40. Three Novel Spin-Labeled Substrates for Enzymatic Incorporation into Nucleic Acid Lattices

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The synthesis of one C(4)-spin-labeled uridine-5′-diphosphate (C(4)-UDP and two nitroxide-containing 2′-deoxyuridine-5′-triphosphate (dUTP) analogs are reported. The C(4)-UDP derivative was incorporated into copolymers by polynucleotide phosphoxylase (PNPase); one of the two dUTP analogs, substituted at position C(4), was a good substrate for TdT, whereas the other one, substituted at position C(5), served as substrate for *E. coli* DNA polymerase (Pol I).

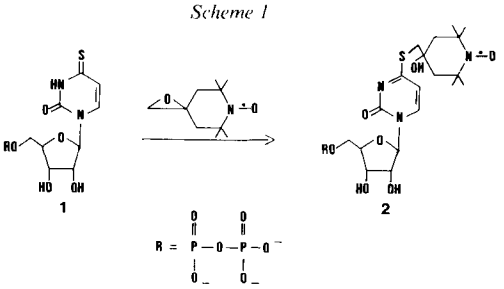
Introduction. – The application of the spin-labeled nucleic acids to investigate problems of biological interest has been well established in this as well as other laboratories [1–4]. Of great interest is the synthesis of spin-labeled ribo- or deoxyribonucleotides which can serve as substrates for enzymes such as PNPase[1]), TdT, and Pol I in order to prepare enzymatically spin-labeled nucleic acids. The former two enzymes have no template requirement and were used in this laboratory to prepare a variety of RNA and DNA homopolymers containing different amounts of enzymatically incorporated spin-labeled ribo- or deoxyribonucleotides [5] [6]. Pol I, on the other hand, has a template requirement and is well suited to make template specific spin-labeled polynucleotides.

We wish to report here the synthesis of the three novel spin-labeled nucleotides pplss^{4}U (**2**), pppls^{4}dU (**6**), and pppDUAT (**12**). pplss^{4}U and pppls^{4}dU served as substrates for PNPase and TdT, respectively, and pppDUAT was readily incorporated by Pol I. The biophysical properties of (lss^{4}U,U)$_n$, (ls^{4}dU,dT)$_n$ and (DUAT,dT)$_n$ were described in [7].

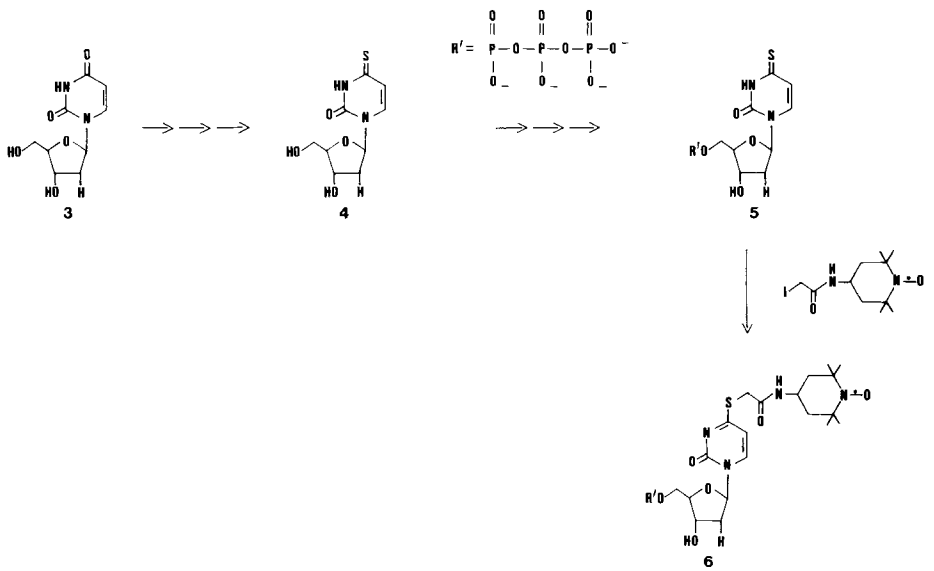
Results and Discussion. – In *Scheme 1* the synthesis of the modified ribonucleotide diphosphate **2** from **1** is outlined. Diphosphate **2** was separated from unreacted starting materials by paper chromatography and used as a substrate for PNPase without further purification. The same ε as already calculated for a similar spin-labeled nucleotide ppls^{4}U [5] was used to determine the concentration of **2** needed for its copolymerization with uridine-5′-diphosphate (ppU).

[1]) *Abbreviations:* PNPase, polynucleotide phosphorylase; TdT, terminal deoxynucleotidyl transferase; Pol I, *E. coli* DNA polymerase I, ls^{4}U, 4-{[*N*-(2,2,6,6-tetramethyl-4-piperidyl-1-oxy)carbamoylmethyl]thio}uridine; p, pp, and ppp, 5′-monophosphate, 5′-diphosphate, and 5′-triphosphate, respectively; α-iodoacetamido-Tempo, 4-(α)-iodoacetamido-2,2,6,6-tetramethylpiperidino-1-oxy; lss^{4}U, 4-{[(4-hydroxy-2,2,6,6-tetramethyl-4-piperidyl-1-oxy)methyl]thio}uridine; ls^{4}dU, 4-{[N-(2,2,6,6-tetramethyl-4-piperidyl-1-oxy)carbamoylmethyl]thio}-2′-deoxyuridine; DUAT, 5-(2,2,6,6-tetramethyl-4-piperidyl-1-oxy)-*N*-allylformamido]-2′-deoxyuridine; (lss^{4}U,U)$_n$, copolymer of lss^{4}U and uridine; (ls^{4}dU,dT)$_n$, copolymer of ls^{4}dU and thymidine; (DUAT,dT-dA)$_n$, alternating copolymer of deoxyadenosine with thymidine or DUAT; epoxy-Tempo, 5,5,7,7-tetramethyl-1-oxa-6-azaspiro[2.5]oct-6-yloxy.

Scheme 1



Scheme 2

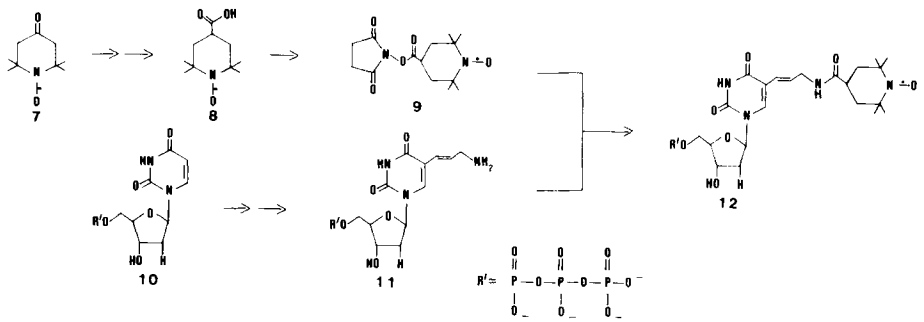


The 4-substituted 2′-deoxynucleotide analog, **6**, was prepared from the nucleotide **3** as shown in *Scheme 2*. 2′-Deoxyuridine (**3**) was first converted to **4** according to published procedures [8–10]. Next, **4** was phosphorylated to **5** by similar methods to those already described [6] [11] in 25% overall yield based on the amount of **4** used. Alkylation of **5** with the activated nitroxide-containing compound, α-iodoacetamido-Tempo, yielded the desired derivative **6**.

Preparation of the 5-substituted Pol I substrate **12**, was achieved by a combination of procedures as shown in *Scheme 3*. The carboxylated spin label 2,2,6,6-tetramethyl-1-oxy-piperidine-4-carboxylate (**8**) was synthesized from **7** according to *Rauckman et al.* [12] and converted to the more active condensing agent **9** after reaction with *N*-hydroxysuccinimide [13]. The modified nucleotide **11** was prepared from **10** by a published procedure [14], but without the HPLC purification step, since it was found more advantageous to purify (HPLC) the final product **12**, which was obtained after condensation of **11** with **9**.

The nucleotides **2**, **6**, and **12** were all well resolved from side products and unreacted starting materials during isolation by paper chromatography. Compound **2** was used

Scheme 3



without further purification, since it was found to be a good substrate for PNPase in this form. Another similarly 4-substituted mononucleoside diphosphate [5] had been incorporated by PNPase after purification by only paper chromatography. The substituted nucleoside triphosphates **6** and **12**, on the other hand, required further purification by *DEAE-Sephadex* column chromatography and HPLC before being well incorporated by TdT and Pol I, respectively.

The nucleotides **2**, **6**, and **12** were characterized by ^{1}H-NMR spectroscopy. Previously reported spectra of nitroxide-containing nucleic-acid building blocks [5] [15–17] were obtained after masking of the free radical by reduction with $Na_2S_2O_4$. Some of these values are again given in the *Table* for comparative purposes. In the reduced 4-substituted RNA analog, pls^{4}U, the H-atoms at the C(1′) position of the sugar were observed as a multiplet at 5.8 ppm, whereas doublets at 6.5 and 8.1 ppm were assigned to the H-atoms at C(5) and C(6) of the base, respectively. Another spectrum of the unreduced pls^{4}U was recorded with about 10% of the material necessary for the reduction method by time averaging for about 1 h. The only difference between the two spectra was a broadening of the peaks of the one containing the paramagnetic nitroxide. The chemical shifts **2**, **6**, and **12** are also reported in the *Table*. Compound **2** showed resonances centered at the same positions as pls^{4}U, except those for H–C(5) and H–C(6) were more broadened which reflects the closer proximity of the paramagnetic probe in the case of **2** as compared to pls^{4}U. The only difference between the spectrum of pls^{4}U and its deoxy derivative pls^{4}dU [18] or its DNA analog **6** was in the H–C(1′) peak, which was shifted slightly downfield.

Table. *^{1}H-NMR Chemical Shifts* (in ppm) *for H-Atoms of Unreduced or Dithionite-Reduced Spin-Labeled Nucleotide Analogs Downfield from TMS*

Compound abbreviation	Pyrimidine		Sugar
	H–C(5)	H–C(6)	H–C(1′)
pls^{4}U[a])	6.5 (*d*)	8.1 (*d*)	5.8 (*m*)
pls^{4}U[b])	6.6 (br.)	8.1 (br.)	5.8 (br.)
pplss^{4}U[b]) (**2**)	6.6 (br.)	8.1 (br.)	5.8 (br.)
pls^{4}dU[b])	6.55 (br.)	8.0 (br.)	6.05 (br.)
pppls^{4}dU[b]) (**6**)	6.6 (br.)	8.1 (br.)	6.05 (br.)
pppDUAT (**12**)	–	7.3 (br.)	5.7 (br.)

[a]) Dithionite reduced according to [5].

[b]) Unreduced, about 0.15 mg of compound in 400 µl of D_2O.

The 5-substituted derivative **12** showed, as expected, no resonance in the H–C(5) region and showed only two broadened peaks in the areas of interest. The one due to H–C(1') was observed at 5.7 ppm and the other, due to H–C(6) at 7.3 ppm.

Compounds **2**, **6**, and **12** were used as substrates for PNPase, TdT, and Pol I, respectively. With PNPase an input ratio of uridine-5'-diphosphate (ppU) to **2** of 20:1 resulted in the approximately 5%-labeled copolymer $(lss^4U,U)_n$. With a pppdT/**6** ratio of 20:1, the nitroxide-containing substrate was incorporated into the nucleic-acid lattice $(ls^4dU,dT)_n$ to an extent of 2 to 3%. The alternating copolymer (DUAT, dT-dA)$_n$ was obtained with Pol I and (dA-dT)$_n$ as template. With an equimolar input ratio pppdT/**12**, the amount of labeled bases per base pairs was of the order of 6%.

Previously, we reported the synthesis of modified ribonucleoside-5'-diphosphates with the spin label attached to position 5 and separated from the base with tethers of varying length [5] [16]. In modifying the ribonucleotide at position 4, we first synthesized pps^4U [5], where the nitroxide was relatively far away from the base. The synthesis of **2** is another example of a ribonucleotide modified at position 4, but this time the label is closer to the base than in pps^4U. In the case of the deoxyribonucleoside-5'-triphosphates, we reported so far the preparations of substrates modified in position 5 [17]. Compound **6** is our first example of a deoxyribonucleotide spin-labeled at position 4. The percentage of enzymatic incorporation of the newly described spin-labeled substrates into nucleic-acid lattices suggests that these modified nucleotides are also of considerable value for the synthesis of macromolecular probes, which can subsequently be detected by ESR in a variety of biological systems.

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Experimental Part

General. Chemicals, with the exception of epoxy tempo, were purchased either from *Aldrich, Alfa, Baker, Fisher*, or *Sigma*. Epoxy-tempo was obtained from the *Josef Stefan Institute* in *Yugoslavia*. Enzymes were procured from *Pharmacia P-L Biochemicals*. Prep. paper chromatography was performed on *Whatman 3MM* paper with abs. EtOH/1M $AcONH_4$ (7:3 *v/v*). Final mononucleotide purification was usually achieved with *DEAE-Sephadex A-25 (Pharmacia)* packed in a 15 cm × 16 mm column and/or HPLC on an instrument from *Bioanalytical Systems Inc.* with a *Bondapak* C_{18} column using a step gradient of 50 mM $(NH_4)_3PO_4$ and EtOH/H_2O (1:1, *v/v*) for elution. Purification of copolymers was accomplished with a 75 cm × 16 mm *Sephacryl S-200* column *(Pharmacia)* at 4° equilibrated with 0.04M NH_4HCO_3. ESR spectra were obtained on a *Varian E-104* spectrometer interfaced to an *Apple II* computer. ^{1}H-NMR spectra were recorded on a *Nicolet NTC 300 FT* instrument.

4-{[(4-Hydroxy-2,2,6,6-tetramethyl-4-piperidyl-1-oxy)methyl]thio}uridine-5'-diphosphate (**2**). To a stirred soln. of 4 mg (0.0074 mmol) of *4-thiouridine-5'-diphosphate* (**1**) in 0.3 ml of 0.5M KH_2PO_4 buffer (pH 7.8) at r.t. were added 10 mg (0.054 mmol) of epoxy-tempo which had been dissolved in 0.3 ml of acetone. After stirring for 3 h, the mixture was streaked onto *Whatman 3MM* paper. The chromatogram was developed and the band corresponding to **2** (R_f 0.56) was eluted from the paper with H_2O and concentrated by lyophilization. The yield was 0.0021 mmol. UV (pH 7.0): 303. ^{1}H-NMR: see the *Table*.

4-Thio-2'-deoxyuridine (**4**). *2'-Deoxyuridine* (**3**; 480 mg, 2.1 mmol) was converted to **4** according to the procedures of *Scheit* [8], *Fox et al.* [9], and *Verheyden et al.* [10]. Purification by prep. TLC with MeOH/$CHCl_3$ (1:9, *v/v*) and recrystallization from abs. EtOH yielded 230 mg (0.94 mmol) of pure **4**. UV (pH 7.0): 331 (22,000).

4-Thio-2'-deoxyuridine-5'-triphosphate (**5**). Compound **4** (25 mg, 0.10 mmol) was phosphorylated as described in [11] using the monofunctional agent 2,2,2-tribromoethylphosphoromorpholino chloridate. The methods of isolation and purification were analogous to those used by *Toppin et al.* [6]. UV (pH 7.0): 331 (22,000).

4-{[N-(2,2,6,6-Tetramethyl-4-piperidyl-1-oxy)carbamoylmethyl]thio}-2'-deoxyuridine-5'-triphosphate (**6**). To 5 mg (0.01 mmol) of **5** in 0.3 ml of 0.5M KH_2PO_4 buffer (pH 7.8) were added 20 mg (0.06 mmol) of α-iodoacetamido-

Tempo which had been dissolved in 0.3 ml of acetone. The soln. was stirred for 3 h, and then **6** was isolated by prep. paper chromatography (R_f 0.34), followed by *DEAE-Sephadex* column chromatography and HPLC. The yield was 0.007 mmol. UV (pH 7.0): 303 (11,700). ^{1}H-NMR: see the *Table*.

2,5-Dioxopyrrolidinyl 2,2,6,6-Tetramethyl-1-oxypiperidine-4-carboxylate (**9**). *2,2,6,6-Tetramethyl-1-oxypiperidin-4-one* (**7**; 5.0 g, 0.03 mol) was converted to *2,2,6,6-tetramethyl-1-oxypiperidine-4-carboxylic acid* (**8**) according to *Rauckman et al.* [12]. The yield was 1.9 g. The acid **8** (500 mg, 2.5 mmol) was stirred with 288 mg (2.5 mmol) of *N*-hydroxysuccinimide in the presence of 515 mg (2.5 mmol) of dicyclohexylcarbodiimide in 20 ml of dry DMF for 18 h at 50°. The suspension was centrifuged, and 705 mg of **9** were isolated from the supernatant after prep. TLC with MeOH/$CHCl_3$ (1:19, *v/v*).

5-[1-(3-Aminoallyl)]-2'-deoxyuridine-5'-triphosphate (**11**). Compound **11** (0.038 mmol) was obtained after modification of 57 mg (0.12 mmol) of **10** according to the procedure of *Langer et al.* [14]. Compound **11** was obtained after *DEAE-Sephadex* chromatography and was used in the next step of the reaction without further purification.

*5-[(2,2,6,6-Tetramethyl-4-piperidyl-1-oxy)-*N*-allylformamido]-2'-deoxyuridine-5'-triphosphate* (**12**). To 0.02 mmol of **11** in 2 ml of 0.1M $Na_2B_4O_7$ buffer (pH 8.7) were added 12 mg (0.04 mmol) of **9** in 0.3 ml of DMF. The soln. was stirred at r.t. for 5 h. Compound **12** was purified by prep. paper chromatography (R_f 0.45), followed by *DEAE-Sephadex* chromatography and HPLC. The yield was 0.014 mmol. ^{1}H-NMR: see the *Table*.

Preparation of $(ls^4U,U)_n$. Compound **2** was copolymerized with ppU in the presence of PNPase according to a procedure described in [5]. The average molecular weight of $(ls^4U,U)_n$ was 100,000–200,000 with a ls^4U/U ratio of 0.005–0.01.

Preparation of $(ls^4dU,dT)_n$. TdT was used to copolymerize **6** with pppdT using the same approach as described in [6]. The amount of $ppls^4dU$ in a stock soln. was calculated based on an ε of 11,700 at 303 nm, the same value as previously determined for $ppls^4U$ [5]. The average molecular weight of $(ls^4dU,dT)_n$ was 100,000–200,000 with a ls^4dU/U ratio of 0.01–0.03.

Preparation of Copolymer $(DUAT,dT\text{-}dA)_n$. A reaction mixture containing 89 µl of 1M KH_2PO_4 buffer (pH 7.5), 12 µl of 10 mM dithiothreitol, 63 µl of 0.12M $MgCl_2$, 1.1 ml of H_2O, 0.5 OD units (260) of $(dA\text{-}dT)_n$, 0.55 µmoles of dATP, 0.27 µmol of pppdT and varying amounts of **12** were preincubated at 37° for 10 min before adding 35 units of Pol I. The polymerization reaction was monitored at 260 nm, and the reaction terminated immediately after the absorbance would reach a minimum by adding 0.4 ml of 3M NaCl/0.04M Na_2EDTA. Deproteinization and isolation of the spin-labeled alternating copolymer was achieved by the methods described in [19].

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