The Chemistry of Pseudouridine. VI. Synthesis of Pseudouridine-3′,5′-diphosphate*

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ABSTRACT: Pseudouridine-3′,5′-diphosphate was synthesized by the following route: A mixture of pseudouridine-2′- and 3′-phosphate was treated with cyanoethyl phosphate and dicyclohexylcarbodiimide to give 5′-cyanoethylphosphorylpseudouridine-2′,3′-cyclic-phosphate in 72% yield.

The cyclic phosphate was opened with ribonuclease and then the cyanoethyl group was removed at 75° with concentrated NH_4OH. This provides a new approach to pyrimidine-3′,5′-diphosphates.

During the course of our work on the chemistry of pseudouridine, it became necessary to prepare pseudouridine-3′,5′-diphosphate (compound IV).[1] Because of the unique properties of pseudouridine, the synthesis of ψ3′,5′DP presented special difficulties, and it was necessary to modify existing methods to solve these problems.

Phosphorylation of unprotected pyrimidine nucleosides with polyphosphoric acid at 60° has been used to produce a mixture of pyrimidine nucleoside-2′,5′- and -3′,5′-diphosphates (Hall and Khorana, 1954; Michelson, 1958). The isomers were separated by ion-exchange chromatography. However, the sugar moiety of pseudouridine is known to isomerize in acid (Cohn, 1960; Chambers *et al.*, 1963; Chambers and Kurkov, 1964). Therefore, phosphorylation of pseudouridine with hot polyphosphoric acid would be expected to lead not only to a mixture of phosphate isomers, but also to isomers differing in the configuration of the sugar residue. For this reason, this procedure did not appear to be desirable for the production of the C isomer of ψ3′,5′DP.[2]

Dibenzylphosphorochloridate has also been used for the synthesis of nucleoside 3′,5′-diphosphates (Baddiley *et al.*, 1958). After phosphorylation of unprotected adenosine with this reagent and removal of the blocking groups by a combination of anionic fission and catalytic hydrogenation, the mixed adenosine diphosphates were separated by ion-exchange chromatography. However, pseudouridine undergoes hydrogenolysis to give the open-chain pseudouridine-H (Cohn, 1960). Thus, removal of the benzyl groups after phosphorylation of pseudouridine with dibenzylphosphorochloridate presented a potential problem, and this approach was not investigated.

Cyanoethyl phosphate (Tener, 1961) and DCC have already been applied to the synthesis of pseudouridine-5′-phosphate (Chambers *et al.*, 1963). Removal of the cyanoethyl blocking group requires moderately vigorous alkaline treatment, and we had observed isomerization of the sugar moiety in the synthesis of ψ5′P. Nevertheless, we chose to explore the use of this method first since it had provided satisfactory results in the past when the other methods had failed (Lengyel and Chambers, 1960).

This method has been used previously for the synthesis of deoxycytidine-3′,5′-diphosphate (Tener, 1961) and adenosine-3′,5′-diphosphate (Fogarty and Rees, 1962).[3] However, before proceeding it seemed desirable to perform a model experiment using uridine, instead of the more difficultly accessible pseudouridine. Unprotected uridine was phosphorylated with CEP and DCC in pyridine. After 3 days the starting material had disappeared. The reaction mixture was worked up as described previously (Chambers *et al.*, 1963) and then treated with concentrated ammonium hydroxide at 60° instead of the usual 75°.[4] A portion of the mixture was then examined by ion-exchange chromatography (Baddiley *et al.*, 1958). A number of products were found and the major product represented only 19% of the total ultraviolet-absorbing material applied to the column. In addition, we observed that much higher salt concentrations were required to elute the compounds from the column than would be expected on the basis of charge alone.

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[1] The following abbreviations are used: ψ5′P, pseudouridine-5′-phosphate; ψ3′,5′DP, pseudouridine-3′,5′-diphosphate; DCC, dicyclohexylcarbodiimide; CEP, cyanoethyl phosphate.

[2] The C isomer has the β-D-ribofuranosyl configuration. For a description of the other isomers and references, see footnote 4 in the accompanying paper (Tomasz *et al.*, 1965).

[3] We should like to thank a referee for calling this reference to our attention.

[4] These milder conditions were used by Tener (1961) to remove the cyanoethyl group from thymidylyl-(3′→5′)-thymidine-3′-cyanoethyl phosphate. We used the lower temperature in the hope that these conditions could be applied to the synthesis of ψ3′,5′DP and would lead to less isomerization than would be expected at 75° (Chambers *et al.*, 1963).

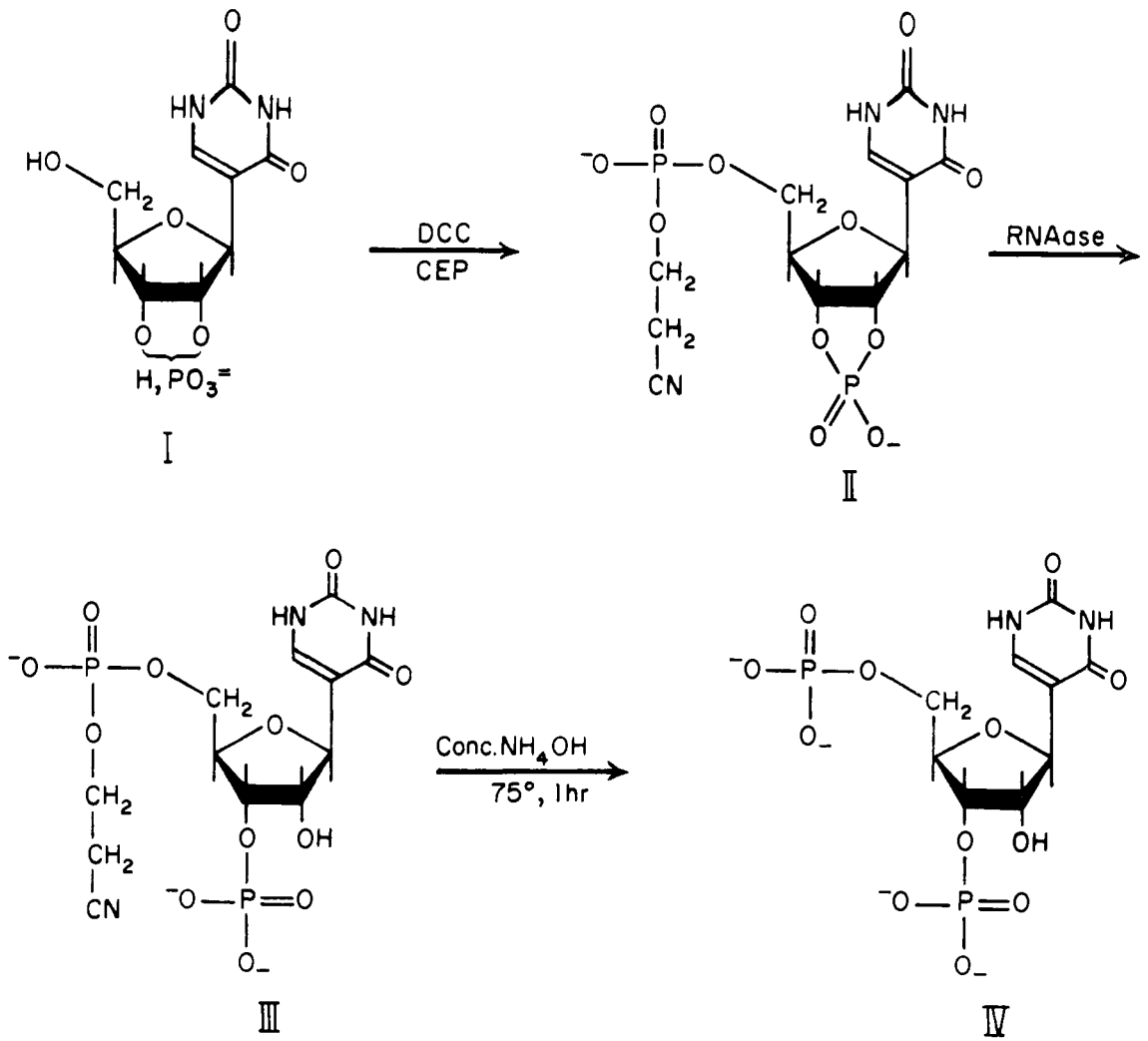

CHART 1: Phosphorylation of pseudouridine.

These results suggested that removal of the cyanoethyl groups had been incomplete and/or that polymeric products were present.[5] Therefore another portion of the product mixture was treated for an additional hour at 60° with concentrated ammonium hydroxide. The ion-exchange pattern was now simpler, but still the major product represented only 45% of the ultraviolet-absorbing material applied to the column. Furthermore, examination of the major product by paper electrophoresis showed that it was a mixture of three components.

These results clearly indicated that it would be impractical to phosphorylate unprotected pseudouridine with CEP and DCC. Therefore an alternative scheme starting from pseudouridylic acid (compound I) was devised (Chart 1).

In the first step of this synthesis, a mixture of pseudouridine-2′- and 3′-phosphate (compound I), obtained from an alkaline hydrolysate of RNA (Cohn, 1961), was treated with CEP and DCC in a mixture of formamide and pyridine. This led not only to phosphorylation of the 5′-hydroxyl group, but also to cyclization of the phosphomonoester group to give compound II in 72% yield. It is probable that the cyclization reaction preceded phosphorylation, giving pseudouridine-2′,3′-cyclic-phosphate in which the only hydroxyl group available for phosphorylation is the 5′-OH. It should be noted that further reaction of the cyclic-phosphate with DCC to give *N*-acylureas (Dekker and Khorana, 1954) was prevented by employing the ammonium salt of pseudouridylic acid.[6]

The major product was separated from the minor components by preparative paper electrophoresis using a sodium phosphate buffer, *p*H 7. Elution of the desired product from the paper with water provided a buffered solution to which ribonuclease was added directly to

[5] For discussion of some of the possibilities see Weimann and Khorana (1962). The nature of these products was not pursued further.

[6] Inhibition of *N*-acylurea formation by bases such as ammonia or triethylamine is well known (Smith *et al.*, 1958). It is interesting that we found it unnecessary to add excess base.

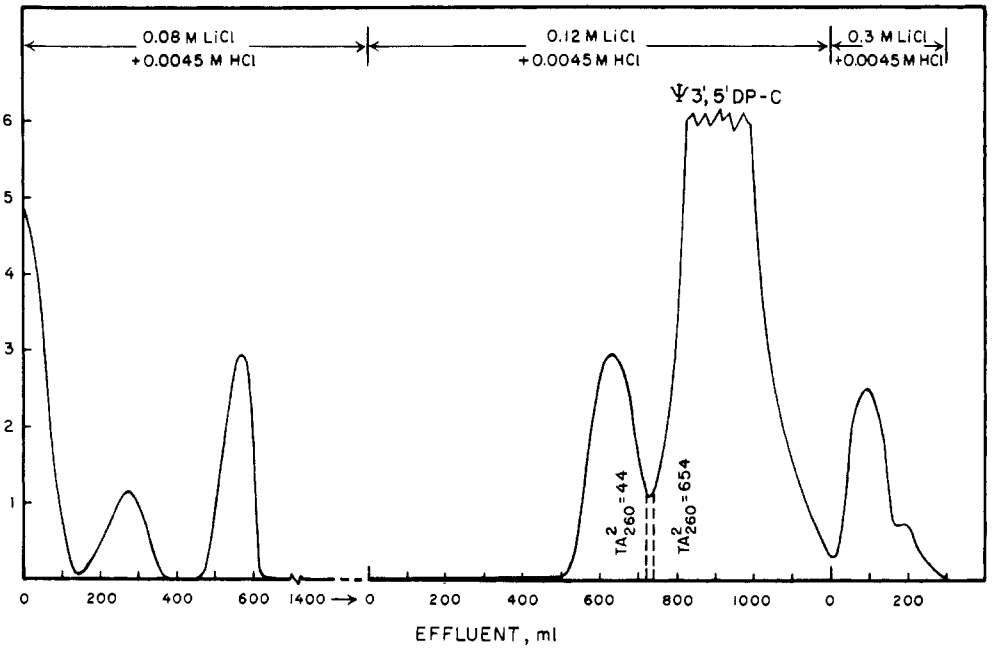

FIGURE 1: Distribution of products in synthesis of pseudouridine-5'-diphosphate.

open the cyclic-phosphate and give compound III. It is necessary to perform this step before attempting to remove the blocking group in order to prevent non-specific opening of the cyclic-phosphate to produce a mixture of 2',5'- and 3',5'-diphosphates during alkaline hydrolysis of the protecting group (Brown and Todd, 1955).

The intermediate, compound III, was treated directly, without isolation, with concentrated ammonium hydroxide at 75° for 1 hour to remove the cyanoethyl groups and give ψ3',5'DP (compound IV). The desired product was isolated by ion-exchange chromatography. The distribution of products is shown in Figure 1.

The ultraviolet spectra of the product are shown in Figure 2. It should be noted that the *p*H 12 spectrum resembles that of ψ5'P and belongs to spectral group I as expected on the basis of our hydrogen bond theory (Chambers *et al.*, 1963).

The product was unaffected by treatment with crude snake venom (*Crotalus adamanteus*), as expected.[7] Treatment with rye grass 3'-nucleotidase (Shuster and Kaplan, 1953) gave a new product quantitatively which was chromatographically identical to ψ5'P (Chambers *et al.*, 1963). This latter material, isolated by paper chromatography, was hydrolyzed completely by crude snake venom to pseudouridine and inorganic phosphate.

These enzymatic results established the positions of the phosphate groups conclusively. They also indicated that the product was entirely the C isomer.[8] In order to establish this latter point conclusively a sample of ψ3',5'-DP was treated with phosphatase and the pseudouridine produced was examined by ion-exchange chromatography as described previously (Chambers *et al.*, 1963). Pseudouridine C was the only product which could be detected. This does not mean that isomerization did not occur in the synthesis since four minor products which were separated from ψ3',5'DP by ion-exchange chromatography were not examined to see if they contained A or B isomers. From our previous experience with ψDP (Chambers *et al.*, 1963), it is probable that the peak just before ψ3',5'DP-C in Figure 1 contains ψ3',5'-DP-B.

Since the purpose of this work was to prepare ψ3',5'-DP for some model chemical studies, no attempt was made to maximize the yield (33% overall) or to scale up the procedure. This synthesis does provide a simple and relatively efficient route to ψ3',5'DP, and it should be equally applicable to other pyrimidine nucleoside 3',5'-diphosphates.

Experimental

Analytical Methods. Procedures used here were identical to those described previously (see Tomasz *et al.*, 1965).

Pseudouridine-3',5'-diphosphate. A mixture of the ammonium salts of pseudouridine-2'- and -3'-monophos-

[7] It has been established that a phosphate group on the 2' or the 3' position blocks the action of the 5'-nucleotidase in crude snake venom (Kornberg and Pricer, 1950).

[8] It has been established that the B isomer of ψ5'P is not attacked by crude *Crotalus adamanteus* venom (Chambers *et al.*, 1963). No information on the corresponding A isomers is available. It should be pointed out that Honjo *et al.* (1964) found that ψ5'P-B *was* hydrolyzed by *Trimeresurus fravoviridis* venom.

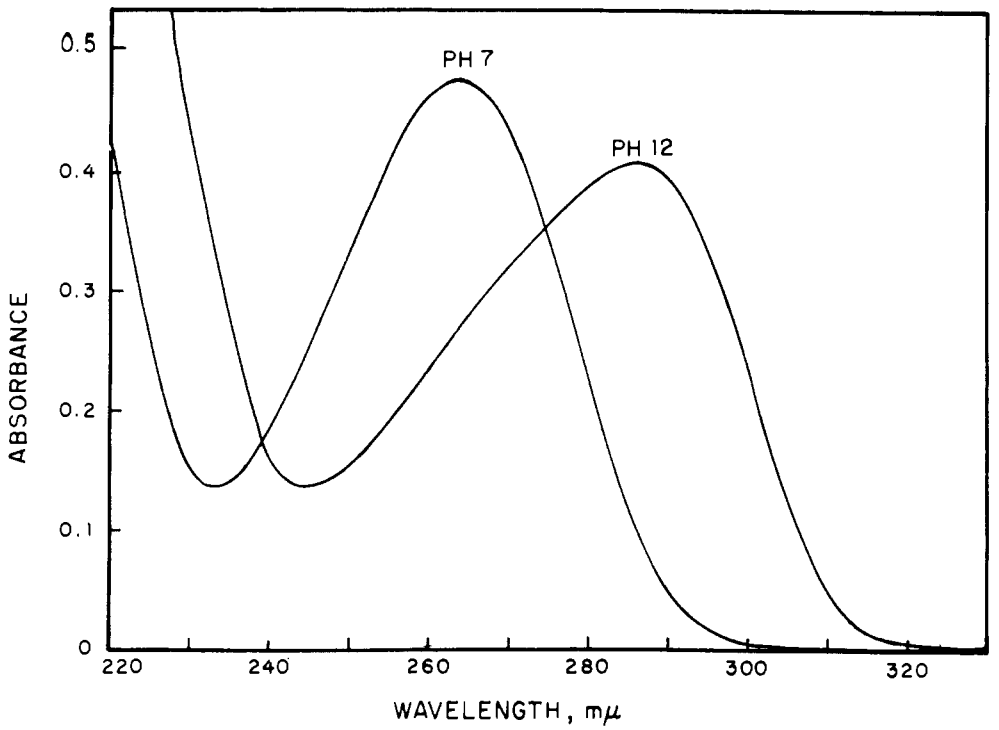

FIGURE 2: Ultraviolet spectra of pseudouridine-3′,5′-diphosphate.

phates (Cohn, 1961) (2240 A^7_{260} units; *ca.* 0.28 mmole) was dissolved in 2.7 ml of formamide. When 50 ml of dry pyridine was added the clear solution became turbid. The pyridine was evaporated *in vacuo* and the process was repeated to remove traces of moisture. Finally, 50 ml of pyridine was again added and the mixture was concentrated to 30 ml. A solution of pyridinium cyanoethyl phosphate (0.62 mmole) in pyridine (2.75 ml) and 880 mg of DCC were added to the above solution and the reaction mixture was shaken in the dark at room temperature. Crystals of dicyclohexylurea soon appeared. After 2 days electrophoresis of an aliquot of the reaction mixture (sodium phosphate buffer, 0.05 M, *p*H 7) indicated the presence of one major and four minor components. Addition of 200 mg of DCC and of cyanoethyl phosphate (0.22 mmole) in pyridine (1.0 ml) and further shaking for 1.5 days did not change the distribution of products. The dark mixture was poured into water and the aqueous solution was extracted three times with ether. The aqueous layer was evaporated *in vacuo* at 37°. The dark formamide solution was fractionated by preparative electrophoresis in five separate runs on 14.5-cm-wide sheets of Whatman 3MM paper. The major ultraviolet-absorbing band was cut from each of the papers and eluted with water. The combined eluates contained 1344 A^7_{260} units.

Part of the above-mentioned solution (1121 A^7_{260} units) was evaporated to dryness *in vacuo*. The residue, containing sodium phosphate from the electrophoresis buffer, as well as cyclic phosphate (compound II), was taken up in 3.5 ml of water. An aliquot of a ribonuclease solution (0.77 ml; 20 mg protein per ml in water) was added and the mixture was incubated at 37° for 4 hours. Electrophoresis of an aliquot from the reaction mixture indicated that the cyclic-phosphate (compound II) had completely disappeared and a new faster-moving product (compound III) had been formed. The reaction mixture was heated at 100° for 4 minutes and then filtered through a Millipore filter to remove the denatured protein. The filtrate was evaporated to dryness and the residue was taken up in 4 ml of concentrated ammonium hydroxide. A considerable amount of inorganic phosphate separated from solution and was removed by filtration. The filtrate was heated at 75° for 1 hour, cooled, and evaporated *in vacuo*. The oily residue (1025 A^2_{260} units) was dissolved in 10 ml of water. Part of this solution (872 A^2_{260} units) was fractionated on a Dowex 1 (chloride) (2% cross-linked, 200–400 mesh) ion-exchange column (2.5 × 19 cm). Stepwise elution gave the product distribution shown in Figure 1. The largest fraction contained the desired product, and ψ3′,5′DP was isolated as its lithium salt (35 mg) by the methanol procedure (Chambers, 1959).

The identity of the product was established by its ultraviolet spectrum (Figure 2) and enzymatic tests. It was shown to be pure C isomer by the procedure described here.

Enzymatic Tests. (a) CRUDE SNAKE VENOM (*Crotalus adamanteus*). A mixture of ψ3′,5′DP (5.6 A^7_{260} units) + 10 μl of 0.03 M $MgCl_2$ + 25 μl of 0.04 M Tris (*p*H 8.8) + 10 μl of the enzyme (Ross Allen's Reptile Farm, Silver Springs, Fla.) was incubated at 37° for 2 hours. Paper chromatography indicated that the material was unchanged. Under these same conditions uridine-5′-

phosphate was hydrolyzed completely to uridine and inorganic phosphate.

(b) 3′-NUCLEOTIDASE (RYE GRASS). A mixture of ψ3′,-5′DP (5.6 A^{7}_{260}) + 0.02 ml of 0.05 M Tris buffer (*p*H 7.6) + 10 μl of enzyme solution (Shuster and Kaplan, 1953) was incubated for 6 hours at 37°. Paper chromatography indicated quantitative conversion of starting material to a new product having an R_F consistent with ψ5′P. This material was eluted from the chromatogram and incubated with crude snake venom as described. After 2 hours it was completely degraded to pseudouridine and inorganic phosphate.

Isomer Distribution of Pseudouridine-3′,5′-diphosphate. ψ 3′,5′ DP (24 A^{7}_{262} in 1 ml 1 M Tris buffer, *p*H 8.5) was mixed with 0.1 ml of alkaline phosphatase (Worthington Biochemical Corp., 18 mg protein per ml). The solution was allowed to stand for 1 hour at room temperature and then was heated at 100° for 4 minutes to stop the reaction. The precipitate was removed by filtration through a Millipore filter. The filtrate was diluted to 100 ml with 0.005 M $Na_2B_4O_7$–0.02 M NH_4OH and absorbed on a 0.8- × 5-cm column of Dowex 1 (chloride) (8×, 200–400 mesh). Elution was carried out with a linear gradient as described previously (Chambers *et al.*, 1963). A single symmetrical peak was eluted at the C-isomer position (500–580 ml of eluent). The spectral properties of material in this peak were identical with those of authentic pseudouridine C.

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