at 96°C for 5 seconds; annealing at 50°C for 5 seconds; extension time at 72°C for 0, 60 or 120 seconds and a final extension at 72°C for 5 minutes. Reaction products from all three time points for each analogue were analysed by electrophoresis on standard 2% agarose gels. The yield of expected product, 237 base pairs long, was determined as a function of the concentration of the analogue triphosphate and the extension time at 72°C. For each set of reactions a control without added template was run at the same time.

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