

Synthesis of Modified Nucleoside 3',5'-Bisphosphates and Their Incorporation into Oligoribonucleotides with T4 RNA Ligase[†]

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ABSTRACT: A simple procedure is described to prepare nucleoside 3'(2'),5'-bisphosphates from the corresponding nucleosides with the use of pyrophosphoryl chloride. This method is rapid, gives nearly quantitative yields and, most importantly, can be used for a variety of nucleosides with base and sugar modifications. Since 3',5'-bisphosphates are donors in the T4 RNA ligase reaction, a single residue can be enzymatically attached to the 3' end of oligoribonucleotides. By these procedures, five different ring-modified nucleosides and one

sugar-modified nucleoside were incorporated onto the 3' end of (Ap)₃C. In two cases, an additional step of synthesis with RNA ligase resulted in the modified nucleotide being located in an internal position in the oligonucleotide. Thus, a general method for the synthesis of oligoribonucleotides containing modified nucleosides is outlined. Since many of the modified nucleosides are fluorescent, oligomers containing them should be useful in a variety of physical and biochemical studies.

The finding that nucleoside 3',5'-bisphosphates are efficient donors in the intermolecular T4 RNA ligase reaction (England & Uhlenbeck, 1978) suggests a convenient method to introduce modified or hypermodified nucleotides into a synthetic oligonucleotide. If a method for the synthesis of the modified 3',5'-bisphosphates were available, RNA ligase could join these molecules onto the 3' end of an oligonucleotide acceptor. After removal of the 3' phosphate the product oligomer containing the modified nucleotide could be used as an acceptor in a subsequent RNA ligase reaction, resulting in a modified residue being placed in an internal position in the sequence. It is likely that substitutions on the base portion of the nucleoside 3',5'-bisphosphate will not affect its efficiency as a donor in the RNA ligase reaction, since RNA ligase shows very little substrate specificity when donors of the type A5'ppX are used in the ATP-independent reaction (England et al., 1977).

We report here that pyrophosphoryl chloride (tetrachloropyrophosphate) (Crofts et al., 1960) can be used for the efficient and general bisphosphorylation of both naturally occurring and highly modified nucleosides to their 3'(2'),5'-bisphosphates and confirm that modified residues can be inserted into the sequence of synthetic oligoribonucleotide with T4 RNA ligase.

Solvents play a critical role in selective phosphorylation with pyrophosphoryl chloride. The reagent has been used successfully in *m*-cresol and in other solvents for the direct and selective phosphorylation of the 5'-hydroxyl group of unprotected nucleosides (Imai et al., 1969; Yoshikawa et al., 1969; Sowa & Ouchi, 1975). Previously, several attempts to phosphorylate primary hydroxyl groups selectively without blocking secondary alcohol functions of nucleosides had not been successful (Barker & Foll, 1957; Ikehara et al., 1963). It was reported that with pyrophosphoryl chloride in the absence of solvent 3'(2'),5'-bisphosphates could be obtained quantitatively (Imai et al., 1969), but the reaction was illustrated only for pGp and

pIp (Honjo et al., 1963). Products that have been obtained using pyrophosphoryl chloride, depending largely upon the experimental conditions, include unsubstituted nucleoside monophosphates (Imai et al., 1969), nucleoside 3'(2'),5'-bisphosphates (Honjo et al., 1963; Simoncsits & Tomasz, 1974; Simoncsits et al., 1975), nucleoside cyclic 2',3'-phosphate 5'-phosphate (Simoncsits & Tomasz, 1975), and highly phosphorylated compounds (Tomasz & Simoncsits, 1975). We have developed conditions for obtaining various 3'(2'),5'-bisphosphates under which the pyrophosphoryl chloride reaction, followed by an extremely simple work-up, becomes both efficient and generally applicable. Moreover, the mixtures of pure bisphosphates can be used directly with the T4-induced RNA ligase since the 3',5' component of the mixture is the substrate and the 2',5' component is neither a substrate nor an inhibitor (England & Uhlenbeck, 1978).

Experimental Section

Chemicals. Adenosine, AMP, pA2'p,¹ pA3'p, and 2'-deoxycytidine were obtained from Sigma Chemical Co.; 2'-*O*-methylcytidine, 3'-*O*-methylcytidine, and pG3'p were from P-L Biochemicals. *lin*-Benzoadenosine was prepared by the method of Leonard et al. (1976), except that the deblocking of the sugar group after ribosidation was better carried out with ethanolic ammonia at room temperature for 24 h. Displacement of the methylthio by an amino group to afford *lin*-benzoadenosine is best accomplished at 150 °C during 24 h. The

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¹ Abbreviations used: one letter abbreviations for oligonucleotides and nucleoside 3',5'-bisphosphates will be used (such as: pA3'p, adenosine 3',5'-bisphosphate; pA2'p, adenosine 2',5'-bisphosphate; pAp, a mixture of the two isomers); methylation of the ribose hydroxyl is indicated by the suffix "m" (so pC3'm2'p is 3'-*O*-methylcytidine 2',5'-bisphosphate); ϵ , etheno (so that ϵ C is 3,*N*⁴-ethenocytidine or 5,6-dihydro-5-oxo-6- β -D-ribofuranosylimidazo[1,2-*c*]pyrimidine (Secrist et al., 1972; Barrio et al., 1972); ϵ A is 1,*N*⁶-ethenoadenosine or 3- β -D-ribofuranosyl[2,1-*i*]purine (Secrist et al., 1972); ϵ G is 1,*N*²-ethenoguanosine or 5,9-dihydro-9-oxo-3- β -D-ribofuranosylimidazo[1,2-*a*]purine (Sattangi et al., 1977)); μ G, 1,*N*²-(2-methylallylidene)guanosine; *lin*A, *lin*-benzoadenosine, which is the trivial name for 1-(β -D-ribofuranosyl)-8-aminoimidazo[4,5-*g*]quinazoline (Leonard et al., 1976); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ³J_{PH} and ⁴J_{PH}, respectively proton-phosphorus coupling constants ³J_{P-O-C-H} and ⁴J_{P-O-C-C-H}.

total yield after these two steps was nearly quantitative. 3,*N*⁴-Ethenocytidine was prepared by the method of Barrio et al. (1972, 1976). *lin*-Benzo-AMP was prepared by the method of Scopes et al. (1977); 1,*N*²-ethenoguanosine, by the method of Sattangi et al. (1977). Pyrophosphoryl chloride was best prepared by reaction of phosphoric oxide, phosphorus trichloride, and chlorine in carbon tetrachloride following the method of Crofts et al. (1960). The compound is stable for at least 1 year when stored desiccated at -20°C . Thin-layer chromatography was performed on Brinkman cellulose F plates using isobutyric acid: NH_4OH : H_2O , 75:1:24. Nucleotides were visualized with ultraviolet light.

Apparatus. Ultraviolet absorption spectra were obtained on a Beckman Acta M VI spectrophotometer. ^{31}P nuclear magnetic resonance spectra were obtained on a Varian Associates XL-100-15 NMR system equipped with a Digital NMR-3 data system, operating at 40.5 MHz for ^{31}P and 100 MHz for ^1H . Broadband proton decoupling centered at about δ 4.0 was used for proton decoupled phosphorus spectra. Deuterium from the D_2O solvent was used for field/frequency stabilization. Phosphoric acid (85%) in a concentric capillary (2-mm O.D.) was used as primary ^{31}P reference. All spectra were obtained by the Fourier transform technique using 16K data points and a 2500-Hz bandwidth.

Enzymes and Enzyme Assays. The source of materials, including the ^3H -labeled oligonucleotide acceptor, $(\text{Ap})_3\text{C}$, and the T4 RNA ligase as well as the procedures for running the reaction and analyzing the products are described in detail in the preceding paper (England & Uhlenbeck, 1978). For the evaluation of a modified pNp as a donor, the 30- μL reaction mixture contained 0.1 mM [*Cyd*- ^3H] $(\text{Ap})_3\text{C}$ (330 Ci/mol), 0.2 mM pNp, 0.5 mM ATP, 50 mM Hepes (pH 8.3), 20 mM MgCl_2 , 3.3 mM dithiothreitol, 10 $\mu\text{g}/\text{mL}$ serum albumin, and various concentrations of T4 RNA ligase. After 60 min at 37°C , the reaction mixtures were spotted on Whatman 3MM paper and a descending chromatogram was run in 60:40 (v/v) 1 M ammonium acetate:ethanol. The yield of the slower moving $(\text{Ap})_3\text{CpNp}$ product spot was calculated as the percentage of ^3H label migrating at that position.

The products were characterized as having had a single modified nucleotide added to $(\text{Ap})_3\text{C}$ by the series of enzymatic degradations used to identify $(\text{Ap})_3\text{CpAp}$ in the preceding paper (England & Uhlenbeck, 1978). Since no ^{32}P label was present in the donor, only the ^3H label in the cytidine can be detected. However, in many cases the nucleotide added is fluorescent and can be seen on the paper chromatogram, thus aiding identification.

$(\text{Ap})_3\text{Cp}\epsilon\text{A}$ and $(\text{Ap})_3\text{CpC2'm}$ were prepared in slightly larger amounts in order to test their effectiveness as acceptors. Each reaction contained 0.25 mM [*Cyd*- ^3H] $(\text{Ap})_3\text{C}$ (285 Ci/mol), 0.5 mM pNp, 0.5 mM ATP, and 250 U/mL RNA ligase in the same buffer used above. After 60 min at 37°C , the products $(\text{Ap})_3\text{CpNp}$ were purified by paper chromatography as above (yield 96% in both cases). After elution from the paper, a portion of each $(\text{Ap})_3\text{CpNp}$ was treated with alkaline phosphatase to form the $(\text{Ap})_3\text{CpN}$, repurified by paper chromatography, eluted, and desalted on Bio-Gel P2 (England & Uhlenbeck, 1978).

The additions of pAp to $(\text{Ap})_3\text{Cp}\epsilon\text{A}$ and to $(\text{Ap})_3\text{CpC2'm}$ were carried out in an analogous manner. Each reaction contained 10–20 μM [*Cyd*- ^3H] $(\text{Ap})_3\text{CpN}$ (285 Ci/mol), 90 μM [$5'$ - ^{32}P]pA3'p (3 Ci/mol), 330 μM ATP, and 250 U/mL RNA ligase in the same buffer used above. After 18 h at 4°C , the reaction mixtures were analyzed by paper chromatography as above.

Nucleoside 3'(2'),5'-Bisphosphates. A mixture of nucle-

oside (0.2 mmol) and pyrophosphoryl chloride (504 mg, 2 mmol) was stirred at -10 to -15°C . After 4–5 h the reaction was quenched by the rapid addition of ice and, immediately thereafter, a chilled solution of 0.5 M triethylammonium bicarbonate, pH 8.0. The colorless solution was evaporated to dryness under vacuum at 20°C . The residue was dissolved and evaporated several times with 10-mL portions of methanol to remove the excess triethylammonium bicarbonate and was then chromatographed on a column of DEAE-cellulose (2.5×40 cm) with a linear gradient of 0.05 to 0.4 M triethylammonium bicarbonate, pH 8.0. The fractions containing the nucleoside bisphosphate were pooled and evaporated to dryness at 20°C as indicated above, giving 85–95% of an unresolved mixture of pure nucleoside 2',5'- and 3',5'-bisphosphates as judged by ^{31}P NMR.

1,*N*²-Ethenoadenosine 3',5'-bisphosphate (p $\epsilon\text{A}3'\text{p}$) was prepared by chloroacetaldehyde modification of pA3'p (Barrio et al., 1972). When the reaction was complete, as judged by thin-layer chromatography, the solvent was evaporated to dryness under vacuum at 20°C . The residue was chromatographed as indicated above in the general preparation of pNp's (yield: 90%).

1,*N*²-(2-Methylallylidene)guanosine 3',5'-bisphosphate (p $\mu\text{G}3'\text{p}$) was prepared by modification of pG3'p with methylmalonaldehyde at pH 4.2 (0.1 M NaOAc buffer) (Moschel & Leonard, 1976). Chromatographic purification was carried out as indicated above for other pNp's (yield: 50%). Thus, in these representative cases, it was satisfactory to modify the 3',5'-bisphosphates rather than to phosphorylate the modified nucleosides.

Results and Discussion

Chemistry. Nucleoside 3'(2'),5'-bisphosphates were obtained selectively by reaction of each unprotected nucleoside with pyrophosphoryl chloride at -10 to -15°C for several hours, rapid hydrolysis by means of ice and triethylammonium bicarbonate, and column chromatography on DEAE-cellulose with a linear gradient of triethylammonium bicarbonate. *The nucleoside 3'(2'),5'-bisphosphates were produced almost exclusively under the conditions described.* They were identified by their spectroscopic, chromatographic, and enzymatic properties, and in representative cases by comparison with authentic samples. Under the conditions we employed, by-products were limited to very small amounts of either 5'-monophosphates or higher phosphorylated products. At least two conditions proved to be critical for efficient reaction: (a) purity of the pyrophosphoryl chloride, which decomposes readily when maintained at room temperature for several days; and (b) temperature of the reaction (Honjo et al., 1963; Tomasz & Simoncsits, 1975).

It is unclear why the pyrophosphoryl chloride method of bisphosphorylation, which was introduced in 1963 for the synthesis of pGp and pIp, has not received more attention. Limited cognizance of the first report (Honjo et al., 1963) or the multiple manipulations described for purification may have contributed. In any case, the presently described directions have considerably simplified and generalized the procedure. For example, 2'-deoxycytidine could be phosphorylated to p dCp in high yield and with little hydrolysis of the glycosidic bond. Other 3'(2'),5'-bisphosphates produced by this method are included in Table I, and one may safely predict extension of the method for obtaining additional deoxyribonucleoside 3',5'-bisphosphates and modified ribonucleoside 3'(2'),5'-bisphosphates as well. The procedure also overcomes the drawbacks of low yields and involved purifications which characterize many of the previous chemical methods for the

TABLE I: ^{31}P NMR Data.^a

Compound	Chemical shifts ^b		
	3'-P	2'-P	5'-P
pA3'p ^c	4.175		3.902
pA2'p		3.696	3.962
plinA3'p	4.077		3.865
plinA2'p		3.475	3.865
pdCp	3.287		3.837
pC2'm3'p	3.399		3.762
pC3'm2'p		3.475	3.837
peC3'p	4.049		3.827
peC2'p		3.721	3.916
peG3'p	4.180		3.878
peG2'p		3.566	3.924
AMP			3.882
lin-BenzoAMP			3.954

^a Spectra recorded in D_2O , EDTA (0.002 M), adjusted to pH ~10 by addition of $\text{Me}_4\text{N}^+\text{OH}^-$. ^b Chemical shifts for proton-decoupled phosphorus signals are expressed in ppm downfield from external 85% H_3PO_4 capillary. In the nondecoupled ^{31}P NMR spectra the 5'-phosphate ^{31}P resonance displays a very characteristic split triplet. The two H_5' protons account for the triplet structure of the phosphate ^{31}P signal. A long-range $^4J_{\text{PH}}$ coupling to the sugar ring H_4 proton is also observed. 3'- and 2'-phosphates ^{31}P show characteristic doublets due to couplings of phosphorus to vicinal protons ($^3J_{\text{PH}3'}$ and $^3J_{\text{PH}2'}$, respectively). Long-range $^3J_{\text{PH}}$ coupling constants are also detected (Cozzzone & Jardetzky, 1976b). ^c In the nondecoupled ^{31}P spectra of pA3'p recorded at 40.48 MHz in the Fourier mode, 5'-P is reported as 0.212 ppm upfield from 3'-P (Lee & Sarma, 1975).

preparation of nucleoside 3'(2'),5'-bisphosphates. These include direct phosphorylation of nucleosides with either dibenzyl phosphorochloridate (Moffatt & Khorana, 1961; Dekker et al., 1953; Cramer et al., 1957), phosphorous oxychloride in triethyl phosphate (Morelli & Benatti, 1974), or *N*-phosphoryl-*N'*-methylimidazolium salts (Takaku et al., 1973). The available enzymatic methods for preparation of 3',5'-bisphosphates include hydrolysis of RNA with venom exonuclease (Richards & Laskowski, 1969) or phosphorylation of 3'-monophosphates by polynucleotide kinase (Richardson, 1971). However, the enzymatic production of various modified pNp's could be limited either by their availability in RNA or the substrate specificity of polynucleotide kinase. Thus, the use of pyrophosphoryl chloride for the preparation of unmodified and modified nucleoside 3'(2'),5'-bisphosphates is presently the most convenient available procedure. The method as described here has also demonstrated promise for the bisphosphorylation of unprotected dinucleoside 3',5'-phosphates. When these are subjected to reaction with pyrophosphoryl chloride at -20°C , 3'(2'),5'-bisphosphorylation is the main reaction, and the products are obtained in acceptable yields. Investigation of this route to pNpNp compounds is continuing.

Mechanisms for the bisphosphorylation reaction with pyrophosphoryl chloride have been established and nucleoside cyclic 2',3'-chlorophosphate 5'-dichlorophosphates have been implicated as intermediates (Tomasz & Simoncsits, 1975). We observed a $65 \pm 5\%$ to $35 \pm 5\%$ ratio of pN3'p to pN2'p in all cases where unsubstituted ribonucleosides were submitted to phosphorylation and work-up conditions described in the Experimental Section. The composition of such a mixture is dictated either by stereoselective nucleophilic attack by water on a cyclic 2',3'-phosphate intermediate (Westheimer, 1968; Tomasz & Simoncsits, 1975) or by a regioselective phosphorylation step (Lynen, 1958; Gruber & Lynen, 1962).

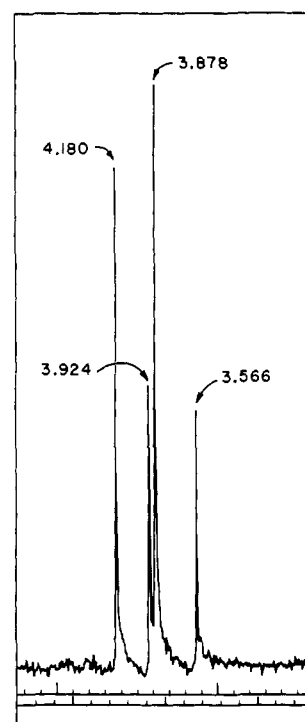


FIGURE 1: Representative proton-decoupled ^{31}P NMR of a mixture of pure nucleoside 3'(2'),5'- and 2',5'-bisphosphates (in this case, 1,*N*²-ethenoguanosine 3'(2'),5'-bisphosphate) obtained after phosphorylation and chromatographic purification of the products (see Experimental Section and Table I for full details).

^{31}P NMR spectroscopy is a simple and powerful tool for the characterization of nucleoside 3'(2'),5'-bisphosphates (Table I). Examination of the proton undecoupled ^{31}P spectra of nucleoside 5'-monophosphates and nucleoside 3'(2'),5'-bisphosphates reveals the striking feature that the 5'-P signals experience very little change from an average value of δ 3.88, downfield from 85% H_3PO_4 , among the compounds listed. Accumulated evidence indicates that the most favored conformation of a ribonucleoside 5'-phosphate is anti at N-C(1') and gauche-gauche along C(4')-C(5'). These are little affected by the addition of a 3'-phosphate, except that this group exhibits greater flexibility than the 5'-phosphate. The ribofuranose ring is in rapid equilibrium between ^2E [C(2')-endo] and ^3E [C(3')-endo] conformations (Sundaralingam, 1969; Remin & Shugar, 1972; Olson & Flory, 1972; Sarma & Myrnes, 1973; Sundaralingam, 1973; Yathindra & Sundaralingam, 1973a,b; Altona & Sundaralingam, 1973; Lee & Sarma, 1974, 1975; Prusiner et al., 1974; Davies & Danyluk, 1974, 1975; Lapper & Smith, 1975; Evans et al., 1975).

With the signal responsible for the 5'-P nearly constant, the 3'-P and 2'-P signals can be assigned readily when the ribonucleoside 3',5'- and 2',5'-bisphosphates are present in different proportions (Figure 1). The 2'-P signals are consistently upfield from the 5'-P resonance. The 3'-P signals are shifted downfield by ca. 0.20 ppm from the 5'-P average position, a result of the 3'-P environment and the equilibrium among the rotamers, $^2\text{E} \rightleftharpoons ^3\text{E}$: trans, gauche (g^-) and gauche (g^+) about C(3')-O(3'), available to the 3'-phosphate group (Lee & Sarma, 1975). Methylation of the 2'-hydroxyl (pC2'm3'p) or its elimination (pdCp) results in a shielding effect on the 3'-phosphate ^{31}P resonance (Table I). In both cases, 2'-OH interaction is removed (Cozzzone & Jardetzky, 1976a) and shifts in the equilibria of the 3'-phosphate rotamer populations are possible (Prusiner et al., 1974; Gorenstein, 1975). When complete re-

TABLE II: Single Addition of pNp to (Ap)₃C.

pNp	RNA ligase concentration					
	7 U/mL	35 U/mL	70 U/mL	105 U/mL	245 U/mL	350 U/mL
pμG3'p	<1 ^a	30	45	54	72	75
pεGp	4	5	9	14	25	40
pεA3'p	10	51	79	88	94	94
plinAp	12	34	54	65	91	94
pεCp	39	87	94	97	96	96
pC2'm3'p	69	92	95	95	95	95
pC3'm2'p	<1	<1	<1	<1	<1	<1
pdCp	25	72	94	98	98	98

^a Yields are given as percent of (Ap)₃C converted to (Ap)₃CpNp.

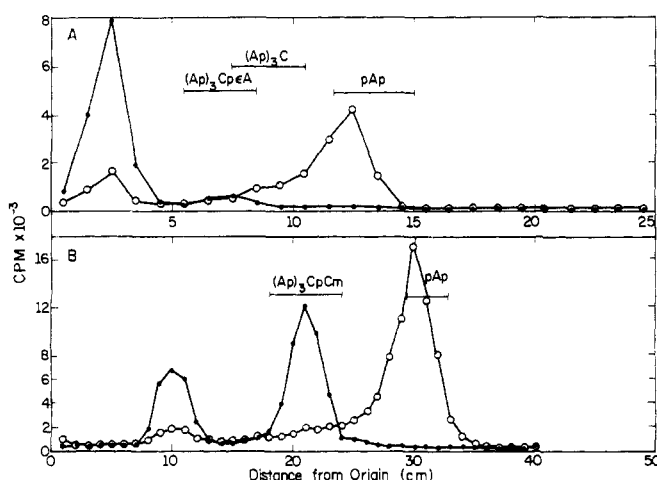


FIGURE 2: Paper chromatographic analysis of the addition of [5'-³²P]pAp to [Cyd-³H](Ap)₃CεA (A) and (Ap)₃CpC2'm (B). Open circles are ³²P radioactivity; closed circles are ³H radioactivity.

laxation of the phosphorus signal is allowed, the ³¹P relative chemical shifts are useful in structure assignment and in the quantitative estimation of proportions in mixtures such as those encountered in the present synthesis of 3'(2'),5'-bisphosphates.

Enzymology. Seven modified nucleoside 3'(2'),5'-bisphosphates were tested for their ability to act as donors with [Cyd-³H](Ap)₃CεA acceptor and T4 RNA ligase (Table II). A twofold excess of donor to acceptor was maintained in each reaction in order to ensure that the 3' isomer was present in sufficient quantity. The nucleoside 2',5'-bisphosphates do not affect the ligation reaction as they are neither substrates nor competitive inhibitors of the RNA ligase reaction (England & Uhlenbeck, 1978). The lack of activity observed for pC3'-m2'p in Table II supports this conclusion. As can also be seen in Table II, all six of the modified pNp's with a 3' phosphate were active donors and modified oligomers of the type (Ap)₃-CpNp were obtained in excellent yields. The identity of these slower moving products was confirmed by digestion of each product with ribonuclease A to give radiolabel which comigrated with (Ap)₃Cp upon analysis by descending paper chromatography and ribonuclease A plus alkaline phosphatase to convert the product back to ³H-labeled (Ap)₃C. Also, in most cases the fluorescence of the modified oligonucleotide could be detected by examining the paper chromatogram in ultraviolet light.

Although the varying proportions of 3' and 2' isomers prevent detailed quantitative comparisons, it is evident that the modified nucleoside bisphosphates are nearly as good substrates as their unmodified counterparts (England & Uhlen-

beck, 1978). This observation is consistent with the remarkable lack of specificity of RNA ligase that was noted when adenylylated pyrophosphates were used as donors (England et al., 1977).

Examples of the addition of pAp to oligonucleotides containing modified nucleosides are shown in Figure 2. In the upper panel the reaction with (Ap)₃CpεA as the acceptor is analyzed. Nearly 85% of the ³H label in the acceptor is converted to a doubly labeled slower moving product, (Ap)₃-CpεApAp. Since the donor [5'-³²P]pAp is in excess, a lesser fraction of the ³²P label is found in the product. In the lower panel, (Ap)₃CpC2'mpAp is obtained in 36% yield under identical reaction conditions for the acceptor (Ap)₃CpC2'm. The lower yield in the latter case strengthens the observation that the reactivity of an acceptor in the RNA ligase reaction is determined by its base composition near the 3' end (England & Uhlenbeck, 1978). In each case, the products were identified by the resistance of ³²P radiolabel to alkaline phosphatase and the production of the expected nucleoside monophosphates [Cyd-³H]Cp and [3'-³²P]εAp or [3'-³²P]C2'm3'p, respectively, upon hydrolysis with spleen phosphodiesterase. Thus, modified nucleosides can be inserted effectively into internal positions in an oligonucleotide sequence.

Our preparation of nucleoside 3'(2'),5'-bisphosphates from the corresponding nucleosides is sufficiently general that a wide variety of modified and hypermodified nucleotides can be obtained. Since the modified bisphosphates are substrates in the RNA ligase reaction, a general method is now available for the synthesis of oligoribonucleotides containing modified bases at specific positions in the sequences and experiments can be designed to clarify the structural and functional roles of modified nucleotides in RNA.

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References

- Altona, C., & Sundaralingam, M. (1973) *J. Am. Chem. Soc.* 95, 2333.
- Barker, G. R., & Foll, G. E. (1957) *J. Chem. Soc.*, 3798.
- Barrio, J. R., Secrist, J. A., III, & Leonard, N. J. (1972) *Biochem. Biophys. Res. Commun.* 46, 597.
- Barrio, J. R., Sattangi, P. D., Gruber, B. A., Dammann, L. G., & Leonard, N. J. (1976) *J. Am. Chem. Soc.* 98, 7408.
- Cozzzone, P. J., & Jardetzky, O. (1976a) *Biochemistry* 15, 4853.
- Cozzzone, P. J., & Jardetzky, O. (1976b) *Biochemistry* 15, 4860.
- Cramer, F., Kenner, G. W., Hughes, N. A., & Todd, A. (1957)

- J. Chem. Soc.*, 3297.
- Crofts, P. C., Downie, I. M., & Heslop, R. B. (1960) *J. Chem. Soc.*, 3673.
- Davies, D. B., & Danyluck, S. S. (1974) *Biochemistry* 13, 4417.
- Davies, D. B., & Danyluck, S. S. (1975) *Biochemistry* 14, 543.
- Dekker, C. A., Michelson, A. M., & Todd, A. R. (1953) *J. Chem. Soc.*, 947.
- England, T. E., & Uhlenbeck, O. C. (1978) *Biochemistry* 17 (preceding paper in this issue).
- England, T. E., Gumpert, R. I., & Uhlenbeck, O. C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 4839.
- Evans, F. E., Lee, C.-H., & Sarma, R. H. (1975) *Biochem. Biophys. Res. Commun.* 63, 106.
- Gorenstein, D. G. (1975) *J. Am. Chem. Soc.* 97, 898.
- Gruber, W., & Lynen, F. (1962) *Justus Liebigs Ann. Chem.* 659, 139.
- Honjo, M., Imai, K., Furukawa, Y., Morijama, H., Yasumatsu, K., & Imada, A., (1963) *Takeda Kenyusikko Nempo* 22, 47.
- Ikehara, M., Ohtsuka, E., & Kodama, Y. (1963) *Chem. Pharm. Bull. (Tokyo)* 11, 1456.
- Imai, K., Fujii, S., Takanohashi, K., Furukawa, Y., Masuda, T., & Honjo, M. (1969) *J. Org. Chem.* 34, 1547.
- Lapper, R. D., & Smith, I. C. P. (1975) *J. Am. Chem. Soc.* 95, 2880.
- Lee, C.-H., & Sarma, R. H. (1974) *FEBS Lett.* 43, 271.
- Lee, C.-H., & Sarma, R. H. (1975) *J. Am. Chem. Soc.* 97, 1225.
- Leonard, N. J., Sprecker, M. A., & Morrice, A. G. (1976) *J. Am. Chem. Soc.* 98, 3987.
- Lynen, F. (1958) *Proc. 4th Int. Congr. Biochem.* 11, 230.
- Moffatt, J. G., & Khorana, H. G. (1961) *J. Am. Chem. Soc.* 83, 663.
- Morelli, A., & Benatti, U. (1974) *Ital. J. Biochem.* 23, 279.
- Moschel, R. C., & Leonard, N. J. (1976) *J. Org. Chem.* 41, 294.
- Olson, W. K., & Flory, P. J. (1972) *Biopolymers* 11, 25.
- Prusiner, P., Yathindra, N., & Sundaralingam, M. (1974) *Biochim. Biophys. Acta* 366, 115.
- Remin, M., & Shugar, D. (1972) *Biochem. Biophys. Res. Commun.* 48, 636.
- Richards, G. M., & Laskowski Sr., M. (1969) *Biochemistry* 8, 4858.
- Richardson, C. C. (1971) *Proced. Nucleic Acid Res.* 2, 815.
- Sarma, R. H. & Mynott, R. J. (1973) *J. Am. Chem. Soc.* 95, 1641.
- Sattsangi, P. D., Leonard, N. J., & Frihart, C. R. (1977) *J. Org. Chem.* 42, 3292.
- Scopes, D. I. C., Barrio, J. R., & Leonard, N. J. (1977) *Science* 195, 296.
- Secrist, J. A., III, Barrio, J. R., Leonard, N. J., & Weber, G. (1972) *Biochemistry* 11, 3499.
- Simoncsits, A., & Tomasz, J. (1974) *Biochim. Biophys. Acta* 340, 509.
- Simoncsits, A., & Tomasz, J. (1975) *Biochim. Biophys. Acta* 395, 74.
- Simoncsits, A., Tomasz, J., & Allende, J. E. (1975) *Nucleic Acids Res.* 2, 257.
- Sowa, T., & Ouchi, S. (1975) *Bull. Chem. Soc., Jpn.* 48, 2084.
- Sundaralingam, M. (1969) *Biopolymers* 7, 821.
- Sundaralingam, M. (1973) *Proc. Jerusalem Symp.*, 5th, 381.
- Takaku, H., Yoshifusa, S., & Hidetoshi, O. (1973) *Chem. Pharm. Bull. (Tokyo)* 21, 1844.
- Tomasz, J., & Simoncsits, A. (1975) *J. Carbohydr. Nucleosides Nucleotides* 2, 315.
- Westheimer, F. H. (1968) *Acc. Chem. Res.* 1, 70.
- Yathindra, N., & Sundaralingam, M. (1973a) *Biopolymers* 12, 297.
- Yathindra, N., & Sundaralingam, M. (1973b) *Biopolymers* 12, 2261.
- Yoshikawa, M., Kato, T., & Takenishi, T. (1969) *Bull. Chem. Soc. Jpn.* 42, 3505.