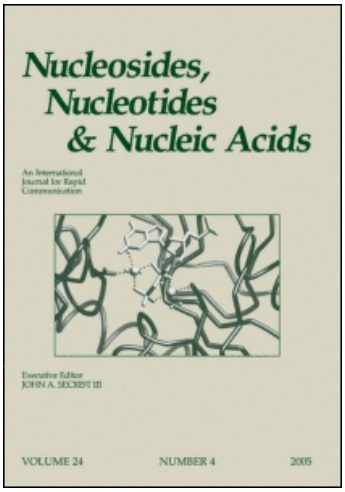

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ANALOGS OF GUANINE NUCLEOSIDE TRIPHOSPHATES FOR SEQUENCING APPLICATIONS

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ANALOGS OF GUANINE NUCLEOSIDE TRIPHOSPHATES FOR SEQUENCING APPLICATIONS

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ABSTRACT

We have synthesized more than 30 different deoxyribonucleosides and triphosphates with modifications either in the base or the phosphate moiety as analogs of 2′-dGTP for DNA sequencing applications. All the modified nucleoside triphosphates were tested as substrates for DNA polymerases, including Sequenase™ T7 DNA polymerase or Thermo Sequenase™ DNA polymerase. Two of the analogs, 7-ethyl-7-deaza-dGTP and 7-hydroxymethyl-7-deaza-dGTP meet our requirements as better sequencing reagents.

One of the most common difficulties in DNA sequencing is known as the "Compression" artifact. This apparently results when sequence-dependent secondary structures form in the DNA as it is being analyzed by gel electrophoresis (1). Fragments with these secondary structures migrate faster than similar-size fragments, which lack the secondary structure. There are several techniques used to eliminate these artifacts including electrophoresis at elevated temperature, the addition of higher concentration of urea and formamide to gels and use of nucleotide analogs. In particular, the dGTP analogs 2′-deoxyinosine-5′-triphosphate (dITP, **22**) and 7-deaza-2′-deoxyguanosine-5′-triphosphate (2) (dZTP, **8**) are commonly used

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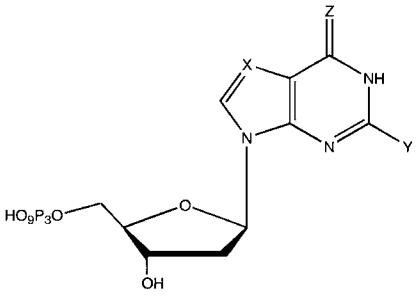
for sequencing. Both these analogs have specific problems, which prompted us to undertake this line of research.

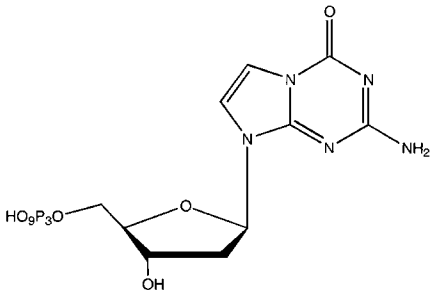
Although dITP has excellent compression resolving properties, eliminating essentially all compressions, the reactivity with both Sequenase™ T7 DNA polymerase and Thermo Sequenase™ DNA polymerase is low (10 to 20% that of dGTP), and uniformity of terminations in the sequencing reactions exhibit sequence specific variations, which can be severe. This makes sequence interpretation more difficult and certain sequencing applications impossible. 7-Deaza-2′-deoxyguanosine-5′-triphosphate has good reactivity with DNA polymerases and reasonable uniformity. However, it resolves only 75–80% of all compressions in slab gels and less in capillary electrophoresis where the denaturing conditions are weaker. Therefore, the goal of this research was to find an analog with better compression resolution than dZTP and better reactivity and uniformity than dITP.

In a research program directed towards finding better substrates for the DNA polymerase than dITP and better compression resolution properties than 7-deaza-dGTP, we have prepared a number of 2′-deoxynucleoside-5′-triphosphates as novel analogs of the guanine bases (Fig. 1). Most of the nucleosides were prepared by using literature procedures with minor modifications and the triphosphates were prepared using $P(O)Cl_3$ and bis(tri-*n*-butylammonium)pyrophosphate in a "one-pot, three-step" phosphorylation procedure (3). The compounds 6-thio dITP (**2**) and 6-dGTP (**3**) were prepared as previously reported (4). The nucleosides 2-bromo- and 2-fluoro-2′-deoxyinosine were synthesized according to the methods developed by Robins (5). 5-Aza-7-deaza-2′-deoxyguanosine (6) and 3,7-dideaza-2′-deoxyguanosine (7) were synthesized using minor modifications of published procedures. 7-Halogenated-7-deaza dGTP analogs have been previously reported (8). The alkynyl series of 7-deaza-2′-deoxyguanosine was prepared according to the literature methods (9,10). The synthesis of 7-alkyl- and 7-hydroxyalkyl-7-deaza-2′ deoxyguanosine as well as 7-substituted-7-deaza-2′-deoxyinosine nucleosides will be reported elsewhere (11). All these nucleoside triphosphates were tested for activity as substrates for DNA polymerase. The analog triphosphates that proved active were also tested for sequencing on templates with known compression artifacts and scored relative to dGTP, dZTP and dITP. Specifically, these analogs were used as substrates replacing dGTP in dye primer cycle sequencing reactions with Thermo Sequenase™ on double stranded pGEM 3Zf(+), starting at the −28 reverse primer. The ratio of an analog to ddGTP was varied in the sequencing reaction mixtures, which were the same as typically used in standard primer labeled sequencing reactions for dGTP or dZTP (12). The results of these experiments are given in Table 1.

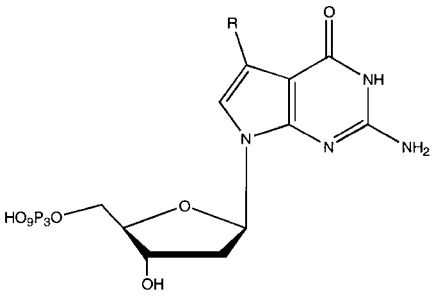
Three characteristics for each analog triphosphate are recorded in Table 1. Relative reactivity is a rough assessment of the compound as substrates for the exonuclease free Thermo Sequenase™ DNA polymerase. These values were determined from the optimal sequencing reaction ratios, reflecting competition between the analog and ddGTP. The comment word "stops" indicate the inability of the polymerase to extend past sites of multiple analog incorporation. Band uniformity is a term used for changes in the sequence specific incorporation of ddGTP, as



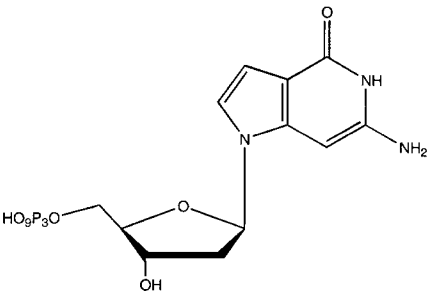
dGTP (**1**) X = N, Y = NH_2, Z = O
6-thio-dITP (**2**) X = N, Y = H, Z = S
6-thio-dGTP (**3**) X = N, Y = NH_2, Z = S
2-bromo-dITP (**4**) X = N, Y = Br, Z = O
2-fluoro-dITP (**5**) X = N, Y = F, Z = O
7-deaza-dGTP (**8**) X = CH, Y = NH_2, Z = O
dITP (**22**) X = N, Y = H, Z = O
7-propynyl-7-deaza-dITP (**23**) X = C-C≡CCH_3, Y = H, Z = O
7-propyl-7-deaza-dITP (**24**) X = C-$CH_2CH_2CH_3$, Y = H, Z = O



5-aza-7-deaza dGTP (**6**)



R = Cl (**9**), I (**10**), CH_3 (**11**), CH_2CH_3 (**12**), $CH_2CH_2CH_3$ (**13**), $CH(CH_3)_2$ (**14**), $(CH_2)_5CH_3$ (**15**), C≡CCH_3 (**16**), C≡$C(CH_2)_3CH_3$ (**17**), CH_2OH (**18**),CH_2CH_2OH (**19**), $CH_2CH_2CH_2OH$ (**20**), C≡CCH_2OH (**21**).



3,7-dideaza dGTP (**7**)

Figure 1.

observed by variations in band intensity. The values reported are the variance of band intensities normalized by an algorithm. Absolute uniformity is scored as zero. Compression scores record the analog's ability to relieve compression artifacts, the larger the number the greater the ability to relieve. The scores of zero for dGTP and four for dITP were arbitrarily set from a limited number of compression artifacts to ascertain the trend of this property.

The nucleoside triphosphates with modifications to the hydrogen bonding face (**2**–**7**) were all unsuitable for sequencing applications. All these compounds, with the exception of 6-thio dGTP (**3**), had very poor to no reactivity with the DNA polymerases tested. Surprisingly, the absence of N1(H) in a G analog, either by design (**6**) or by ionization (**5**) led to inactivity. The nucleotide, 2-F dITP (**5**) was found to have a pKa value of 5.2 as determined by UV absorbance titration and curve fitting. The optimal sequencing conditions for Thermo Sequenase™ DNA polymerase is at pH = 9.5. At this pH, extension with this analog is almost non-existent, however

Table 1. Results of Primer Labeled Sequencing Using Thermo Sequenase™ Polymerase

Compound	Relative Reactivity	Band Uniformity[1]	Compression Score[2]
1. dGTP	1	0.15	0
2. 6S-dITP	0.1(stops)		
3. 6S-dGTP	0.9(stops)	0.66	0
4. 2Br-dITP	0.1(stops)		
5. 2F-dITP	0.1 to 0.2 (at pH = 9.5 to 7.5)		
6. 5aza-7deaza-dGTP	0		
7. 3,7 dideaza-dGTP	0		
8. 7-Deaza-dGTP(dZTP)	1	0.19	1
9. 7-Chloro-dZTP	1	0.23	−1
10. 7-Iodo-dZTP	1.1	0.23	−1
11. 7-Methyl-dZTP	1	0.18	2
12. 7-Ethyl-dZTP	1	0.19	3
13. 7-Propyl-dZTP	0.85	0.21	3.5
14. 7-Isopropyl-dZTP	0.2(stops)		4
15. 7-Hexyl-dZTP	0.1(stops)		4
16. 7-Propynyl-dZTP	1.25	0.21	0
17. 7-Hexynyl-dZTP	0.6(stops)		1
18. 7-Hydroxymethyl-dZTP	1	0.16	1.5
19. 7-Hydroxyethyl-dZTP	0.2(stops)		
20. 7-Hydroxypropyl-dZTP	0.6	0.64	3.5
21. 7-Hydroxypropynyl-dZTP	0.5	0.28	0
22. dITP	0.2	0.33	4
23. 7-Propyl-7-deaza dITP	0.4	0.25	4
24. 7-Propynyl-7-deaza dITP	0.1 (stops)	0.3	4

[1]Lower numbers more uniform heights.
[2]Fewer compressions, higher score.

somewhat better activity is observed upon lowering the pH to 7.5. A related pH phenomenon is observed in the thermodynamic properties of duplexes containing 2-fluoro-2′-deoxyinosine (13).

Compound **7** is an example of the importance of the nitrogen at the N-3 position for polymerase substrate recognition (14). Although, 6-thio dGTP (**3**) exhibits good activity under our sequencing conditions, stops or pauses were observed at multiple runs of G incorporation. Such behavior renders this analog unsuitable as a replacement for dGTP in most sequencing applications (4).

Nucleotide derivatives of 7-deaza-2′-deoxyguanosine triphosphate with substitution at the 7-position (**9**–**21**) yield an exciting class of polymerase substrates. 7-Halogenated (8) (**9**–**10**) as well as 7-propynyl dZTP (**16**) are excellent substrates, which easily sequence out to 500 bases with intermediate band uniformity. Using these analogs unfortunately made compression artifacts as bad or worse than dGTP. It is likely that the structural properties that impart duplex stability to double stranded DNA containing these bases (9,15), are also responsible for stabilizing the structures which result in compression artifacts. Increasing the size of the substituent on the triple bond as in compounds **17** and **21** lowered substrate activity.

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with 7-hexynyl dZTP (**17**) exhibiting abortive behavior at multiple G incorporation sites.

Alkyl derivatives of 7-deaza-2′-deoxyguanosine triphosphate (**11**–**15**) display a trend where increasing size gives increasing compression resolution but decreasing polymerase reactivity. 7-Methyl (**11**) and 7-ethyl dZTP (**12**) have a relative reactivity that matches the parent compound (**8**). 7-Propyl dZTP (**13**) shows less reactivity and more background noise in primer sequencing reactions. However, the ability to resolve compression artifacts is in the order: propyl > ethyl > methyl > hydrogen. The increase in steric bulk at the 7-position, such as with isopropyl (**14**) or chain length as with hexyl (**15**) diminishes the reactivity greatly and elongation at specific sites is terminated.

The hydroxyalkyl series (**18**–**20**) has very interesting polymerase behavior. 7-Hydroxymethyl-7-deaza-2′-deoxyguanosine-5′-triphosphate (**18**) with reactivity and uniformity as good as 2′-dGTP (**1**) and a better compression score than dZTP (**8**) was by far the best sequencing analog tested. Under difficult template conditions, this analog out-performed any other analog tested in this study. Extending the alkyl chain by one methylene group, to hydroxyethyl (**19**), led to poor reactivity and polymerase stops. Hydroxypropyl dZTP (**20**) did not terminate polymerase extentions but has large uniformity problems. We believe that the hydrogen bonding capability in these analogs is responsible for their polymerase behavior and more studies are underway to understand this class of molecules.

Two compounds (**23**–**24**) were prepared as analogs of 7-substituted-7-deaza-dITP. Initial results indicate that the 7-propynyl derivative (**23**) is as good or better a substrate as 2′-dITP for Thermo Sequenase™ DNA polymerase. The propynyl substitution also helps band uniformity while retaining an excellent compression score. Further testing of this analog will be done to evaluate its utility for routine sequencing with both dyelabeled primers and dye-labeled terminators in slab gels and capillary electrophoresis. The alkyl addition to 7-deaza-7-dITP, namely 7-Propyl-7-deaza-dITP (**24**) worsened reactivity of an already poor substrate.

In conclusion, we have synthesized a number of analogs of 2′-deoxyguanosine triphosphate. Our results show that 7-ethyl, 7-propyl, and 7-hydroxymethyl-7-deaza-2′-deoxyguanosine triphosphates are good replacements for 2′-dGTP in sequencing reactions, eliminating compression artifacts to a greater degree than the parent compound, 7-deaza-2′-deoxyguanosine triphosphate. We are now in the process of determining why certain analogs relieve compression artifacts better than others do and examining other biological activities for our novel nucleosides and nucleotides.

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