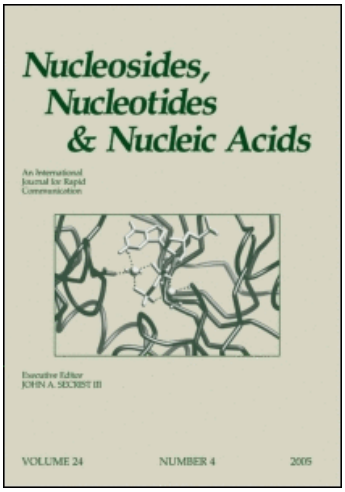

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NOVEL NUCLEOSIDE TRIPHOSPHATE ANALOGS FOR THE ENZYMATIC LABELING OF NUCLEIC ACIDS

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ABSTRACT

We have evaluated several novel nucleotide analogs suitable for enzymatic labeling of nucleic acid targets for a variety of array-based assays. Two new reagents in particular, a C4-labeled 1-(2′,3′-dideoxy-β-D-ribofuranosyl) imidazole-4-carboxamide 5′-triphosphate **5** and an N1-labeled 5-(β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione 5′-triphosphate **3**, were found to be excellent substrates for labeling with terminal deoxynucleotidyl transferase and T7 RNA polymerase, respectively.

High-density DNA probe arrays are proving to be a valuable tool for hybridization- based genetic analysis (1). These assays currently involve labeling of nucleic acid molecules with fluorescent or otherwise detectable molecules in order to detect hybridization to the arrays (2). We have evaluated several novel nucleotide analogs suitable for enzymatic labeling of nucleic acid targets for a variety of array-based assays. Our goal was to identify reagents suitable for use in two particular labeling procedures: (i.), 3′-end labeling of fragmented, DNA targets with *terminal deoxynucleotidyl transferase (TdT);* and (ii.), template-directed internal labeling of RNA targets using *in vitro* transcription with *T7 RNA polymerase (T7)*.

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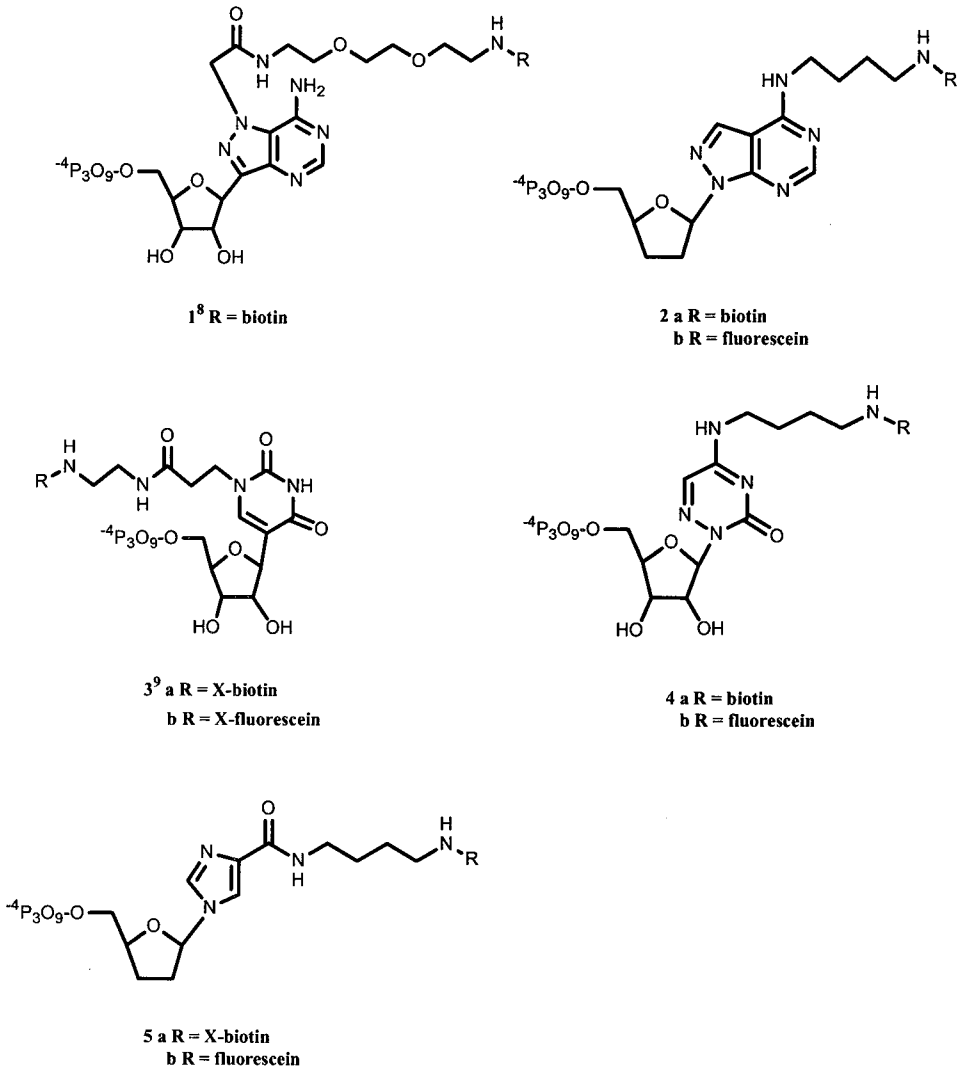

Figure 1.

The analogs selected for this study were nucleotides in which the native heterocyclic base was substituted with the following: 1-(imidazole-4-carboxamide), 2-(1,2,4-triazine-3,5[2H,4H]-dione), 5-(2,4[1H,3H]-pyrimidinedione), 3-(pyrazalo-[4,3-d]pyrimidine), and 1-(pyrazalo-[3,4-d]pyrimidine). Labeled versions of these molecules were designed and synthesized (**3**) for evaluation of their relative incorporation efficiency and suitability for use in array-based assays.

A rapid and quantitative assay using HPLC (4), indicated that several analogs (Fig. 1) were good substrates for *TdT* labeling of DNA The data are summarized in Table 1. The fluorescein- and biotin-labeled 1-(2′,3′-dideoxy-β-D-ribofuranosyl)

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Table 1. *TdT* Incorporation Efficiencies (HPLC) of Analog Triphosphates Labeled with Biotin and 5-carboxyfluorescein

Labeled NTP Analog	*TdT* Incorporation (%)	
	40 u	160 u
1	54	94
2a	48	100
b	58	98
3a	98	96
b	61	88
4a	47	85
b	67	98
5a	100	–[10]
b	60	97

imidazole-4- carboxamide 5′-triphosphates **5a** and **5b** (5) were incorporated most efficiently by *TdT*, and showed excellent performance when used in GeneChip® hybridization assays. Table 2 summarizes the assay performance results using **5a**, **b** in the GeneChip® p53 array assay. The data indicate that the signal intensities are generally higher and the base calling accuracy for both strands is equivalent to that obtained with the corresponding control reagents, N6 fluorescein and biotin-labeled ddATP (6).

The N1-fluorescein-labeled 5-(β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione 5′-triphosphate **3b**, proved to be the best substrate for *in vitro* transcription labeling of target RNA using *T7 RNA polymerase*. HPLC labeling assay data (not shown) indicated that this nucleotide analog could be incorporated with equivalent or better efficiency than the control, in this case, C5-fluorescein-labeled UTP (6). The functional hybridization performance in a commercial HIV GeneChip® assay was equivalent to that of the control, as indicated by the average hybridization intensity and call accuracy data in Table 3 (7).

In conclusion, it was found that *TdT* was generally tolerant of changes in base structure, and we have independently confirmed that ribonucleotides are consistently incorporated about as efficiently as 2′-deoxy, and 2′,3′-dideoxynucleotides

Table 2. Hybridization Data for Analog **5** in a p53 GeneChip® DNA Functional Assay (11)

Labeled NTP Analog	Median Signal Intensity(cps) for 1300 Calls	Call Accuracy(%)	
		Sense	Antisense
*Bio-ddATP(control)	5175	95.0	97.8
FI-ddATP (control)	1226	81.0	92.9
5a	8278	94.2	97.2
5b	1328	85.9	90.7

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Table 3. Hybridization Data for Analog **3b** in an HIV GeneChip® RNA Functional Assay (12)

Labeled NTP Analog	Median Signal Intensity(cps) for 1041 Calls	Call Accuracy (%)	
		Sense	Antisense
FI-12-UTP (control)	2742	97.6	96.6
3b	2437	96.7	96.2

(data not shown). In contrast, *T7 RNA polymerase* was relatively intolerant of changes in the heterocyclic base, with the exception of **3b**. Thus, two new reagents, the C4-labeled 1-(2′,3′-dideoxy-β-D-ribofuranosyl) imidazole-4-carboxamide 5′-triphosphates **5**, and the N1-labeled 5-(β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione 5′-triphosphates **3**, were found to be excellent substrates for labeling with *TdT* and *T7 RNA polymerase*, respectively. In addition, data was obtained which confirms that the use of these nucleotide analogs in standard GeneChip® assays can provide assay performance equivalent to that obtained using the conventional labeling reagents.

ACKNOWLEDGMENTS

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1. Lipshutz, R.J.; Fodor, S.; Gingeras, T.R.; Lockhart, D.J. *Nature Genet.* **1999**, *21*, 20–24, and references cited therein.
2. McGall, G.H. in *Biochip Arrays and Integrated Devices for Clinical Diagnostics* (Hori, H. editor), International Business Communications Library Series, **1997**, Chapter 2, and references cited therein.
3. The synthesis of these analogs is too lengthy to appear here, and will be described in a separate paper. All spectral characterization data were consistent with the assigned structure of intermediates and the final nucleoside triphosphates.
4. The incorporation efficiency for TdT labeling of $d(pT)_{16}$ was determined by IE-HPLC analysis, from the ratio of peak areas of the unlabeled and labeled oligonucleotide (absorbance detection at 260 nm). For IVT labeling of RNA using T7 RNA polymerase, a 1.2 kb transcript from a crude IVT reaction was resolved from unincorporated nucleotides by IE-HPLC, and the incorporation of the fluorescein was determined from peak area ratios by simultaneous absorbance detection of the RNA transcript at 260 nm and 495 nm.

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5. The desired N1-β isomer was identified by comparison of NMR chemical shift and NOE data with that reported for related nucleosides (Pochet, S.; Dugue', L.; Meiera, A; Marlie're, P. *Biorg. Med. Chem. Lett.* **1995**, *5*, 1679–1684).
6. Commercially available from NEN.
7. Labeling reaction conditions were not optimized.
8. Prepared from formycin A, following procedures similar to those reported in reference 10.
9. Prepared from β-D-ribofuranosyl-2,4(1H,3H)-pyrimidinedione, according to Muhlegger, K.; et.al. **1996**, WO 96/28640.
10. These values could not be determined reliably because of competing disproportionation activity at higher enzyme concentrations.
11. The labeling of target DNA followed the recommended procedure for the commercially available assay, except that 4-times the amount of *TdT* (100 u) was used to that for the control (25 u).
12. The labeling of RNA target with **3b** followed the recommended procedure for the commercially available assay, except that the UTP:analog triphosphate ratio was decreased from 1:2 to 1:5.

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