194. Synthesis of Unique Spin-Labeled Nucleic Acids by Combined Enzymatic and Chemical Approaches

by **Charles R. Toppin, Ingrid E. Thomas** and **Albert M. Bobst***

Department of Chemistry, University of Cincinnati, Cincinnati, Ohio 45221, U.S.A.

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Summary

The synthesis of uridine-5′-diphosphate analogs, spin-labeled either at C(4) or C(5) is reported as well as their enzymatic incorporation into ribonucleic acids, some of which had previously been shown to be potent interferon inducers upon annealing with poly(inosinic acid). Also, the synthesis of spin-labeled poly(cytidylic acid) obtained by chemical acylation is presented.

Introduction. – Nitroxide spin-labels have been extensively used in recent years for studying the interactions in many different biological systems [1]. Spin-labeled nucleic acids, either enzymatically synthesized with PNPase[1]), terminal deoxynucleotidyl transferase, or prepared directly by limited chemical modification of nucleic acids, have been used in this laboratory to monitor nucleic acid-nucleic acid and nucleic acid-protein interactions [2–4].

We wish to report the synthesis of spin-labeled polyribonucleotides whose pyrimidine bases contain the nitroxide radical in a well-defined position with respect to the base as well as the preparation of spin-labeled $(C)_n$, $(lC,C_x)_n$, which was obtained by chemical acylation of $(C)_n$. The site specificity was achieved by first synthesizing 4- or 5-nitroxide-labeled uridine diphosphate analogs and then copolymerizing the modified nucleotides with uridine- or cytidine-diphosphate in the presence of PNPase. The interferon-inducing capability of some of the spin-labeled polyribonucleotides reported here was determined upon annealing with $(I)_n$ [5].

Results and Discussion. – The 5-substituted uridine-5′-diphosphate derivative **4** was obtained from **1**, according to *Scheme 1*. The *S*-substituent was introduced into

[1]) Abbreviations: PNPase, polynucleotide phosphorylase; $(C)_n$, poly(cytidylic acid); $(I)_n$, poly(inosinic acid); α-iodoacetamido tempo, 4-(α)-iodoacetamido-2,2,6,6-tetramethylpiperidino-1-oxy; $(RUTT,U)_n$, copolymer of RUTT and uridine; ls^4U, 4-{[*N*-(2,2,6,6-tetramethyl-4-piperidyl-1-oxy)-carbamoylmethyl]thio}uridine; $(ls^4U,U)_n$, copolymer of ls^4U and uridine; $(ls^4U,C)_n$, copolymer of ls^4U and cytidine; $(lC,C_x)_n$, $(C)_n$ chemically modified with the anhydride of 3-carboxy-2,2,5,5-tetramethylpyrrolinyl-1-oxy; RUTT, 5-{[*N*-(2,2,6,6-tetramethyl-4-piperidyl-1-oxy)carbamoylmethyl]thio}uridine; p or pp, 5′-monophosphate or 5′-diphosphate.

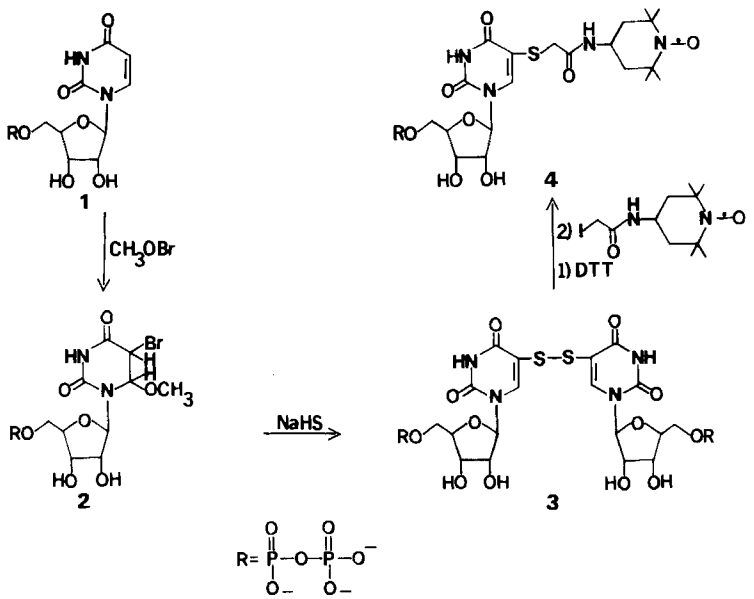
Table 1. *^{1}H-NMR Chemical Shifts (in ppm) for H-Atoms of Dithionite-Reduced Spin-Labeled Uridine-5′- Monophosphate Analogs Downfield from TMS*

Compound Abbreviation	Piperidine			Leg	Pyrimidine		Sugar	
	CH	CH_2	CH_3	CH_2	H–C(5)	H–C(6)	H–C(1′)	H–C(2′), H–C(5′)
pRUTT	3.05–3.4 (*m*)	1.8 (*d*)	1.2 (*s*), 1.3 (*s*)	3.05–3.4 (*m*)	–	8.0 (*s*)	5.7 (*d*)	3.9–4.5 (*m*)
pls^{4}U	3.7–4.4 (*m*)	1.8 (*d*)	1.2 (*s*), 1.3 (*s*)	3.7–4.4 (*m*)	6.5 (*d*)	8.1 (*d*)	5.8 (*m*)	3.7–4.4 (*m*)

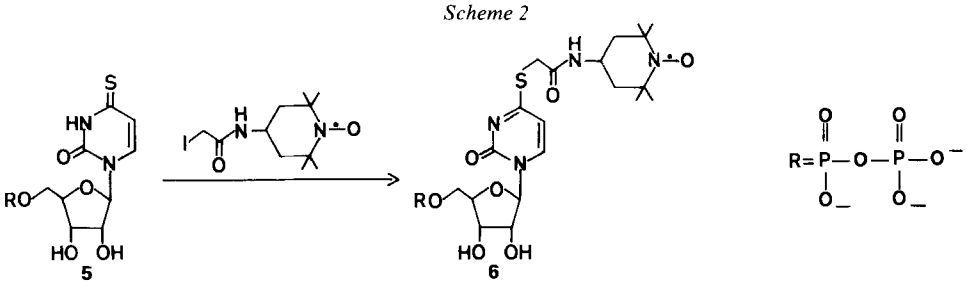
2 as described in [6] and the thiolated nucleotide was isolated as disulfide **3**. Compound **3** was quantitatively converted to **4** by alkylation with α-iodoacetamido tempo in the presence of dithiothreitol [7]. For the purpose of characterization the 5′-monophosphorylated analog of **4** was also synthesized. The ^{1}H-NMR data of this derivative in its reduced form [8] in D_2O are summarized in *Table 1*. Both, the monophosphate derivative of **4** and the diphosphate **4**, yielded the same digestion product with bacterial alkaline phosphatase and the resulting nucleoside of **4** gave the expected molecular-ion peak by electron-impact mass spectrometry.

In *Table 1* the singlet at 8.0 was assigned to H–C(6) and the doublet at 5.7 ($J(1,2)=4$) to H–C(1′). The unresolved peaks of the sugar protons occurred between 3.9 and 4.5. The 12 non-equivalent ring CH_3-protons were observed as two singlets at 1.2 and 1.3. A comparison of the values shown in *Table 1* with the published data of some other C(5)-substituted spin-labeled uridine analogs [8] conclusively indicate that the attachment of the spin-label occurs in position 5 and that there is one nitroxide per base.

Scheme 1



Scheme 2



In *Scheme 2* the synthesis of the 4-substituted derivative **6** from **5** is outlined. For the purpose of NMR characterization the 5′-monophosphorylated derivative of **6** was synthesized and the data are listed in *Table 1*. Both, **6** and its monophosphorylated derivative, gave the same digestion product with bacterial alkaline phosphatase. In *Table 1* the doublet centered at 8.1 ($J(1,2)=8$) was assigned to the H–C(6), and the other doublet at 6.5 ($J(1,2)=7$) to H–C(5). The 2 singlets at 1.2 and 1.3 were assigned to the 12 non-equivalent piperidine CH_3-protons.

The enzymatic formation of $(RUTT,U)_n$, $(ls^4U,U)_n$, and $(ls^4U,C)_n$ was achieved with PNPase according to [9] [10] and is summarized in *Scheme 3*. The properties of the spin-labeled polyribonucleotides are shown in *Table 2*. The molecular weight of the copolymers were all in the 100,000 dalton range and the amount of labeled residues in the polynucleotide chain strongly depended on the input ratio of unmodified to modified nucleoside-5′-diphosphate. It is apparent from *Table 2* that the 4-substituted derivatives were considerably better incorporated by PNPase than the 5-substituted ones.

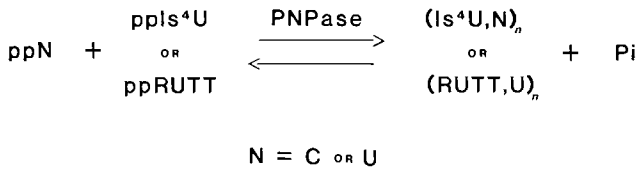
In *Scheme 4* the chemical acylation of $(C)_n$ with the anhydride of 3-carboxy-2,2,5,5-tetramethylpyrrolinyl-1-oxy to form $(lC,C_x)_n$ is outlined. Depending on the reaction conditions x was of the order of 16 and 1000 as determined by ESR using standard procedures [11]. As already noted [5] $(lC,C_x)_n$ is not very stable and

Table 2. *Molecular Weights, Percent Yields and Labeling of Macromolecular RNA Formed from Copolymerization of Unmodified and Spin-Labeled Nucleoside-5′-diphosphates at Various Input Ratios*

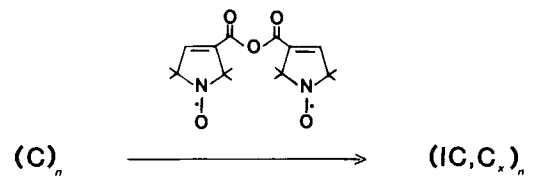
Polymer	N/N*[a])	N*/N[b]) ($\times 10^2$)	Yield (%)	M.W. ($\times 10^3$)
$(RUTT,U)_n$	4	1.5	30–40	100–200
$(RUTT,U)_n$	12	1	40–50	100–200
$(RUTT,U)_n$	25	0.5	20–30	100–200
$(ls^4U,U)_n$	7	12	20–30	75–150
$(ls^4U,U)_n$	10	10	30–40	100–200
$(ls^4U,U)_n$	14	6	30–40	100–200
$(ls^4U,U)_n$	29	3	30–40	75–150
$(ls^4U,C)_n$	7	12	20–30	> 100
$(ls^4U,C)_n$	14	4	30–40	> 100
$(ls^4U,C)_n$	28	2–3	20–30	> 100

[a]) Input ratio of unmodified nucleoside-5′-diphosphate.
[b]) Ratio of labeled to unlabeled residues in polymer.

Scheme 3



Scheme 4



has the tendency to release some of the nitroxide radicals, when it is incubated in neutral buffers for several days.

In conclusion, a combination of chemical and enzymatic procedures are best suited for the synthesis of stable spin-labeled polyribonucleotides which can serve as valuable macromolecular spin probes in complex biological systems.

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Experimental Part

General. Nucleotide starting materials and other chemicals were obtained either from *Aldrich, Alfa, Baker, Fisher* or *Sigma.* Enzymes were purchased from *P & L Biochemicals* or *Sigma.* Preparative paper chromatography was performed on *Whatman* 3-mm paper with abs. EtOH/1M $AcONH_4$ (7:3 *v/v*). Anion exchange chromatography was achieved with *DEAE-Sephadex A-25 (Pharmacia)* packed in a 15 cm × 16 mm column. Polynucleotide purifications were done on a 75 cm × 16 mm *Sephacryl S-200* column *(Pharmacia)* at 4° equilibrated with 0.04M NH_4HCO_3. ^{1}H-NMR spectra were obtained on a *Nicolet NTC 300 FT* instrument. ESR spectra were recorded on an *E-104* spectrometer interfaced to an *Apple II* computer.

Bis-uridine-5'-diphosphate 5-disulfide (**3**). To a suspension of 100 mg (0.19 mmol) of **1** in 4 ml of cold anh. MeOH were added 4 ml of freshly prepared methyl hypobromite (1.6 mmol). The mixture was stirred at 0° to 4° until the suspension changed to a clear yellow solution; 20 ml of cold Et_2O were added and precipitation allowed to proceed for 20 min. The precipitated nucleotide **2** was isolated by centrifugation, washed 3 times with cold Et_2O and dried with a stream of N_2. To **2**, dissolved in 3 ml of cold freshly distilled *N,N*-dimethylacetamide, were added 25 mg (0.45 mmol) of anh. sodium hydrosulfide. The solution was stirred at 4° for 5 h and at r.t. for 5 h after which 20 ml of Et_2O were added. The precipitate was collected after centrifugation, dissolved in 0.5 ml of 0.05M NH_4HCO_3 and purified by anion exchange chromatography using a linear gradient of 0.05M to 0.5M NH_4HCO_3 (200 ml in each chamber); 20 to 30 mg of **3**, which eluted from the column between 0.36M and 0.50M salt strength, were concentrated by lyophilization and stored in a dry state at −20°. The compound was characterized by its UV spectrum and used without further purification.

5-{[N-(2,2,6,6-Tetramethyl-4-piperidyl-1-oxy)carbamoylmethyl]thio}uridine-5'-diphosphate (**4**). To 15 mg (0.015 mmol) of **3** in 0.2 ml of 0.5M KH_2PO_4 buffer (pH 7.8) were added 3.6 mg (0.023 mmol) of dithiothreitol and the mixture was stirred at r.t. for 30 min. *α*-Iodoacetamido tempo (30 mg, 0.09 mmol) in 0.2 ml of acetone was added and stirring continued for 3 h. The reaction was stopped by streaking

the entire solution onto *Whatman 3MM* paper. The chromatogram was developed with abs. EtOH/1M $AcONH_4$ (7:3, *v/v*). The band corresponding to **4** was eluted from the paper and chromatographed on a *DEAE-Sephadex* column using a linear gradient of 0.05M to 0.4M NH_4HCO_3 (200 ml in each chamber). Excess NH_4HCO_3 was removed from **4** by a combination of lyophilization and desalting on a *Bio-Gel P2* column with H_2O. The yield of **4** was 10 mg. The UV spectrum (pH 7.0) showed a band from 245 to 270 nm ($\varepsilon = 6200$ at 260 nm). ^{1}H-NMR of pRUTT (synthesized similarly to ppRUTT using uridine-5′-monophosphate instead of **1**): *Table 1*. MS: 487 (M^+) (for alkaline phosphatase digestion product of **4**, RUTT).

4-{[N-(2,2,6,6-Tetramethyl-4-piperidinyl-1-oxy)carbamoylmethyl]thio}uridine-5′-diphosphate (**6**). α-Iodoacetamido tempo (20 mg, 0.06 mmol) in 0.3 ml of acetone was added to 4 mg (0.0074 mmol) of **5** dissolved in 0.3 ml of 0.5M KH_2PO_4 buffer (pH 7.8). Compound **6** was isolated from the mixture and purified by the same techniques as those used for **4**. UV (pH 7.0): 303 nm ($\varepsilon = 11{,}700$). ^{1}H-NMR of pls^{4}U (synthesized similarly to **4** using 4-thiouridine-5′-monophosphate instead of **5**): *Table 1*.

Enzymatically Prepared Copolymers, $(ls^4U, U)_n$, $(ls^4U, C)_n$ and $(RUTT, U)_n$. Polymerizations were set up using 50 µl of 1M Tris buffer (pH 8.7), 40 µl of bovine serum albumin (3 mg/ml), 80 µl of 0.02M $MnCl_2$, 40 µl of cytidine-5′-diphosphate (100 mg/ml) or uridine-5′-diphosphate (100 mg/ml), varying amounts of **4** or **6** and 2.25 units of PNPase. The mixture was incubated at 46° before deproteinization with $CHCl_3$/isoamyl alcohol (5:2, *v/v*) and isolation of the copolymers was achieved on a *Sephacryl S-200* column. The spin-labeled polynucleotides were lyophilized and stored at −20° as stock solutions (50 absorbance units/ml).

Preparation of $(lC, C_{1000})_n$. $(C)_n$ (4 mg) dissolved in 200 µl H_2O and 800 µl formamide was evacuated for 4 h (10^{-2} Torr) before adding 125 µl Bu_3N and 11.5 mg of the anhydride of 3-carboxy-2,2,5,5-tetramethylpyrrolinyl-1-oxy. The solution was stirred for 16 h at 4° and subsequently 1 ml of H_2O was added before dialyzing the mixture first against 0.2M NaCl, 0.001M EDTA, 0.01M Tris (pH 8.2) and then against H_2O.

Preparation of $(lC, C_{16})_n$. $(C)_n$ (4 mg) dissolved in 200 µl and 800 µl formamide were evacuated for 4 h (10^{-2} Torr) before adding 200 µl Bu_3N and 18 mg of the anhydride of 3-carboxy-2,2,5,5,-tetramethylpyrrolinyl-1-oxy. After stirring for 16 h at r.t. an additional amount of spin-label anhydride (31 mg) was added, and the mixture was then stirred for another 44 h before adding 1 ml of H_2O and dialyzing the mixture first against 0.2M NaCl, 0.001M EDTA, 0.01M Tris (pH 8.2) and subquently against H_2O.

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