Synthesis of Nucleotides from O^2,2′-Cyclouridine[1]

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Procedures for the synthesis of protected O^2,2′-cyclouridines and their incorporation into dinucleoside monophosphates have been developed. The properties of these molecules with snake venom and spleen phosphodiesterases have been investigated. The cyclonucleotides are easily converted into arabinouridine nucleotides and thus provide a convenient route to these compounds.

On a développé des méthodes pour synthèse de cyclouridines-O^2,2′ protégés et pour leur incorporation dans des monophosphates de dinucléoside. On a examiné les propriétés de ces molécules vis à vis le venin de serpent et les phosphodiestérases de la rate. Les cyclonucléotides sont facilement transformés en nucléotides d'arabinouridine et fournissent ainsi une méthode simple d'arrivée à ces composés.

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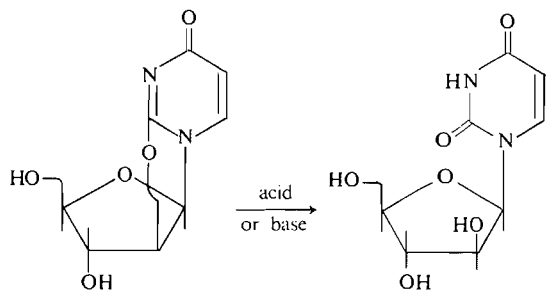
Introduction

A great deal of interest has developed in arabinonucleotides. The activity of arabinonucleotides toward enzymes (1–4), their antitumor and antiviral activities (5, 6), and the role of the arabino 2′-hydroxyl group in nucleotide structure and activity (7, 8) have generated a need for the rapid synthesis of oligoarabinonucleotides. While there have been several reports on the synthesis of nucleotides containing arabinonucleoside units (1, 9, 10), there has been much less attention paid to the stepwise synthesis of oligoarabinonucleotides of predetermined sequence.

It had occurred to us that the cyclopyrimidine nucleosides as intermediates might provide an attractive route to the synthesis of oligoarabinonucleotides. For example O^2,2′-cyclouridine (1), which is readily available in high yield from uridine (11), closely resembles a deoxynucleoside in the carbohydrate portion of the molecule. Further it is known that mild acid (12) or base (13) treatment readily converts **1** into arabinouridine (**2**). Thus it would seem that the versatile methods which are available for the synthesis of oligodeoxynucleotides (14, 15), should be directly applicable to the synthesis of oligocyclopyrimidines which should be easily converted by mild acid or base into the corresponding oligoarabinonucleotides. Further, the cyclonucleotides themselves are of interest due to the modification of the pyrimidine ring.



1 **2**

In this manuscript we describe the synthesis of dinucleoside monophosphates containing cyclouridine units, their interaction with spleen and snake venom phosphodiesterases, and, their conversion to arabinouridine nucleotides. A preliminary account of one of these reactions has previously been reported (16).

Except for our initial report (16) O^2,2′-cyclopyrimidines have not been used in a stepwise synthesis of nucleotides. Nagyvary and Provenzale (10, 17) have converted existing pyrimidine nucleotides into arabinonucleotides via the cyclopyrimidine intermediate. Schramm and Ulmer-Schurnbrand (18) have converted uridylic acid into polyarabinouridylic acids via the O^2,2′-cyclouridylate intermediates. Ikehara and Tezuka (19) have described the synthesis of O^6,2′-cyclouridylyl-(3′–5′)-O^6,2′-cyclouridine (**3**).

[1]Part XII in a series on anhydronucleosides. Part XI is ref. 20.

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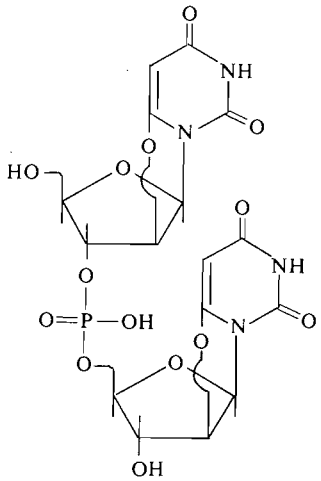


3

The properties of this latter nucleotide toward phosphodiesterases are of interest when compared to the corresponding nucleotide of O^2,2′-cyclouridine (**15**, see below). There have also been two major reports on the incorporation of 8,2′-thioanhydropurine nucleosides into dinucleoside monophosphate units (20, 21).

Results and Discussion

Synthesis of O^2,2′-Cyclouridine Nucleotides

The same general approach as used in the synthesis of deoxynucleotides (14, 15) was considered for the cyclouridines: the phosphorylation of a suitable 5′-protected cyclonucleoside (**4**) followed by condensation with a 3′-protected cyclonucleoside (**6**).

The triphenylmethyl protecting groups have been generally used (22, 23) for selectively protecting the 5′-hydroxyl groups in nucleosides. When **1** was treated with triphenylmethyl chloride (Scheme 1) in hot pyridine **4***a* was not obtained. Rather the cyclolinkage had been destroyed and after removal of the trityl group 2′-chloro-2′-deoxyuridine was obtained. This compound could also be obtained by treating **1** with pyridinium hydrochloride. Compound **4***a* was obtained by treating 5′-*O*-trityluridine (**7***a*) with diphenyl carbonate in DMF (11). The trityl group itself turned out to be too resistant to acid hydrolysis to be generally useful since hydrolysis of the cyclolinkage occurred simultaneously with hydrolysis of the trityl group.

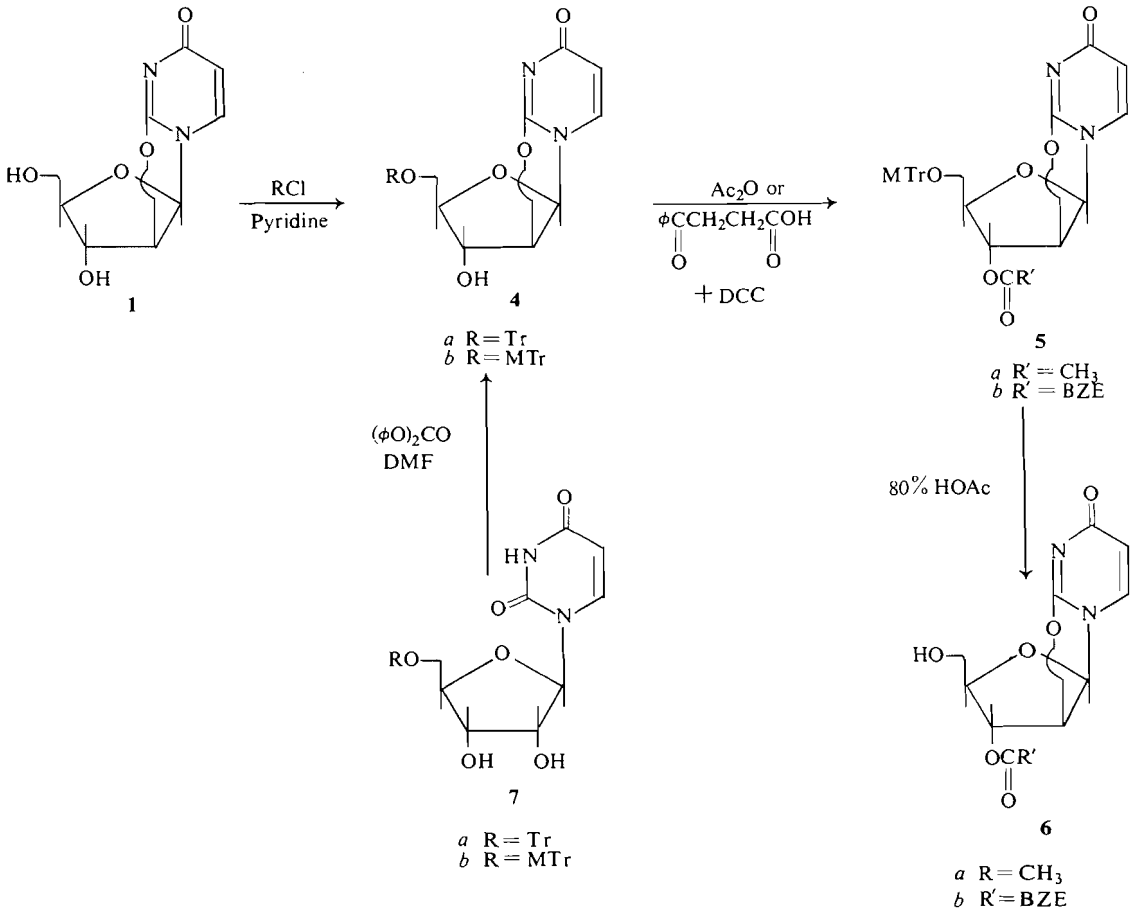
Compound **4***b* was obtained from **7***b* as above or by treating **1** with monomethoxytrityl chloride at room temperature in pyridine. Conditions for removal of the monomethoxytrityl group (80% HOAc, 1½ h at room temperature (23)) did not affect the cyclouridine linkage.

Two base labile protecting groups which have been used in nucleotide synthesis are the acetyl (24) and the β-benzoylpropionyl (25) groups. Both of these groups can be removed with mild base while the β-benzoylpropionyl group can be selectively removed with hydrazine. Thus **4***b* was converted into **5***a* and *b* which yielded **6***a* and *b* on treatment with 80% acetic acid at room temperature.

With the protected cyclouridines **4** and **6** being readily available, the phosphorylation of these compounds was investigated. In the first sequence (Scheme 2), **4***b* and β-cyanoethyl phosphate (βCEP) were condensed using dicyclohexylcarbodiimide (DCC) and the resulting intermediate was condensed directly with isopropylidineuridine (U-ISP) using triisopropylbenzenesulfonyl chloride (TPS). The cyanoethyl derivative of 5′-*O*-monomethoxytrityl-O^2,2′-cyclouridylyl-(3′–5′)-isopropylidineuridine (**8***a*) was obtained in 27% isolated yield. On treatment with 80% acetic acid at room temperature followed by pyridine – ammonium hydroxide (5:2) **8***a* was converted into **9***a*. The treatment of the nucleotides containing cyclouridine units with phosphodiesterases will be discussed together below. Compound **9***a* was converted to **10***a* or *b* on treatment with sodium hydroxide and/or hot acetic acid. Both **10***a* and *b* were completely degraded by phosphodiesterases to the expected products. These results confirm the identity of compound **9**.

The β,β,β-trichloroethyl group has also been used as a phosphate protecting group (26). As a result compound **8***b* was synthesized from **4***b* using a procedure (Scheme 2) very similar to the one used by Neilson and Werstiuk in the ribonucleotide area (27). Removal of the monomethoxytrityl group with acetic acid produced **9***b*. Treatment of **8***b* with hot acetic acid followed by treatment with zinc and acetic acid gave **10***b*.

Compound **9***a* was obtained in an overall yield of 40% from **4***b* by (*1*) phosphorylating **4***b* with trichloroethyl phosphate using TPS, (*2*) removing the trichloroethyl group at this point with copper zinc in DMF, and (*3*) condensing the product with U-ISP and triphenyl phosphine-2,2′-dithiodipyridine (TPP-PDS (28)). Removal of the monomethoxytrityl group gave **9***a*. It is interest-



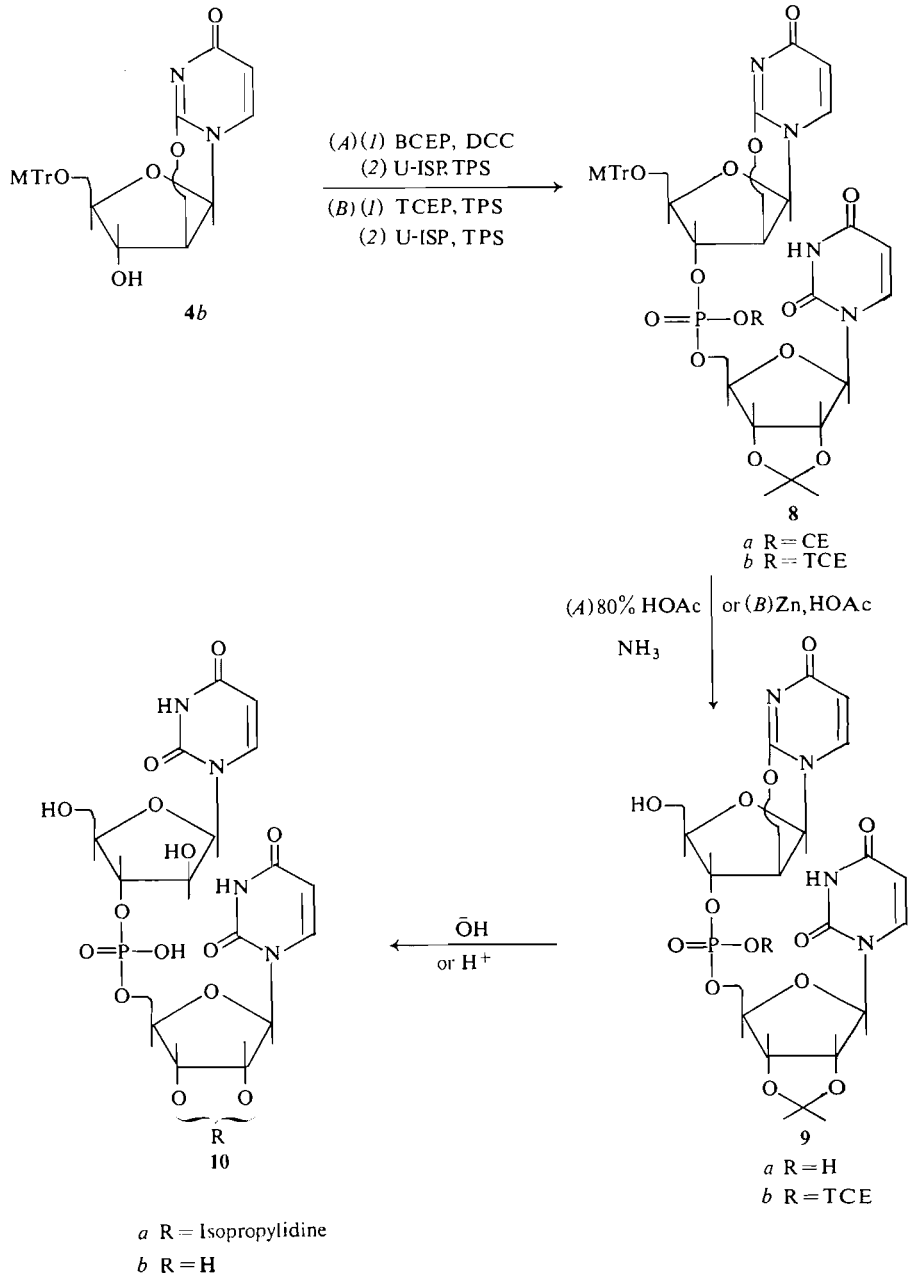
Tr = Triphenylmethyl
MTr = *p*-Methoxyphenyldiphenylmethyl
BZE = β-Benzoylethyl
DCC = Dicyclohexylcarbodiimide

SCHEME 1

ing to note that in these syntheses only small amounts of cyclonucleoside 3′-phosphates were detected in the final products. This might indicate that condensation yields were quite high but poor recovery of products was experienced. Most losses probably occur at extraction stages in the procedures. However, we have not been able to significantly improve isolated yields.

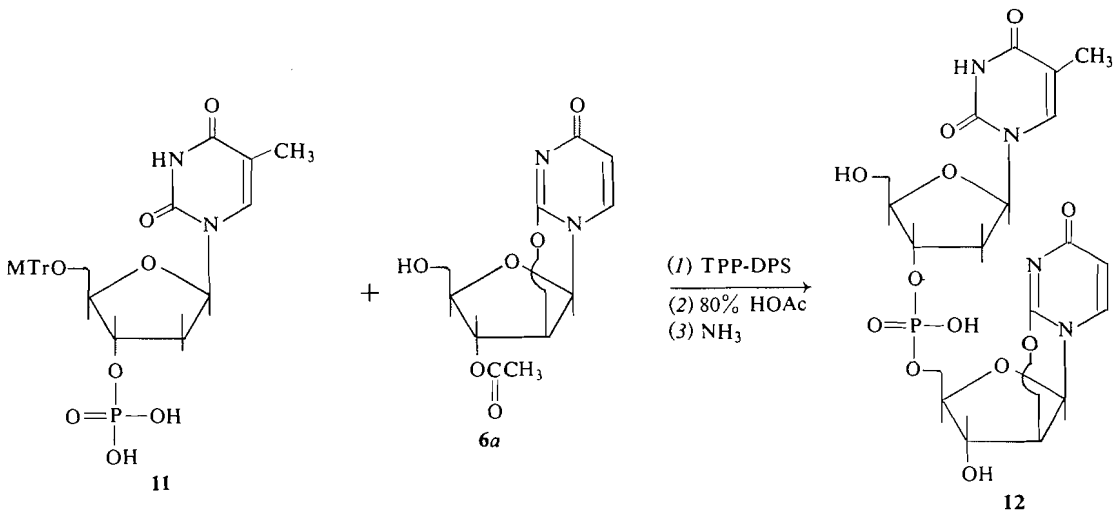
To determine whether the cyclonucleoside unit could be added to the 3′-end of a nucleotide chain, compound **12** was synthesized. The condensation of **11** with **6***a* using TPP-DPS followed by removal of protecting groups gave **12** in 23% yield. Compound **12** was treated with hot acetic acid to produce thymidylyl-(3′–5′)-arabinouridine (TpaU) which gave complete degration to thymidine 3′-phosphate and arabinouridine with spleen enzyme. With snake venom enzyme TpaU was degraded to the extent of 72% after 24 h. The undegraded material was completely degraded with either spleen enzyme or on prolonged treatment with snake venom. Previous reports have indicated (1) that dinucleoside monophosphates containing arabinonucleosides required more enzyme and longer incubation times than the corresponding ribonucleotides in order to achieve complete degradation.

The route to the dicyclouridine monophosphate (**15**) is outlined in Scheme 3. The important intermediate in the reaction is compound **14** which is readily obtained from **13** using copper–



SCHEME 2

zinc in DMF. Compound **13** was prepared by condensing the cyclohexylammonium salt (**26**) of β,β,β-trichloroethyl phosphate with **4***b* using TPS. We were unable to obtain any **13** using either DCC or TPP-DPS as condensing agents. Apparently cyclohexylamine is too strong a base to be displaced using these latter reagents. All three condensing agents gave quantitative phosphorylation of **4***b* by the pyridinium salt of βCEP. However, the conditions required to remove the cyanoethyl group (7 *M* NH_4OH (29) or 1 *N* NaOH (23)) also cause hydrolysis of the cyclonucleoside. Methanol–pyrrolidine (9:1) has been used (30) to remove the cyanoethyl group from phosphodiesters but this reagent also caused degradation of **1**.



Condensation between **14** and **6***a* followed by removal of protecting groups gave 39 and 46% isolated yields of **15** using DCC or TPP-DPS as condensing agents. Again no cyclouridine 3′-phosphate was observed in the products of the reaction. Compound **15** was converted to arabinouridylyl-(3′–5′)-arabinouridine (**16**) by sodium hydroxide. Compound **16** was completely degraded to arabinouridine 3′-phosphate and arabinouridine by spleen enzyme and was slowly degraded by snake venom enzyme to arabinouridine 5′-phosphate and arabinouridine.

Enzyme Studies on Nucleotides Containing Cyclouridine

cUpU-ISP (**9***a*) was completely degraded by snake venom enzyme to cU and pU-ISP. However, spleen enzyme which operates from the 5′-end of a nucleotide did not degrade the molecule at all. TpcU (**12**) on the other hand was completely degraded by spleen enzyme but was not degraded by snake venom. However after 7 h (at 37 °C and p*H* 9.2) with snake venom 7% of **12** had been converted to the arabinonucleotide which is degraded by the enzyme to thymidine and arabinouridine 5′-phosphate. After 24 h 35% of the cyclouridine nucleotide had been hydrolyzed by the basic medium to the arabinonucleotide which was degraded by the enzyme. The nucleotide cUpcU (**15**) was not degraded by either spleen or snake venom. Clearly the presence of a cU moiety in the chain blocks the recognition ability of the enzymes and prevents degradation. These results make an interesting contrast to those obtained by Ikehara and Tezuka (19) for compound **3**; they reported that this molecule was degraded by snake venom phosphodiesterase.

The difference in reactivities of **3** and **15** might imply that snake venom phosphodiesterase requires the anti conformation of the base for recognition. In **3** the base is nearly *anti* while in **15** it is close to the *syn* conformation. Of course there is a significant change in electronic and H-bonding characteristics of **15** as compared to **3**. Further it has been observed that the cyclopurine nucleotides are not substrates for either snake venom or spleen phosphodiesterases (20). In the cyclopurines the base is constrained in a near *anti* conformation.

We have found (35) that **12**, **1**, and **9***a* are weak competitive inhibitors of snake venom phosphodiesterase with their effectiveness as inhibitors being in the ratio of 1:2:25. They are also weak competitive inhibitors of spleen phosphodiesterase in the ratio 10:1.2:1.

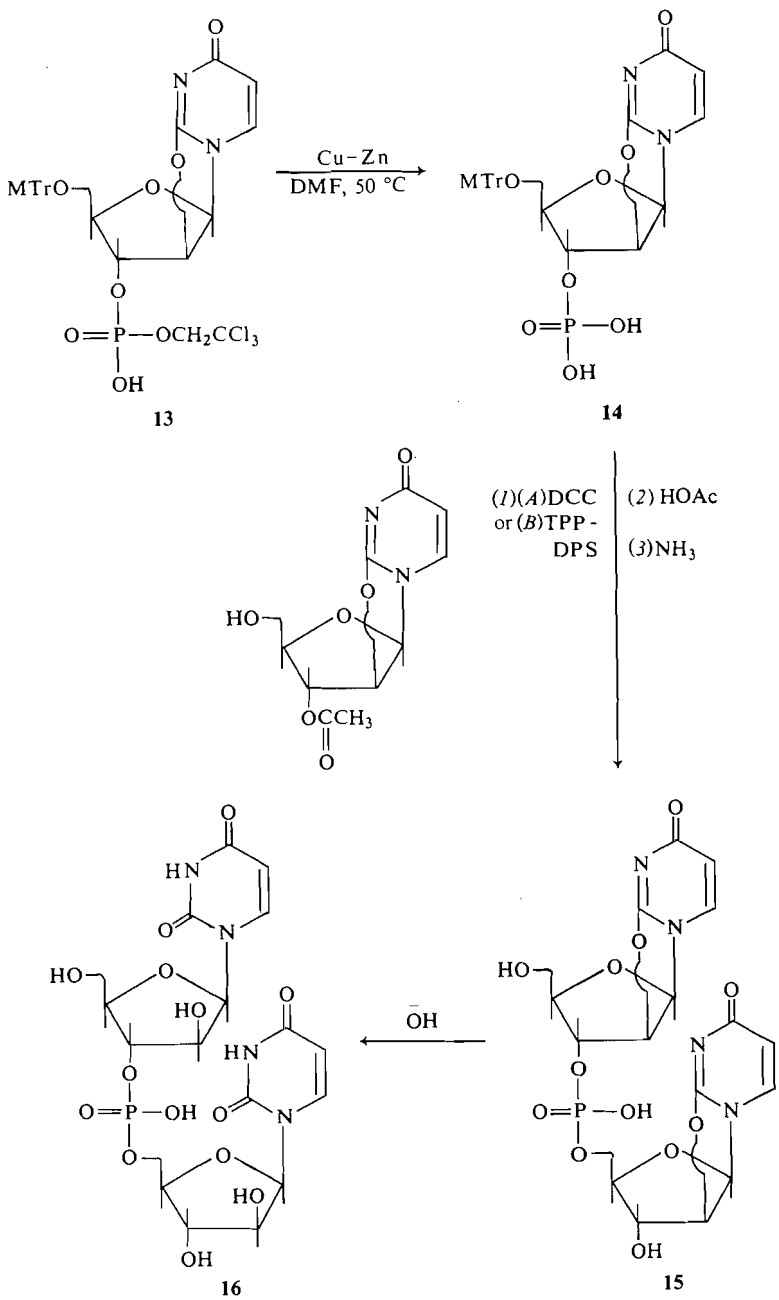
In summary, methods have been developed which incorporate cyclouridine into dinucleoside monophosphate units. These compounds are of interest as analogues of natural nucleotides and provide a convenient route to arabinouridine nucleotides.

Experimental

A complete description of all our general methods, reagents and chemicals, enzyme assays, and general procedures has been given in ref. 20 with the following exceptions.

General Methods

Column chromatography was carried out on silica gel



SCHEME 3

(60–200 mesh) from Fisher Scientific. A slurry of silica gel was allowed to settle under its own weight at the desired flow rate. The eluant was monitored by thin-layer chromatography.

For aqueous solutions containing nucleosides, the absorption maxima and corresponding extinction coefficients used were: O^2,2′-cyclouridine (250 nm, 7820 (13)); uridine (262 nm, 10 100 (30)); arabinouridine (263 nm, 10 500 (12)); and thymidine (267 nm, 9650 (30)). The term O.D. unit refers to the extinction of a nucleotide solution at neutral p*H* in 1 ml of solution using a 1-cm light path quartz cell. A number in subscript refers to the wavelength used in determination of the O.D. unit. Mass spectra were obtained on a Finnigan 1015 mass spectrometer.

Reagents and Chemicals

The dicyclohexylammonium salt of β,β,β-trichloroethyl

phosphate was prepared by dropping the phosphorodichloridate into a cooled solution of water–dioxane (1:1). After removal of the solvents, the residue was dissolved in ethanol–dioxane (1:1) and cyclohexylamine was added. The precipitated product was recrystallized from ethanol–dioxane (m.p. 194–198 °C (26)).

O^2,2′-Cyclouridine (m.p. 244–248 °C, 83% (11)), 5′-*O*-trityluridine (m.p. 114.5–116 °C, 89%, (22)), 5′-*O*-monomethoxytrityluridine (m.p. 98–102 °C, 76%, (31)), 2′,3′-*O*-isopropylidineuridine (m.p. 161–162.5 °C, 69%, (32)), and 5′-*O*-monomethoxytritylthymidine (m.p. 103–105 °C, 85%, (23)) were prepared by established procedures.

2′-Chloro-2′-deoxyuridine (dU-Cl)

(*A*) O^2,2′-Cyclouridine (3.4 g, 15 mmol) and trityl chloride (4.6 g, 16.1 mmol) were dissolved in pyridine (50 ml) and the solution was refluxed for 2 h. The solution was poured into ice water (500 ml) with stirring. The precipitate was collected by filtration and washed with petroleum ether (40 ml). The precipitate was dissolved in chloroform and dried over anhydrous sodium sulfate The solvents were removed under reduced pressure and the residue was crystallized from ethyl alcohol to yield 3.9 g (52%) of material (m.p. 164–166.5 °C, R_f 0.70 (ethylacetate)) assumed to be 5′-*O*-trityl-2′-chloro-2′-deoxyuridine ($\lambda_{max}^{(EtOH)}$, 259 nm (10 700)). A sample of this material (200 mg) was dissolved in 80% acetic acid and the solution heated on a steam bath for 20 min. The solvents were removed at reduced pressure and the last traces of acetic acid were removed by evaporation of ethanol. The residue was crystallized from ethanol (EtOH) to yield 69 mg (66%) of 2′-chloro-2′-deoxyuridine (m.p. 202–206 °C, lit. (33) m.p. 205–206 °C; R_f 0.21 (ethylacetate); $\lambda_{max}^{(EtOH)}$ 260 nm).

(*B*) O^2,2′-Cyclouridine (50 mg) was dissolved in pyridine (4 ml), 1 drop of concentrated hydrochloric acid was added, and the solution was heated on a steam bath for 2 h. The solvents were removed at reduced pressure and the residue was crystallized from ethyl acetate to give 25 mg of dU-Cl (mixture m.p. 200–205 °C).

As further proof of the identity of dU-Cl, 25 mg of the material and methanol (0.3 ml) containing sodium methoxide (8.1 mg) were dissolved in dimethylformamide and the solution was heated at 70–75 °C for 2 h. The solution was cooled and triturated with ether – petroleum ether (2:3, 50 ml). The solvent was decanted and the residue crystallized from ethanol to give O^2,2′-cyclouridine (5.2 mg, 24%).

5′-O-Trityl-O^2,2′-cyclouridine (4a)

5′-*O*-Trityluridine (670 mg, 1.36 mmol) was dissolved in dimethylformamide (1.5 ml) and treated with diphenyl carbonate (390 mg, 1.8 mmol) and sodium bicarbonate (10 mg). The mixture was heated at 150° for 30 min and then was poured into ether. The precipitated gum was crystallized from ethanol to yield 400 mg (62.5%) of **4***a* (m.p. 2.5-218°, lit. (13) m.p. 217–219 °C).

Stability of the Cyclolinkage to Acid

10% Acetic Acid

Compound **4***a* (10 mg) was dissolved in 10% acetic acid (1 ml) and the solution was heated on a steam bath for 10 min. In addition to unreacted **4***a*, two non-trityl containing nucleosides were present and identified as **1** and **2** in the ratio of 2:1.

80% Acetic Acid

Compound **4***a* (10 mg) was dissolved in 80% acetic acid (1 ml) and the solution was stirred at room temperature for 21 h. In addition to **4***a*, compounds **1** and **2** were present in a ratio of 7:1.

5′-O-Monomethoxytrityl-O^2,2′-cyclouridine (4b)

(*A*) O^2,2′-Cyclouridine (4.35 g, 19.2 mmol) and monomethoxytrityl chloride (6.0 g, 19.5 mmol) were stirred in pyridine (100 ml) for 4 days. The solvent was removed at reduced pressure and the residue was dissolved in a mixture of chloroform–water (600 ml, 1:1). The chloroform layer was separated and washed with water (2 × 50 ml). The organic solution was dried over anhydrous sodium sulfate and then concentrated. Crystallization occurred to yield 5.70 g (60%) of **4***b*; m.p. 155–158 °C; $\lambda_{max}^{(EtOH)}$ 229 nm (16 800); R_f 0.26 (tetrahydrofuran); principal bands in the i.r. spectrum (KBr disk) occurred at 6.06, 6.13, and 14.2 μ.

Anal. Calcd. for $C_{29}H_{26}H_2O_6$: C, 69.87; H, 5.26; N, 5.62. Found: C, 70.23; H, 5.28; N, 5.53.

(*B*) 5′-*O*-Monomethoxytrityluridine (730 mg, 1.41 mmol) was dissolved in dimethylformamide (0.7 ml) and was treated with diphenyl carbonate (392 mg, 1.82 mmol) and sodium bicarbonate (7 mg). The mixture was heated at 150° for 30 min. After cooling, the mixture was poured into ether. The precipitated gum was crystallized from ethanol to yield 91 mg of **4***b*.

5′-O-Monomethoxytrityl-3′-O-acetyl-O^2,2′-cyclouridine (5a)

Compound **4***b* (2.5 g, 5.0 mmol) and acetic anhydride (0.8 ml, 8.5 mmol) were dissolved in pyridine (15 ml) and the solution was stirred for 18 h and poured into ice water (500 ml) with stirring. The precipitated product was collected by filtration and dissolved in chloroform. Concentration of the solution to a small volume followed by the addition of hexane yielded 2.57 g (95%) of **5***a*; m.p. 104–107 °C; $\lambda_{max}^{(EtOH)}$ 230 nm (19 000); R_f 0.36 (THF); principal bands in the i.r. spectrum (KBr disk) occurred at 5.70, 6.08, and 14.2 μ.

Anal. Calcd. for $C_{31}H_{28}N_2O_7$: C, 68.88; H, 5.22; N, 5.18. Found: C, 68.78; H, 5.14; N, 5.08.

3′-O-Acetyl-O^2,2′-cyclouridine (6a)

Compound **4***b* (21.7 g, 40 mmol) was dissolved in 80% acetic acid and the solution was stirred for 4 h. The solvent was removed at reduced pressure and the last trace of acetic acid was removed by evaporation of ethanol. The residue was dissolved in ethanol–chloroform and the solution was poured into ether. The precipitate was filtered to yield 8.35 g (83%) of **6***a*; m.p. 205–208 °C; $\lambda_{max}^{(EtOH)}$ 249 (8220) and 224 nm (9600); R_f 0.13 (THF); principal band in the i.r. spectrum (KBr disk) occurred at 5.73 and 6.10 μ.

Anal. Calcd. for $C_{11}H_{12}N_2O_6$: C, 49.25; H, 4.51; N, 10.44. Found: C, 49.10; H, 4.50; N, 10.26.

5′-O-Monomethoxytrityl-3′-O-(β-benzoylpropionyl)-O^2,2′-cyclouridine (5b)

Compound **4***b* (4.0 g, 9.0 mmol), 3-benzoylpropionic acid (4.28 g, 24.0 mmol), and dicyclohexylcarbodiimide (6.6 g, 32.0 mmol) were dissolved in pyridine (80 ml) and the solution was stirred for 13 h. Cold water (24 ml) was added and the mixture was stirred for 6 h. The insoluble dicyclohexylurea was removed by filtration and was

washed with pyridine–water (1:1, 50 ml). The filtrate and washings were extracted with chloroform (3 × 200 ml) and the aqueous layer discarded. The chloroform layer was extracted with saturated sodium bicarbonate solution (2 × 150 ml), washed once with water, concentrated to a small volume, and applied to a silica gel column (3.5 × 46 cm) in ether. The column was eluted first with ether (4.5 l) followed by tetrahydrofuran (3.5 l). Pure **5***b* was obtained in the tetrahydrofuran fractions. Concentration of the solution followed by precipitation of the product with hexane yielded 4.3 g (82.5%) of **5***b*; m.p. 94.97 °C; $\lambda_{max}^{(EtOH)}$ 236 nm (29 000); R_f 0.42 (THF); principal bands in the i.r. spectrum occurred at 5.71, 5.93, 6.06, and 14.2 μ.

Anal. Calcd. for $C_{39}H_{34}N_2O_8$: C, 71.11; H, 5.20; N, 4.25. Found: C, 70.68; H, 5.27; N, 4.42.

3′-O-(β-Benzoylpropionyl)-O²,2′-cyclouridine (6b)

Compound **5***b* (4.02 g, 6.1 mmol) was dissolved in 80% acetic acid and the solution was stirred for 4.5 h. The solvent was removed at reduced pressure and the last trace of acetic acid was removed by evaporation of ethanol. The residue was dissolved in ethanol and applied to 15 thick-layer plates. The plates were developed in ethyl acetate and in tetrahydrofuran. The nucleoside band at R_f 0.15 was eluted from the plates with tetrahydrofuran. Concentration of the solution followed by addition of hexane to precipitate the product yielded **6***b* (2 g, 85%); m.p. 178–180 °C; $\lambda_{max}^{(EtOH)}$ 244 and 18 700); R_f 0.16 (THF); principal bands in the i.r. spectrum occurred at 5.72, 5.92, 6.17, and 13.3 μ.

Anal. Calcd. for $C_{19}H_{18}N_2O_7 \cdot \frac{1}{2}H_2O$: C, 57.72; H, 4.84; N, 7.08. Found: C, 57.61; H, 4.70; N, 7.03.

The β-Cyanoethyl Derivative of 5′-O-Monomethoxytrityl-O²,2′-cyclouridylyl-(3′–5′)-2′,3′-O-isopropylideneuridine (8a)

β-Cyanoethyl phosphate (9.0 mmol) was dried by evaporation of pyridine (2 × 10 ml). 5′-*O*-Monomethoxytrityl-*O*²,2′-cyclouridine (1.0 g, 2.0 mmol), dicyclohexylcarbodiimide (6.6 g, 32 mmol), and pyridine (10 ml) were added and the mixture was stirred for 3 days. After the addition of cold water (14 ml), stirring was continued for 16 h. The mixture was filtered to remove dicyclohexylurea and the residue was washed with pyridine–water (1:1, 35 ml). The combined filtrates were extracted with chloroform–ethanol (5:1, 400 ml). After separation of the layers, the organic layer was concentrated to a volume of ~75 ml. The organic layer was extracted with saturated aqueous sodium chloride (10 ml) and then concentrated at reduced pressure. Pyridine (10 ml) was added to dissolve the MTr-cUp(CE). The mixture was filtered to remove undissolved salts and dicyclohexylurea and the filtrate was concentrated to a gum at reduced pressure.

The gum was dried by evaporation of pyridine (2 × 10 ml). 2′,3′-*O*-Isopropylideneuridine (900 mg, 3.16 mmol) was added and the mixture was dried by evaporation of pyridine (2 × 10 ml). Triisopropylbenzenesulfonyl chloride (1.2 g, 4.0 mmol) and pyridine (10 ml) were added and the solution was concentrated to a volume of ~5 ml. The solution was stirred for 18 h. Pyridine (7 ml) was added to increase the volume of the solution. After the addition of cold water (8 ml), stirring was continued for 1 h. The solution was extracted with chloroform and the layers were separated. The organic layer was concentrated to a small volume and the solution was poured into ether with stirring. The residue was dissolved in tetrahydrofuran and applied to 20 thick-layer, plates. The plates were developed in ethyl acetate and tetrahydrofuran. The nucleotide band with R_f 0.23 was eluted with tetrahydrofuran and ethanol followed by concentration and addition of hexane to yield 480 mg (27%) of **8***a*; m.p. 140–150 °C with softening at 134 °C (34); $\lambda_{max}^{(EtOH)}$ 232 (25 500), 250 nm (sh); R_f^A 0.94; principal bands in the i.r. spectrum (KBr disk) occurred at 4.42, 5.89, 6.05, and 14.1 μ.

Anal. Calcd. for $C_{44}H_{44}N_5O_{14}P$: C, 58.86; H, 4.94; N, 7.80. Found: C, 58.63; H, 5.03; N, 7.75.

O²,2′-Cyclouridylyl-(3′–5′)-2′,3′-O-isopropylideneuridine (9a)

Compound **8***a* (30 mg) was dissolved in 80% acetic acid (0.5 ml) and the solution was stirred for 2 h. The solvent was removed at reduced pressure. The residue was dissolved in pyridine ammonium hydroxide (5:2) and the solution was stirred for 30 min. The solution was applied to Whatman papers and developed in Solvent B. The product **9***a* (R_f 0.69) was eluted with water and applied to Whatman electrophoretic paper. The product (R_m^{Tp} 0.33) was eluted with water and identified through its paper chromatographic and electrophoretic properties and its u.v. spectrum (Table 1). Snake venom enzyme degraded **9***a* to cU (R_f^B 0.71; 2.80 O.D.$_{250}$ units) and isopropylidineuridine 5′-phosphate (pU-ISP) (R_f^B 0.60; 3.65 O.D.$_{260}$ units) with pU-ISP/cU = 1.01. Spleen enzyme did not degrade **9***a* at all. The structure of **9***a* was further confirmed by its conversion to the arabinonucleotides **10***a* and **10***b* below.

Arabinouridylyl-(3′–5′)-2′,3′-O-isopropylideneuridine (10a)

Compound **9***a* (4.80 O.D.$_{255}$ units) was dissolved in 1 *N* sodium hydroxide and the solution was stirred for 1 h. The solution was neutralized with Dowex 50W-X8 resin, filtered, and applied to Whatman paper developed in solvent A. The product (R_f^A 0.28) was eluted with water and characterized by its paper chromatographic and electrophoretic properties (Table 1) and its hydrolysis by

TABLE 1. Physical properties of cyclouridine nucleotides

Compound	R_f Values on paper chromatography* Solvent A	Solvent B	R_m†	$\lambda_{max}^{(H_2O)}$ (nm)
9*a*	0.30	0.66	0.35	255
10*a*	0.28	0.66	0.38	262
10*b*	0.15	0.60	0.38	262
12	0.17	0.61	0.35	260
TpaU	0.14	0.60	0.36	264
15	—	—	0.37	250, 223
16	0.16	—	0.36	263

*Whatman 3 MM paper. Solvent A, isopropyl alcohol – concentrated ammonium hydroxide – water (7:1:2, v/v/v); solvent B, ethanol–water (7:3, v/v).

†Electrophoretic mobility relative to thymidine 3′-phosphate (Tp) in triethylammonium bicarbonate buffer (p*H* 7.5).

spleen phosphodiesterase. Spleen enzyme degraded **10***a* to arabinouridine 3′-phosphate (aUp) (2.45 O.D.$_{263}$ units; R_f^A 0.03) and isopropylidineuridine (U-ISP) (2.17 O.D.$_{262}$ units; R_f^A 0.72) with aUp/U-ISP = 1.1.

*Arabinouridylyl-(3′–5′)-uridine (**10***b*)

(*A*) Compound **8***a* (16 mg) was dissolved in 80% acetic acid (5 ml) and the solution was refluxed for 2 h. The solvent was removed at reduced pressure, the residue was dissolved in pyridine – ammonium hydroxide (5:2, 2 ml), applied to Whatman paper, and developed in solvent A. The product **10***b* was eluted from the paper with water and characterized by its chromatographic and electrophoretic properties and by its u.v. spectrum (Table 1). Snake venom degraded **10***b* to arabinouridine (**2**) (5.24 O.D.$_{263}$ units; R_f^A 0.49) and uridine 5′-phosphate (pU) (5.79 O.D.$_{262}$ units; R_f^A 0.04) with pU/**2** = 1.1.

(*B*) Compound **9***a* was heated with 80% acetic acid on a steam bath for 2.5 h. The product, **10***b*, was identical with the material produced by method A above.

The β,β,β-*Trichloroethyl Ester of 5′-O-Monomethoxytrityl-O*^{2}*,2′-cyclouridylyl-(3′–5′)-2′,3′-O-isopropylideneuridine (**8***b*)

β,β,β-Trichloroethyl phosphate (420 mg, 1.0 mmol cyclohexylammonium salt) was dissolved in dry pyridine (25 ml) and converted to the pyridinium salt by evaporation of pyridine (2 × 25 ml). Triisopropylbenzenesulfonyl chloride (450 mg, 1.5 mmol) and pyridine (25 ml) were added and the solution was stirred for 30 min. 5′-*O*-Monomethoxytrityl-*O*^{2},2′-cyclouridine (250 mg, 0.5 mmol) was added and the solution was stirred for 4 h. After the addition of more TPS (225 mg, 0.75 mmol), the solution was stirred for 17 h. The solution was cooled, cold water (5 ml) was added, and the resulting solution was stirred for 30 min and then poured into ice water (50 ml). The solution was extracted with methylene chloride (4 × 25 ml) and the combined methylene chloride extracts were washed with water (4 × 25 ml) and concentrated to a gum at reduced pressure.

The gum was dried by evaporation of pyridine at reduced pressure (2 × 25 ml). TPS (225 mg, 0.75 mmol) and pyridine (25 ml) were added and the solution was stirred for 30 min. 2′,3′-*O*-Isopropylideneuridine (280 mg, 1.0 mmol) was added and the solution was stirred for 6 h. After the addition of more TPS (100 mg, 0.30 mmol), stirring was continued for 19 h. Cold water (5 ml) was added and after stirring for 30 min the solution was poured into ice water (50 ml). After extraction with methylene chloride (4 × 25 ml), the combined methylene chloride extracts were washed with water (4 × 25 ml) and concentrated at reduced pressure to a small volume. The solution was applied to 10 thick-layer plates and developed in ethyl acetate and tetrahydrofuran. The band with R_f 0.43 was eluted with tetrahydrofuran which was concentrated and upon the addition of hexane a white precipitate formed, identified as **8***b* (111 mg, 23%, m.p. 140–150 °C). Physical properties are listed in Table 1.

Anal. Calcd. for $C_{43}H_{42}Cl_3N_4O_{14}P$: C, 52.91; H, 4.34; N, 5.74. Found: C, 53.06; H, 4.46; N, 5.61.

The β,β,β-*Trichloroethyl Derivative of O*^{2}*,2′-Cyclouridylyl-(3′–5′)-2′,3′-O-isopropylidineuridine (**9***b*)

Compound **8***b* (48 mg) was dissolved in 80% acetic acid and the solution was stirred for 4.5 h. After removal of the solvent at reduced pressure the residue was dissolved in a small volume of ethanol and applied to Whatman paper which was developed in solvent C. The nucleotide band appeared at R_f^C 0.62 and was eluted with water which on lyophilization gave **9***b* (30 mg, 86%); R_f 0.20 (THF); $\lambda_{max}^{(H_2O)}$ 255 nm. The structure was further confirmed by conversion of **9***b* to **9***a* below.

O^2*,2′-Cyclouridylyl-(3′–5′)-2′,3′-O-isopropylideneuridine (**9***a*)

Compound **9***b* (4.6 mg) was dissolved in 80% acetic acid (0.5 ml). Zinc (5 mg) was added and the mixture was stirred for 1 h. After filtration, the solvent was evaporated at reduced pressure. The residue was dissolved in ethanol–water and applied to Whatman electrophoresis paper. A nucleotide band occurred at R_m^{Tp} 0.28 and was eluted with water to yield **9***a*. Spleen enzyme did not affect this nucleotide but snake venom degraded a sample to **1** (3.5 O.D.$_{250}$ units) and pU-ISP (4.5 O.D.$_{262}$ units) with pU-ISP/**1** = 0.99.

One-half of the above product was hydrolyzed with base to **10***a* which was completely degraded by both snake and spleen enzymes in the correct manner and ratios.

*Direct Synthesis of **9***a *from **4***b

Compound **4***b* (50 mg) was phosphorylated with β,β,β-trichloroethyl phosphate (cyclohexylammonium salt) using TPS (150 mg) in pyridine (5 ml) as described above. After quenching the reaction in the normal way the product was extracted into methylene chloride. Thin-layer chromatography showed no **4***b* remaining. After removal of solvents, the residue was treated with Cu–Zn in DMF at 50 °C for 30 min. The solution was filtered, the residue washed with 50% aqueous pyridine (100 ml), and the combined washings and filtrate were passed through a Dowex 50W-X8 (pyridinium form) column. The eluant was concentrated to a small volume and added dropwise to ether (250 ml). The precipitate was collected by filtration and dried overnight over P_2O_5.

This residue was further dried by evaporation of pyridine (3 × 2 ml) and condensed with isopropylidineuridine (0.11 mmol) using triphenylphosphine (130 mg) and 2,2′-dithiodipyridine (110 mg) in pyridine (1 ml) at room temperature. After 18 h, water (2 ml) was added and stirring continued for 2 h. The reaction mixture was then poured into ether, the solvents decanted, and the residue treated with 80% HOAc for 4 h at room temperature. The solution was applied to Whatman paper which was developed in solvent C. The nucleotide band at R_f^C 0.12 was eluted and identified as **9***a* (0.04 mmol determined spectroscopically). Very little (< 10%) cUp was detected. Presumably most of the loss of material occurs at the extraction steps.

Thymidylyl-(3′–5′)-O^2*,2′-cyclouridine (TpcU, **12***)

5′-*O*-Monomethoxytritylthymidine (80 mg, 0.15 mmol) was phosphorylated with β-cyanoethyl phosphate (0.3 mmol) and mesitylenesulfonyl chloride (MS) (130 mg, 0.6 mmol) in the usual way. The cyanoethyl group was removed with 7 *M* ammonium hydroxide at 70 °C for 1.5 h. The product was dried by evaporation of pyridine and condensed with **6***a* (20 mg, 0.3 mmol) in pyridine (0.7 ml). The solution was stirred for 6.5 h. After the addition of

cold water (0.7 ml), stirring was continued for 16 h whereupon the solvents were removed at reduced pressure and the residue was dissolved in chloroform (4 ml). The chloroform solution was washed with water (3 × 1 ml) and then evaporated. The residue was treated with 80% HOAc at room temperature for 2.5 h to remove the trityl group and the products were isolated by chromatography on Whatman paper in solvent B. The product appeared at R_f^B 0.52 and was separated from a trace of Tp by preparative paper electrophoresis at p*H* 7.5. To remove the acetyl group, this product was treated with 15% ammonium hydroxide in ethanol for 90 min. Removal of solvents gave TpcU (**12**, 23%, see Table 1).

Compound **12** was completely degraded by spleen enzyme to Tp (R_f^B 0.58; 7.75 O.D.$_{267}$ units) and cU (R_f^B 0.69; 6.28 O.D.$_{250}$ units) with Tp/cU = 1.01. Incubation of TpcU with snake venom enzyme partially hydrolyzed the TpcU to TpaU and yielded TpcU (R_f^B 0.61; 8.2 O.D.$_{260}$ units; 93%), and paU (R_f^B 0.44; 0.4 O.D.$_{263}$ units) and T (R_f^B 0.75; 0.3 O.D.$_{267}$ units) with paU/T = 1.23. Incubation of **12** with snake venom for 24 h caused a 35% hydrolysis to TpaU which was degraded to T and paU with paU/T = 0.86.

Thymidylyl-(3′–5′)-arabinouridine (TpaU)

Compound **12** was treated with 80% HOAc on a steam bath for 2 h. and the product, TpaU, was isolated by paper chromatography in solvent A. The product was completely degraded by spleen enzyme to Tp (R_f^A 0.05; 4.87 O.D.$_{267}$ units) and arabinouridine (aU) (R_f^A 0.52; 4.80 O.D.$_{263}$ units) with Tp/aU = 1.1. TpaU was slowly hydrolyzed to arabinouridine 5′-phosphate (paU) (R_f^A 0.02; $\lambda_{max}^{(H_2O)}$ 263 nm) and T (R_f^A 0.63) with paU/T = 0.99. For example after 24 h, TpaU was 72% hydrolyzed by snake venom. The remaining material could be completely degraded either by further treatment with snake venom or by treatment with spleen enzyme and to the expected products.

Studies on the Phosphorylation of 4b

(*A*) Compound **4***a* (100 mg, 0.2 mmol) and β,β,β-trichloroethyl phosphate (cyclohexylammonium salt, 0.4 mmol) were condensed using TPS (0.8 mmol) in pyridine (10 ml) as described previously. The product was treated with Cu–Zn in DMF at 50 °C for 30 min. Paper electrophoresis showed complete conversion of the initial product **13** (R_m^{Tp} 0.13) to compound **14** (R_m^{Tp} 0.55). No other nucleoside material was present. Compound **14** was converted to cyclouridine 3′-phosphate (R_m^{Tp} 1.0; $\lambda_{max}^{(H_2O)}$ 250 nm) on treatment with 80% HOAc at room temperature for 3 h.

(*B*) Compound **4***b* could not be phosphorylated with β,β,β-trichloroethyl phosphate (cyclohexylammonium salt) using either DCC or TPP-DPS as condensing agents in the standard procedures.

(*C*) All three condensing agents (TPS, DCC, and TPP-DPS) quantitatively phosphorylated **4***b* using the pyridinium salt of β-cyanoethyl phosphate in the standard procedures.

Treatment of Cyclouridine with Methanol–Pyrrolidine

Cyclouridine (5 mg) was treated with methanol–pyrrolidine (9:1) under gentle reflux (80–85 °C) for 4 h. After 4 h the products were isolated by chromatography on Whatman paper in solvent C. None of the products contained the cyclouridine structure since the $\lambda_{max}^{(H_2O)}$ of all products was near 260 and not 250 nm.

*Synthesis of O^2,2′-Cyclouridylyl-(3′–5′)-O^2,2′-cyclouridine (**15**, cUpcU)*

(*A*) 5′-*O*-Monomethoxytrityl-O^2,2′-cyclouridine (250 mg, 0.5 mmol) was phosphorylated with β,β,β-trichloroethyl phosphate (cyclohexylammonium salt) (1.0 mmol) using triisopropylbenzenesulfonyl chloride (2.0 mmol) in pyridine (25 ml) in the usual manner. After the addition of cold water (5 ml), the solution was poured into ice water (50 ml), and the product extracted into methylene chloride (4 × 25 ml) which was washed with water (4 × 25 ml). The solvents were removed by evaporation at reduced pressure and the residue was dissolved in dimethylformamide (10 ml); Cu–Zn was added and the mixture was heated, with stirring, at 50° for 30 min. After filtration, the residue was washed with 50% aqueous pyridine (25 ml). The combined filtrates were applied to a Dowex 50W-X8 (pyridinium form) column and the product, MMTr-cUp, was eluted with 50% aqueous pyridine (200 ml). The solution was concentrated to a small volume and slowly dropped into ether (500 ml) with stirring. The ether was decanted and the residue was dissolved in pyridine (10 ml).

The solvent was removed by evaporation at reduced pressure and the gum was dried by evaporation of pyridine (3 × 10 ml), 3′-*O*-Acetyl-O^2,2′-cyclouridine (125 mg, 0.46 mmol) was added and the compounds were dried by evaporation of pyridine (1 × 10 ml). Dicyclohexylcarbodiimide (10 mmol) and pyridine (10 ml) were added and the mixture was stirred for 11 days. Cold water (10 ml) was added and stirring was continued for 12 h. After filtration, the residue was washed with 50% aqueous pyridine (10 ml) and the combined filtrates were evaporated at reduced pressure. The residue was dissolved in 80% acetic acid and the solution was stirred for 4 h. The solvent was removed by evaporation at reduced pressure and the residue was dissolved in ammonium hydroxide – ethanol (1:3). After stirring for 1 h, the solution was applied to Whatman papers and developed in solvent C for 3 days. The nucleotide band with R_f^C 0.16 was eluted with water and lyophilized to yield 93 mg (39%) of cUpcU ($\lambda_{max}^{(H_2O)}$ 250 and 223 nm, and λ_{min} 240 and 213 nm). The product was not degraded by spleen or snake venom phosphodiesterase.

(*B*) 5′-*O*-Monomethoxytrityl-O^2,2′-cyclouridine 3′-phosphate (MTr-cUp) was synthesized by phosphorylation of 5′-*O*-monomethoxytrityl-O^2,2′-cyclouridine (250 nm, 0.5 mmol) with trichloroethyl phosphate (cyclohexylammonium salt, 1 mmol) followed by treatment with Cu–Zn in dimethylformamide at 50° in the manner described in method *A*.

MMTr-cUp was dried by evaporation of pyridine (3 × 10 ml), 3′-*O*-acetyl-O^2,2′-cyclouridine (270 mg, 1.0 mmol) was added, and the mixture was dried by evaporation of pyridine (1 × 10 ml). Triphenylphosphine and 2,2′-dithiodipyridine (2.5 mmol each) were added with pyridine (5 ml) and the solution was stirred for 12 h. Cold water (2 ml) was added to the cooled solution and stirring was continued for 2 h. The solution was dropped into ether with stirring and the precipitate was dissolved in 80% acetic acid. The solution was stirred for 4 h, the solvent was removed by evaporation at reduced pressure,

and the residue was dissolved in ammonium hydroxide – ethanol (1:3). After stirring for 1 h, the solution was chromatographed on Whatman paper in solvent C for 3 days. The nucleotide band with R_f^C 0.12 was eluted with water and lyophilized to yield 118 mg (46%) of cUpcU ($\lambda_{max}^{(H_2O)}$ 250 and 223 nm, and λ_{min} 240 and 214 nm). The product was not degraded by snake venom or spleen phosphodiesterase.

*Arabinouridylyl-(3′–5′)-arabinouridine (aUpaU, **16**)*

cUpcU (15.8 mg) was dissolved in 2 *N* sodium hydroxide (1 ml) and the solution was stirred for 20 min. After neutralization with Dowex 50W-X8 resin (pyridinium form) and filtration, the solution was applied to Whatman papers and developed in solvent A. The band with R_f^A 0.15 was eluted with water to yield aUpaU ($\lambda_{max}^{(H_2O)}$ 260 nm). Incubation of aUpaU (10 O.D. units) with spleen enzyme gave complete degradation to aUp (R_f^A 0.09; 5.01 O.D. units) and aU (R_f^A 0.59; 4.84 O.D. units) with aUp/aU = 1.03. Incubation with snake venom enzyme (for 7 h) gave paU (R_f^A 0.07; 1.63 O.D. units), aU (R_f^A 0.55; 1.71 O.D. units), and aUpaU (R_f^A 0.16; 6.76 O.D. units; 68%) with paU/aU = 0.95. The undegraded aUpaU could be completely degraded either by prolonged treatment with snake venom enzyme or by spleen enzyme to give the expected products.

Effect of Cyclouridines on Snake Venom Enzymes (35)

Snake Venom

The *p*-nitrophenyl ester of thymidine 5′-phosphate was used as reference compound for snake venom hydrolyses ($K_m = 4.78 \times 10^{-4}$ *M* with $V_{max} = 7.25$ μmol/min, mg). Lineweaver–Burke plots and regression line analysis showed that **12**, **1**, and **9***a* were all weak, competitive inhibitors of snake venom phosphodiesterase with **12** < **1** < **9***a* (1:2:25).

Spleen Enzyme

A simlar analysis of spleen phosphodiesterase using the *p*-nitrophenyl ester of thymidine 3′-phosphate as reference ($K_m = 4.55 \times 10^{-3}$ *M*, with $V_{max} = 0.33$ μmol/min) showed that **12**, **1**, and **9***a* were weak competitive inhibitors of the enzyme with **12** > **1** > **9***a* (10:1.2:1). Full details of these studies will be reported elsewhere.

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