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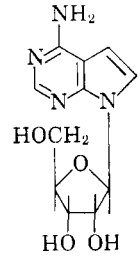
Nucleic Acids. V. Nucleotide Derivatives of Tubercidin (7-Deazaadenosine)*

Arthur R. Hanze

ABSTRACT: The syntheses of tubercidin 3′,5′-cyclic phosphate (cyclic 3,5-TuMP), the methyl ester of tubercidin 5′-phosphate (MepTu), tubercidinyl-(2′→5′)-thymidine (Tu^pT), tubercidinyl-(3′→5′)-thymidine (TupT), and thymidylyl-(3′→5′)-tubercidin (TpTu) are reported. An improved synthesis of tubercidin 5′-phosphate is given. The biological properties of these compounds are compared with those of tubercidin.

Tubercidin[1] I (7-deazaadenosine), a nucleoside described by Susuki and Marumo (1960), possesses both antitumor and antiviral activity and is highly cytotoxic (Acs *et al.*, 1964; Duvall, 1963; Owen and Smith, 1964).



Acs *et al.* (1964) studied the metabolism of tubercidin (Tu) in mouse fibroblasts and showed that Tu is incorporated into both RNA and DNA. They also reported that tubercidin 5′-triphosphate (TuTP) could replace ATP in the *Escherichia coli* RNA polymerase reaction and that it was used efficiently in heteropolymer as well as homopolymer formation. Nishimura *et al.* (1966) found that TuTP could replace ATP in the *E. coli* polymerase reaction with some thymidine-containing synthetic deoxypolynucleotides as templates but not with others. Ikehara and Ohtsuka (1965) prepared the trinucleoside diphosphate tubercidinyl-(3′→5′)-adenylyl-(3′→5′)-adenosine (TupApA) and showed that it stimulated the binding of Lys-$tRNA^{Lys}$ and Thr-$tRNA^{Thr}$ to ribosomes. In light of these interesting activities of Tu, its similarity to adenosine on the one hand and its high activity as an antitumor and antiviral agent on the other, we instituted a program to prepare some derivatives which might possess greater selectivity of action in biological systems.

One derivative in which we were particularly interested was the tubercidin analog of adenosine 3′,5-cyclic phosphate (cyclic AMP). Cyclic AMP has been shown (Sutherland and Rall, 1960) to occur widely in animal tissues. It promotes the accumulation of active phosphorylase in the tissues, causing increased glycogenolysis. For the synthesis of the analogous tubercidin 3′,5′-cyclic phosphate (cyclic 3,5-TuMP), tubercidin 5′-phosphate (pTu) was required. Although the synthesis of the latter compound has already been reported (Pike *et al.*, 1964), the yield was low and the isolation difficult. By protecting the amine function, we were able to double the over-all yield of pTu and isolate it by direct crystallization. The synthesis of this compound and its conversion into cyclic 3,5-TuMP is outlined in Figure 1. 2′,3′-Isopropylidenetubercidin (II) was benzoylated to give N^6,N^6,5′-tribenzoylisopropylidenetubercidin (III). Selective O debenzoylation gave N^6-benzoylisopropylidenetubercidin (IV). Phosphorylation of this compound by the Tener procedure (Tener, 1961) yielded the crystalline 5′-cyanoethyl phosphate (V), which was converted into isopropylidenetubercidin 5′-phosphate (VI) by treatment with aqueous methanolic ammonium hydroxide. Removal of the isopropylidene group with 80% aqueous acetic acid gave crystalline pTu VII directly. Reaction of VII with DCC in pyridine in the presence of 4-mor-

* From the Biochemical Research Division, The Upjohn Company, Kalamazoo, Michigan 49001. *Received October 2, 1967.* Paper IV of this series: Hanze (1967).

[1] Abbreviations used: Tu, tubercidin; cyclic 3,5-TuMP, tubercidin 3′,5′-cyclic phosphate; MepTu, methyl ester of tubercidin-5′-phosphate; Tu^pT, 7-deazaadenylyl-(2′→5′)-thymidine; TupT, 7-deazaadenylyl-(3′→5′)-thymidine; TpTu, thymidylyl-(3′→5′)-tubercidin; pTu, tubercidin 5′-phosphate; pCpCpA, 5′-phosphorylcytidylyl-(3′→5′)-cytidylyl-(3′→5′)-adenosine; MT, methoxytrityl; pTAc, 5′-thymidylic acid 3′-acetate; ATP, adenosine triphosphate; DCC, dicyclohexylcarbodiimide.

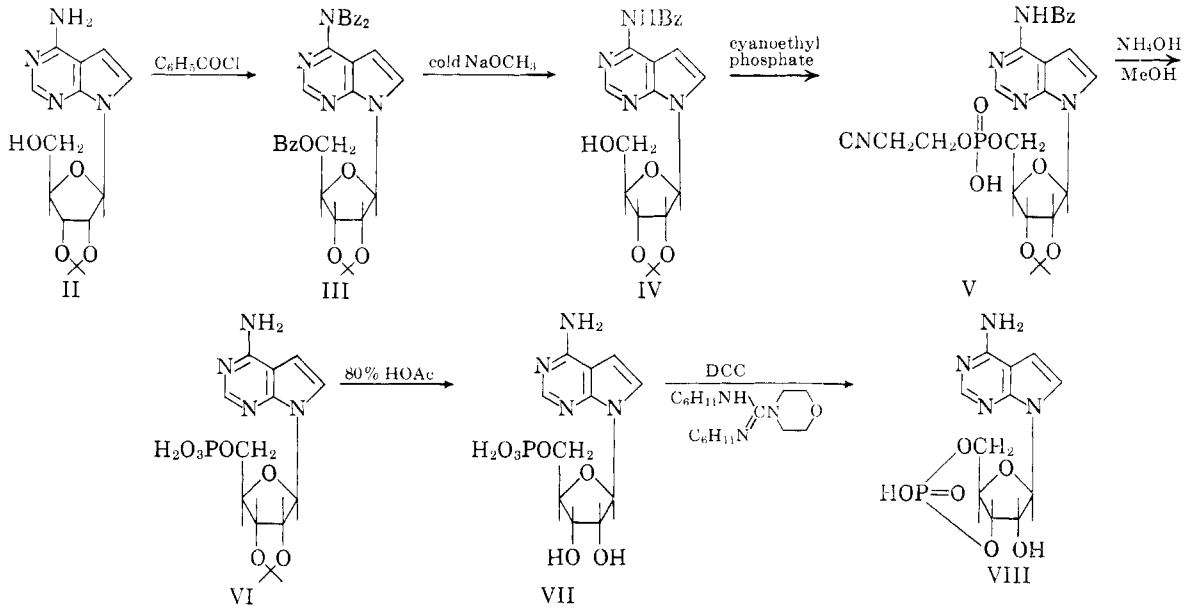


FIGURE 1.

pholine-*N*,*N*′-dicyclohexylcarboxamidine according to the procedure of Smith *et al.* (1961) gave crystalline cyclic 3,5-TuMP, VIII. In addition to analysis, ultraviolet spectrum, and nuclear magnetic resonance, proof of structure included its hydrolysis with crude snake venom (*Crotalus adamanteus*) to give tubercidin and tubercidin 3′-phosphate. The venom phosphodiesterase in the crude venom split the cyclic phosphate bond to give 75–80% 5′-phosphate and 20–25% 3-phosphate, the 5′-phosphate being further split by the 5′-nucleotidase in crude venom to give tubercidin.

Since the biological activity of nucleosides and nucleotides may possibly be modified by incorporation into dinucleoside phosphates and higher oligomers, we initiated a program designed to incorporate tubercidin into such systems. The compounds initially chosen for synthesis were TupT, Tu^{p}T, and TpTu.

The synthesis of TupT and Tu^{p}T is outlined in Figure 2. Tubercidin was benzoylated to give the pentabenzoyl IX, which was selectively O debenzoylated to N^6-benzoyltubercidin (X). Treatment of X with monomethoxytrityl chloride gave the 5′-methoxytrityl-N^6-benzoyltubercidin (XI). Condensation of XI with the 5′-acetate of 3′-thymidylic acid gave, after removal of the benzoyl and isopropylidene groups, a mixture of the 3′→5′- and 2′→5′-dinucleoside phosphates XII and XIII.

These two compounds were separated by gradient elution on a Dowex 1-X2 formate column following the procedure of Taylor and Hall (1964). Surprisingly, the two dinucleosides were also separated cleanly on a DEAE-cellulose (carbonate) column, using gradient elution with bicarbonate buffer (Figure 3).

Both compounds are cleaved by purified venom phosphodiesterase to give Tu plus pT, whereas only the compound coming off the column last is split with spleen diesterase. This latter compound must therefore possess a 3′→5′ ester linkage and the former the 2′→5′ bond since the resistance of a 2′→5′ phosphoesterase linkage toward spleen diesterase has been shown (Michelson *et al.*, 1956).

Thymidylyl-(3′→5′)-tubercidin (TpTu) was synthesized as shown in Figure 4. Treatment of 5′-cyanoethylphosphoryl-N^6-benzoylisopropylidenetubercidin(V) with dilute sodium hydroxide at 0° for 30 min selectively removed the cyanoethyl group to give the N^6-benzoyl-2′,3′-isopropylidenetubercidin 5′-phosphate (XIV). Condensation of XIV with 5′-tritylthymidine followed by removal of the benzoyl and isopropylidene groups and purification on a DEAE-cellulose column gave purified TpTu. The structure of this compound was proved by its splitting with spleen phosphodiesterase to give 3′-thymidylic acid plus tubercidin and by purified venom phosphodiesterase to give thymidine and tubercidin 5′-phosphate.

Smith *et al.* (1966) have shown that greater than 95% of Tu, 92% of pTu, and 60% of 7-deazainosine are absorbed by mammalian blood cells *in vitro*, with the tubercidin retained therein as a mixture of phosphates. Some dinucleoside phosphates, on the other hand, are excluded to the extent of approximately 50%. In the hope that a simple ester of a nucleotide would show a distribution similar to a dinucleoside phosphate, we prepared the methyl ester of tubercidin 5′-phosphate (MepTu) XVI. Using the procedure developed by Chambers and Moffatt (1958) this was synthesized (Figure 4) from an intermediate already on hand, N^6-benzoylisopropylidenetubercidin 5′-phosphate. Reaction of XIV with methyl alcohol and DCC gave the methyl ester of N^6-benzoyl-2′,3′-isopropylidenetubercidin 5′-phosphate which was converted into MepTu XVI by treatment with ammonia followed by aqueous acetic acid. MepTu is split quantitatively with venom phosphodiesterase to give pTu and is unaffected by spleen phosphodiesterase, as expected, since spleen splits only esters of nucleoside 3′-phosphates.

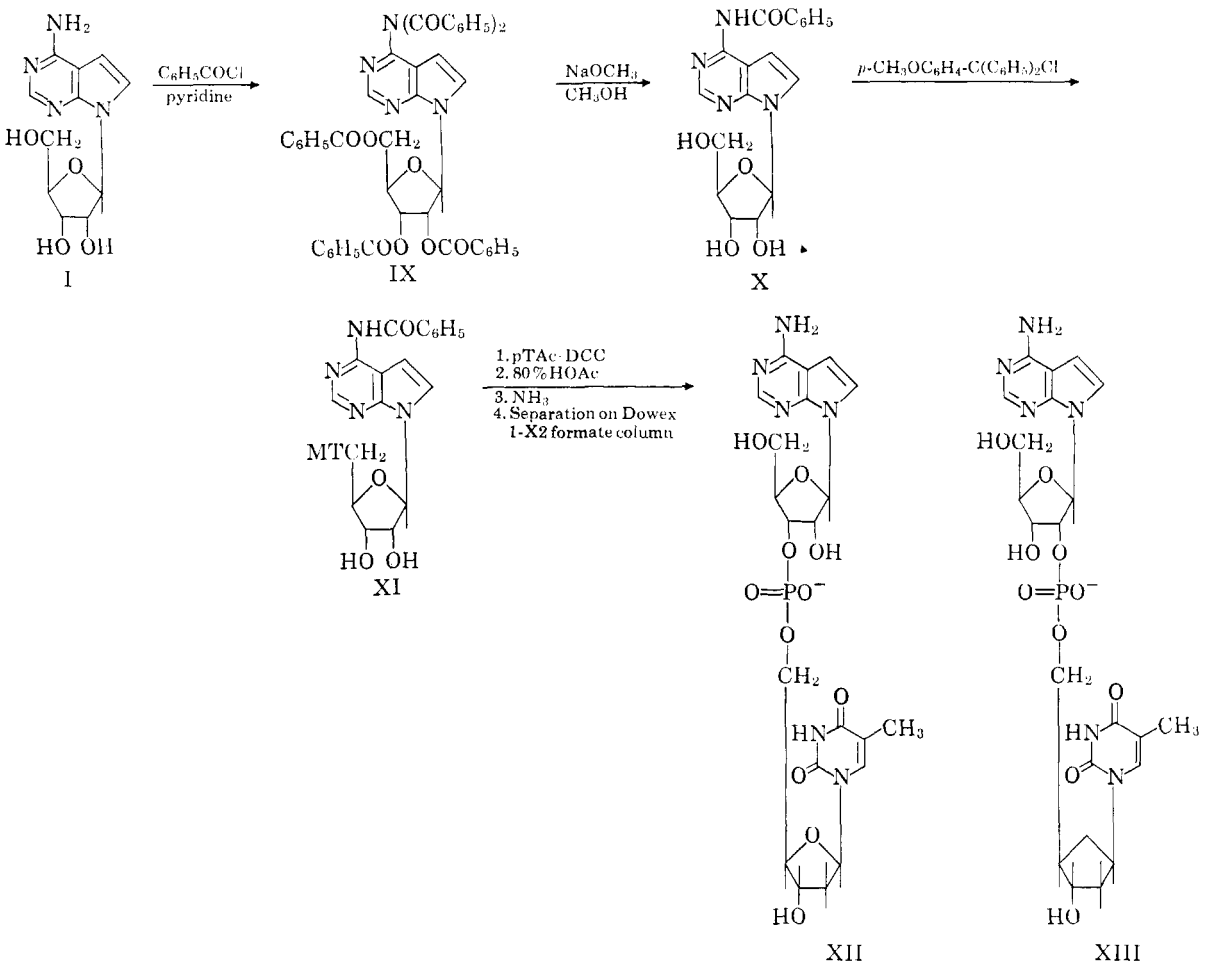


FIGURE 2.

Biological Activity

Cytotoxicity. Tu, pTu, and MepTu show approximately the same relative degrees of cytotoxicity to growing KB cells (ID_{50} = 0.006–0.01 μg/ml). The dinucleoside phosphates, TupT and Tu^pT, and cyclic 3,5-TuMP were approximately one-sixth to one-eighth as cytotoxic as tubercidin itself.

Uptake by Blood Cells. Whereas Tu and pTu are almost completely absorbed by human red blood cells *in vitro* (Smith *et al.*, 1966), MepTu is approximately 90% and tubercidin 3′→5′-cyclic phosphate 50–60% excluded. The dinucleoside phosphates are also excluded to the extent of about 50%.

Acute Toxicity in Mice. Tu, pTu, and tubercidin 3′,5′-phosphate have values of LD_{50} in mice of 18–40 mg/kg. MepTu is considerably less toxic with a value of 180 mg/kg. The complete biological data on the above derivatives of tubercidin has been published (Smith *et al.*, 1967).

Phosphorylase Activation. Cyclic 3,5-TuMP has an activity equivalent to that of cyclic AMP in the phosphorylase activation assay (Butcher *et al.*, 1965) (private communication from R. W. Butcher and E. W. Sutherland, School of Medicine, Vanderbilt University).

Experimental Section

Materials and Methods. Melting points were taken on a Thomas-Hoover apparatus. Nuclear magnetic resonance spectra were recorded on a Varian A-60A nuclear magnetic resonance spectrometer. Thin-layer chromatography was run on microscope slides coated with silica gel, Camag Kieselgel DF-5. Paper chromatograms were run on Whatman No. 3MM paper using system A (isopropyl alcohol–concentrated NH_4OH–H_2O, 7:1:2) unless otherwise noted.

Enzymatic Hydrolyses. The effect of snake venom phosphodiesterase was studied using Worthington Purified venom phosphodiesterase (VPH) (11 μg/ml; potency 0.15). The reaction mixture contained enzyme (0.03 ml), Tris buffer (pH 9, 0.1 M) (0.01 ml), and substrate (0.5–1.0 μmole) in a final volume of 0.1 ml, and was incubated at 37° for 17 hr, heated in a steam bath for 1 min, and spotted on Whatman No. 3MM paper. Paper chromatography was run in system A and Tu, pTu, and MepTu were used as controls. Hydrolyses with crude snake venom (Ross Allen Reptile Institute) were run in similar fashion.

The splitting of the dinucleoside phosphates with spleen phosphodiesterase was performed according to

the method of Razzell (1963) using enzyme preparations purchased from Worthington Biochemical Corp. The incubation mixtures contained 0.02 ml of 1 M ammonium acetate (pH 5.7), 0.005 ml of 1% Tween 80, 0.02 ml of enzyme (specific activity 20–30 units/ml), and 0.5–1.0 μmole of substrate in a final volume of 0.1 ml. After incubation for 2 hr at 37°, the reaction mixture was heated in a boiling-water bath for 1 min to precipitate protein, streaked on Whatman No. 3MM paper, and chromatographed in system A with suitable controls. The spots were eluted and the amounts were determined by ultraviolet measurement.

N^6,N^6,5′-Tribenzoylisopropylidenetubercidin (III). To a solution of 1.75 g (5 mmoles) of isopropylidenetubercidin II (Pike *et al.*, 1964) in 50 ml of pyridine in an ice bath was added 4.35 g (31.0 mmoles) of benzoyl chloride. The reaction mixture was stirred in an ice bath for 90 min and then poured onto 150 ml of ice and water. The mixture was acidified with 2 N hydrochloric acid and filtered. The solid was recrystallized from acetone–water to yield 3.28 g (two crops) of product melting at 178–180°. Recrystallization from acetone–water yielded 2.78 g (78.7%) of analytically pure material melting at 131.5–133°. The difference in melting points appears to be due to polymorphism: λ_{max}^{EtOH} 226 (ε 34,300), sh 274 (9700), sh 281 (9500), and sl sh 303 mμ (6900).

Anal. Calcd for $C_{25}H_{31}N_4O_7$ (619.63): C, 67.84; H, 5.04; N, 9.04. Found: C, 67.31; H 5.04; N, 9.13.

N^6-Benzoylisopropylidenetubercidin (IV). To a partial solution of 0.5 g (0.81 mmole) of N^6,N^6,5′-tribenzoylisopropylidenetubercidin (III) in 25 ml each of anhydrous tetrahydrofuran and anhydrous methanol in an ice bath was added with stirring 0.2 ml of 25% sodium methylate in methanol. The mixture was removed from the ice bath and the reaction was followed by thin-layer chromatography on silica gel with 50% acetone–Skellysolve B. After 25 min, an additional 0.2 ml of 25% sodium methylate was added. After 1 hr most of the starting material was gone. The solution was set in the refrigerator overnight and then treated with Dowex 50W-X8 (pyridinium form) until the pH of the solution was 5–6. The mixture was filtered and the filtrate was concentrated under reduced pressure (bath temperature 40–45°) to yield a syrup which was chromatographed on 50 g of silica gel with 25% acetone–Skellysolve B, taking 7-ml fractions. Fractions 80–115 were combined and concentrated to give 210 mg (63.7%) of amorphous IV: λ_{max}^{EtOH} 224 (ε 26,800), sh 236 (22,400), and sh 304 mμ (9600). A chromatographic fraction from a later run was crystallized from ether–Skellysolve B, mp 106.5–109°.

Anal. Calcd for $C_{21}H_{22}N_4O_5$ (410.41): C, 61.30; H, 5.63; N, 13.62. Found: C, 61.05; H, 5.64; N, 13.43.

5′-O-Cyanoethylphosphoryl-N^6-benzoylisopropylidenetubercidin (V). A solution of 12 mmoles of freshly prepared cyanoethyl phosphate in 12.0 ml of pyridine was dried by repeated concentration under reduced pressure with a bath temperature of 35°, introducing only dry air between additions. After the final concentration, 2.05 g (5.0 mmoles) of N^6-benzoylisopropylidenetubercidin (IV) was added, followed by 100 ml of especially dried pyridine (Jacob and Khorana, 1964). The concentration was repeated as above and the residue was dissolved in 40 ml of the same pyridine. Dicyclohexylcarbodiimide (6.19 g, 30 mmoles) was added and the mixture was shaken in the dark at room temperature for 4 days. Water (4.0 ml) was added and the mixture was shaken for an additional 6 hr. The mixture was filtered, the filtrate was stirred an additional 30 min with 40 ml of water, and filtered. The filtrate was concentrated under reduced pressure in a 35° bath to yield a syrup which was dissolved in 50 ml of water and extracted with ether. The aqueous layer was then lyophilized. Upon dissolving the lyophilized solid in water, crystallization occurred. The crystals were filtered and dried in a vacuum dessicator over $CaCl_2$: yield 1.43 g (52.7%), mp 210–211°, λ_{max}^{EtOH} 303 mμ (ε 12,000). A small sample (100 mg) was recrystallized from 12 ml of MeOH and 12 ml of water, mp 219.5–220°.

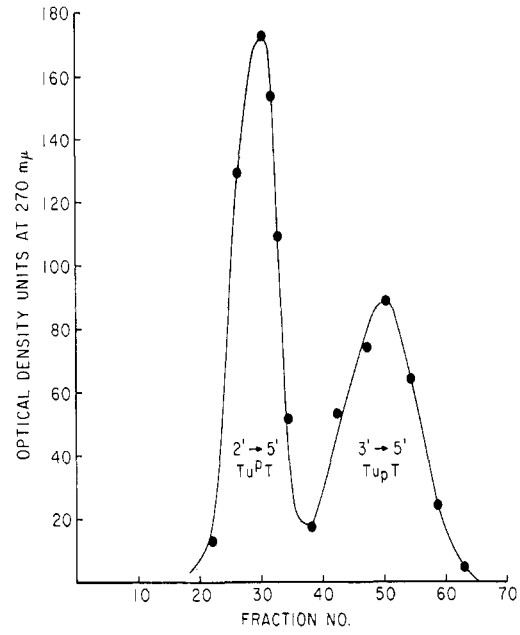


FIGURE 3: Ion-exchange chromatographic separation of tubercidinyl-(2′→5′)-thymidine (TuPT) and tubercidinyl-(3′→5′)-thymidine on a DEAE-cellulose carbonate column (18 × 21 cm). The gradient elution was 0.08–0.125 N $(C_2H_5)_3N^+H\ HCO_3^-$.

Anal. Calcd for $C_{24}H_{26}N_8O_8P$ (543.47): C, 53.03; H, 4.82; N, 12.89; P, 5.70. Found: C, 53.01; H, 4.14; N, 12.47; P, 5.69.

Tubercidin 5′-Phosphate (VII). 5′-Cyanoethylphosphoryl-N^6-benzoyl-2′,3′-isopropylidenetubercidin (V) (6.53 g, 12 mmoles) was dissolved in 50 ml of methanol and 50 ml of concentrated ammonium hydroxide. After 16 hr, there were three spots, including starting material, on a thin-layer chromatography separation on silica gel (system A or 50% MeOH–$CHCl_3$).

An additional 25 ml of concentrated ammonium hydroxide was added. After another 48 hr (*ca.* 3 days in all) there was only one product by thin-layer chromatography (silica gel–system A). The solution was concentrated to dryness under reduced pressure in a 35° bath to give a semicrystalline solid (VI).

The solid was dissolved in 200 ml of 80% acetic acid

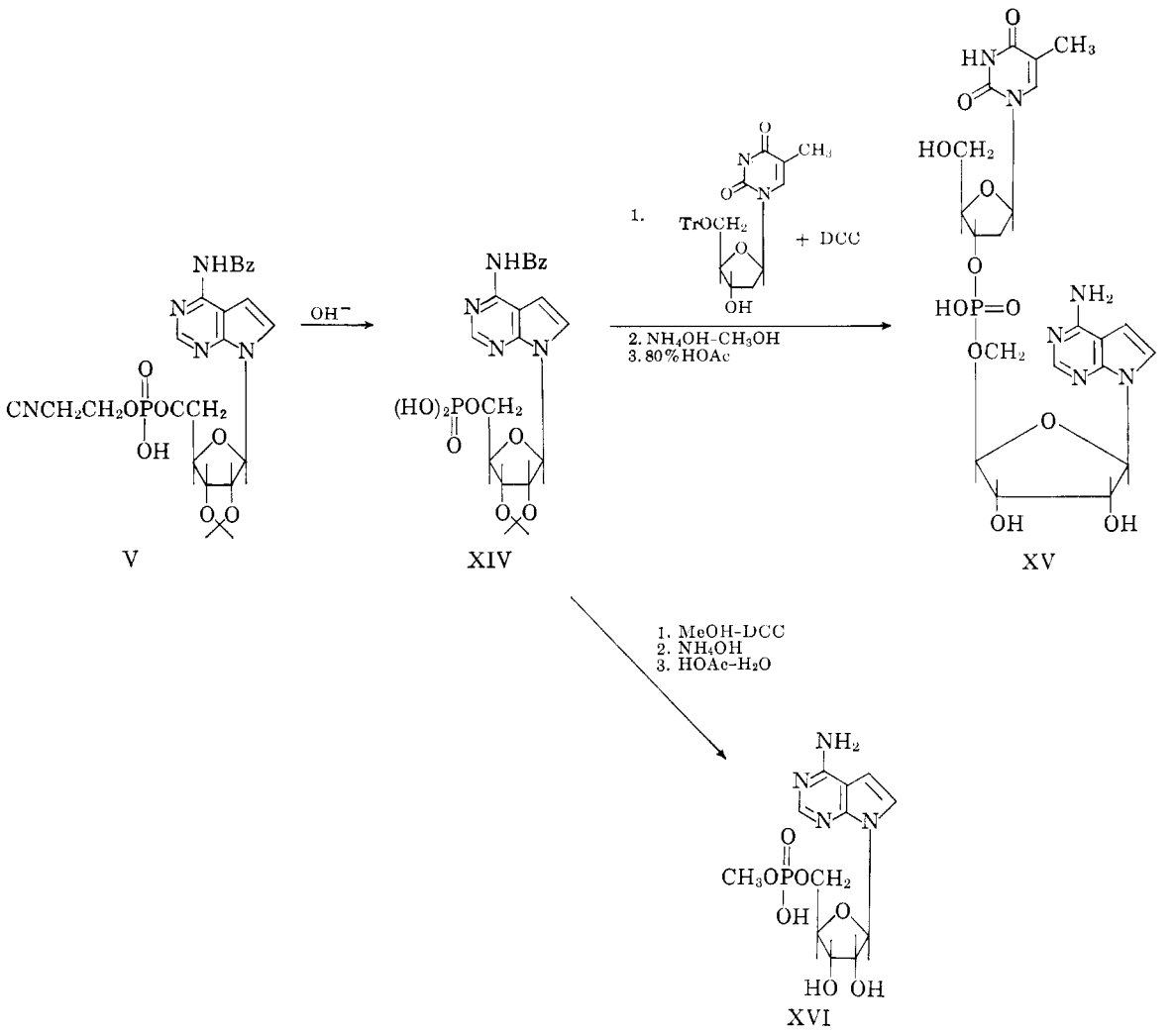


FIGURE 4.

and kept at room temperature for 5 days. By thin-layer chromatography (silica gel–system A) there was still a considerable amount of starting material. The solution was then heated on a steam bath with stirring for 2 hr and 45 min. By thin-layer chromatography there was one main spot with traces of two others. The solution was concentrated in the usual manner to yield a semi-crystalline solid. Trituration with a mixture of 10 ml of acetic and 10 ml of water gave a crystalline solid VII on centrifugation. The solid was washed five times with water, slurried with water, and freeze dried, yield 2.63 g (63.3%), mp 250–260°. By thin-layer chromatography (silica gel–system A) the product is identical with an authentic sample of pTu obtained from Dr. P. F. Wiley of these laboratories: $\lambda_{max}^{0.1\ N\ HCl}$ 228 mμ (ε 23,600) and 272 mμ (ε 10,400).

Anal. Calcd for $C_{11}H_{15}N_4O_7P$ (346.28): C, 38.16; H, 4.37; N, 16.16; P, 8.95. Found: C, 38.72; H, 4.74; N, 15.92; P, 8.55.

Tubercidin 3',5'-Cyclic Phosphate (VIII). A solution of 346 mg (1.0 mmole) of pTu (VII) and 293 mg (1.0 mmole) of 4-morpholine-*N,N'*-dicyclohexylcarboxamidine (Smith *et al.*, 1961) in 5 ml of water and 20 ml of pyridine was concentrated under reduced pressure in a 35–40° bath to dryness. Anhydrous pyridine was added and the concentration was repeated. This operation was repeated three more times. The syrupy residue was taken up in 100 ml of anhydrous pyridine and added dropwise through a condenser to a boiling solution of 412 mg (2.0 mmoles) of dicyclohexylcarbodiimide in 100 ml of anhydrous pyridine over 2 hr and 20 min, keeping the whole system anhydrous. The reaction mixture was refluxed an additional 80 min and then concentrated to dryness under reduced pressure in a 40° bath. The residue was taken up in 100 ml of water and 100 ml of ether and shaken or stirred vigorously for 2 hr. The mixture was then filtered and the aqueous layer was lyophilized to yield 0.6 g of a white fluffy solid. The solid was dissolved in 4 ml of water and clarified by filtration. By thin-layer chromatography on a cellulose plate, using system A, the product has an R_F of *ca.* 0.5 whereas starting material (pTu) remained at the origin.

The aqueous solution (4 ml) was treated with a small amount of charcoal (Darco G-60) and clarified by the use of filter aid (Celite). The filtrate was adjusted to pH 2–3 with dilute HCl, seeded with crystals previously obtained, and set in the refrigerator over the weekend. The mixture was filtered, washed with a little water, and

dried in a vacuum dessicator over P_2O_5: yield 250 mg (70.5%), mp 260–290° dec, $\lambda_{max}^{0.01N\,HCl}$ 226 mμ (ε 20,800) and 270 mμ (ε 9470). The compound had a satisfactory nuclear magnetic resonance.

Anal. Calcd for $C_{11}H_{13}N_4O_6P \cdot 1.5H_2O$ (355.24): C, 37.2; H, 4.54; N, 15.75; P, 8.73. Found: C, 37.22; H, 4.53; N, 16.07; P, 8.58.

Additional proof of structure of the product consisted of examining the products of its splitting with crude snake venom (*C. adamanteus*). Since the crude venom contains some 5′-nucleotidase, any 5′-phosphate formed by the splitting of the cyclic bond with the phosphodiesterase, which is present in the venom, will be further split to give the free nucleoside, Tu. Any 3′-phosphate formed by the phosphodiesterase will remain as such. Hydrolysis of cyclic TuMP with crude snake venom (23.5 mg/ml) for 23 hr at 37° gave tubercidin and tubercidin 3′-phosphate in a ratio of *ca.* 3 to 1. Thus, the venom phosphodiesterase splits the cyclic bond to give 75–80% 5′-phosphate and 20–25% 3′-phosphate.

N^6,N^6-2′,3′,5′-Pentabenzoyltubercidin (IX). Benzoyl chloride (38 ml) was added to a solution of 1.25 g of tubercidin in 25 ml of dry pyridine at 0–5°. The reaction mixture was allowed to warm to room temperature. After 40 hr (a subsequent run was worked up in 1.5 hr on the basis of thin-layer chromatography on silica gel using 50% acetone–Skellysolve B) the solution was poured into ice and water and the solid was filtered, dried, and recrystallized from acetone–water, crude 3.56 g, mp 180–182°. Recrystallization from acetone (40 ml)–water (4 ml) gave 2.7 g (two crops) of analytically pure material: mp 187–188°; λ_{max}^{EtOH} 230 (ε 56,750), sh 274 (12,450), sh 283 (11,800), and sh 303 mμ (7500).

Anal. Calcd for $C_{46}H_{34}N_4O_9$ (786.76): C, 70.22; H, 4.36; N, 7.12. Found: C, 70.40; H, 4.46; N, 7.40.

N^6-Benzoyltubercidin (X). A solution of 0.5 g (0.73 mmole) of N^6,N^6-2′,3′,5′-pentabenzoyltubercidin (IX) in 25 ml each of anhydrous methanol and anhydrous tetrahydrofuran was treated in an ice bath with 0.5 ml of 25% sodium methylate in methanol. The reaction was followed by thin-layer chromatography on silica gel (50% acetone–Skellysolve B). After 20 min there was no starting material but three slower moving materials were found. After 6 hr there was essentially one spot. The solution was kept in the freezing compartment of the refrigerator overnight and then the sodium ions were removed with Dowex 50W-X8 (pyridinium form). The filtrate was concentrated to yield a syrup (350 mg) which was crystallized from isopropyl alcohol and then methyl alcohol–isopropyl alcohol to yield 65 mg (24%) of analytically pure material: mp 181–182°; λ_{max}^{EtOH} 225 (ε 27,100), sh 238 (22,950), sl sh 288 (8300), and 305 mμ (9200).

Anal. Calcd for $C_{18}H_{18}N_4O_5$ (370.36): C, 58.37; H, 4.90; N, 15.13. Found: C, 58.42; H, 4.96; N, 14.96.

N^6-Benzoyl-5′-O-p-anisyldiphenylmethyltubercidin (XI). A solution of 1.5 g (4.05 mmoles) of N^6-benzoyltubercidin (X) and 1.8 g (6.08 mmoles) of *p*-anisyldiphenylmethyl chloride was treated with 30 ml of pyridine for 4 hr at 25°. The solution was concentrated to dryness under high vacuum and the residue was dissolved in ethyl acetate. The ethyl acetate solution was washed with water, dried over anhydrous sodium sulfate, filtered, and concentrated to dryness under reduced pressure. The residue was crystallized from benzene to yield 1.37 g (52.8%, three crops) of product melting at 168–171°. Recrystallization from benzene gave an analytical sample, mp 170–171°.

Anal. Calcd for $C_{38}H_{34}N_4O_6$ (642.68): C, 71.01; H, 5.33; N, 8.72. Found: C, 70.86; H, 5.58; N, 9.38, 9.34.

7-Deazaadenylyl-(3′→5′)- (XII) and (2′→5′)-Thymidine (XIII). A solution of 920 mg (2 mmoles) of N^6-benzoyl-5′-*O*-*p*-anisyldiphenylmethyltubercidin (XI) and 1.29 g (2 mmoles) of 3′-*O*-acetylthymidylic 5′-acid, prepared according to Gilham and Khorana (1958), in 70 ml of dry pyridine in a 100-ml round-bottom flask equipped with an addition funnel protected from moisture, was concentrated under reduced pressure in a 40° bath to dryness. Dry pyridine was added through the addition funnel without opening the system to air and the concentration was repeated. This was repeated a third time. The system was brought to atmospheric pressure with dry air and 20 ml of dry pyridine was added under dry nitrogen. To this especially dried solution was added 2.06 g (10 mmoles) of dicyclohexylcarbodiimide and the mixture was shaken in the dark at room temperature for 3 days. Water (10 ml) was added and the mixture was stirred for 23 hr. The mixture was filtered, washed with water, and the filtrate was concentrated under high vacuum at a bath temperature of 38° to give a syrupy solid. The mixture was taken up in water, and the solution was extracted with petroleum ether (bp 30–60°). The aqueous layer was lyophilized, yield, 2.2 g.

For the removal of the benzoyl and acetyl groups, 1.1 g of the above lyophilized solid was dissolved in 8 ml of water, 5 ml of methanol, and 16 ml of concentrated ammonium hydroxide and stirred overnight at room temperature. The reaction was followed by the disappearance of the 303-mμ *N*-benzoyl peak and the appearance of a 264-mμ peak. The mixture was concentrated to dryness under high vacuum with a bath temperature of 35°.

The 5′-*O*-*p*-anisyldiphenylmethyl group was then removed by hydrolysis of the above syrup in 80% aqueous acetic acid for 18 hr. The acetic acid was then removed under high vacuum at a bath temperature of 30°. The residue was dissolved in 20 ml of water, and the solution was adjusted to pH 8 with 3 N NH_4OH and extracted with ether. The aqueous layer was concentrated slightly to remove the dissolved ether and diluted to mark in a 25-ml volumetric flask, to give 17,000$A_{269}^{pH\,8.0}$ units. The aqueous solution was then lyophilized to give an amorphous solid.

The above solid (60 mg) in water was fractionated on a 1.1 × 17 cm Dowex 1-X2 formate column, using a linear salt gradient increasing 0.03 N sodium formate to 0.08 N sodium formate (total 4 l., 20-ml fractions; flow rate 1.0 ml/3 min). Since the compounds were still not completely eluted, elution was continued with 0.08 N sodium formate. Fractions 84–114 were combined, lyophilized, and run over a Dowex 50W-X8 (H^+ form) column, the eluate immediately being lyophilized (fraction A). Fractions 220–264 were similarly combined

and desalted (fraction B). Fraction A contained 537 $A_{268}^{pH\,8.0}$ units and B had $232A_{268}^{pH8.0}$ units. At three different levels of enzyme concentration, the former was totally resistant to enzymatic splitting by spleen phosphodiesterase whereas the latter was completely split. Both were split to varying degrees with venom. Thus, fraction A is the 2′→5′-phosphodiester (XIII) and B is the 3′→5′ compound, since it is known (Michelson *et al.*, 1956) that 2′→5′-phosphodiester linkages are not affected by spleen phosphodiesterases, but are split by venom phosphodiesterase at high enzyme concentrations.

Purification of the remainder of the material was effected on a DEAE-cellulose column (Figure 3). A solution of 170 mg in water at pH 7.5 was chromatographed on a 1.8 × 21 cm DEAE-cellulose column in the carbonate form, taking 5-ml fractions and eluting by gradient, using 2 l. each of 0.08 and 0.125 N triethylammonium bicarbonate at a flow rate of 1 ml/3 min.

Fractions 23–35 were combined, lyophilized, redissolved twice in water, and lyophilized to give $1300A_{270}^{pH\,8.0}$ units; R_F 0.56 on system A. This material is identical with fraction A above. Fractions 42–58 were similarly treated to give material identical with fraction B, 1050 $A_{270}^{pH\,8.0}$ units.

5′-O-Phosphoryl-N⁶-benzoylisopropylidenetubercidin (XIV). To an ice-cold solution of 544 mg (1 mmole) of 5′-*O*-cyanoethylphosphoryl-*N*⁶-benzoylisopropylidenetubercidin in 5.5 ml each of water and pyridine was added 11.0 ml of 1.0 N sodium hydroxide. The solution was stirred in an ice bath for 30 min and then adjusted to pH 6 with freshly prepared Dowex 50W-X8 (pyridinium form). The mixture was filtered, the resin was washed with water, and the combined filtrates were lyophilized to yield 500 mg of product which was used as is in the next step.

Thymidylyl-(3′→5′)-tubercidin (XV). A mixture of 500 mg (1 mmole) of 5′-*O*-phosphoryl-*N*⁶-benzoylisopropylidenetubercidin (XIV) (see above) and 485 mg (1 mmole) of 5′-*O*-tritylthymidine (Gilham and Khorana, 1958) was dried by repeated concentrations under reduced pressure at 30° with pyridine purified according to Jacob and Khorana (1964). Dry air was introduced into the flask, followed by 5 ml of specially purified dry pyridine and 1.03 g (5 mmoles) of dicyclohexylcarbodiimide. The mixture was shaken in the dark at room temperature for 4 days. Water (5 ml) was added and the mixture was stirred for an additional 24 hr. The mixture was filtered and the filtrate was concentrated under reduced pressure to a syrup.

For removal of the benzoyl group, the above syrup was treated overnight with 8 ml of water, 20 ml of concentrated NH_4OH, and 20 ml of MeOH. The mixture was concentrated to dryness under high vacuum at 30–35°, triturated with acetone, and a small amount (90 mg) of dicyclohexylurea was removed by filtration. The filtrate was concentrated to dryness and treated with 12 ml of 80% acetic acid for 2.5 days at room temperature to remove the trityl and isopropylidene groups. The mixture was filtered and the filtrate was concentrated under reduced pressure at 35° to yield a syrup. The syrup was dissolved in 20 ml of water, the pH was adjusted to 8 with 3 N NH_4OH, and the solution was extracted with ether. The dissolved ether was removed by concentration and the volume was made up to 25 ml, $6600A_{270}^{pH\,8.0}$ units. The total amount was chromatographed on a 3 × 51 cm DEAE-cellulose (carbonate form) column taking 9-ml fractions and eluting gradiently with 2 l. each of 0.02 and 0.125 N triethylammonium bicarbonate at a flow rate of 1.0 ml/min.

Fractions 66–77 were combined ($1600A_{267}^{pH\,8.0}$ units). The solution was lyophilized, the residue was dissolved in a minimum of water, streaked on four 6 × 22.5 in. Whatman No. 3MM sheets, and developed with system A to give two bands. The slower moving band (R_F 0.23, $205A_{267}$ units) was split by venom phosphodiesterase into thymidine and tubercidin 5′-*O*-phosphate and hence is the desired TpTu. The faster moving band was shown to be the isopropylidene derivative of TpTu by hydrolysis to TpTu with 80% acetic acid for 48 hr at 24°.

Tubercidin 5′-Phosphate, Methyl Ester (MepTu) (XVI). A solution of 6.9 g (14.0 mmoles) of *N*⁶-benzoyl-2′,3′-isopropylidenetubercidin 5′-phosphate (XIV) in 50 ml of anhydrous purified pyridine was concentrated to dryness under reduced pressure at 35°. This procedure was repeated twice to ensure complete dryness. To this syrup was added 280 ml of anhydrous purified pyridine, 16.8 ml of tributylamine, 560 ml of anhydrous methanol, and 28.9 g of dicyclohexylcarbodiimide and the mixture was stirred in the dark for 6 days at room temperature. The mixture was concentrated to a very small volume under reduced pressure at 35° and extracted twice with 250 ml of anhydrous ether, and the ether was decanted. To the residual liquid was added 500 ml of water and the mixture was refrigerated overnight. The precipitated dicyclohexylurea was removed by filtration, the filtrate was stirred an additional 2 hr at room temperature, concentrated to *ca.* 70 ml, and refiltered. There were $87{,}500A_{302}^{pH\,8.0}$ units. The solution was concentrated to a syrup at 35° and dissolved in 270 ml of concentrated NH_4OH and 270 ml of methanol and stirred for 22 hr at room temperature. The reaction mixture was concentrated at 35° to a syrup which was dissolved in 400 ml of 80% acetic acid and heated on the steam bath for 1 hr to give $99{,}000A_{272}^{pH\,8.0}$ units. By paper chromatography on system A, there were six spots with the following R_F's: 0.14, 0.36 (phosphorescent), 0.52, 0.58, 0.76 (phosphorescent), and 0.89. Three spots, none of which was pTu, were observed on electrophoresis on 5 × 25.5 in. sheets for 1 hr at 5000 V, 80–85 mA at pH 3.6 (0.05 M formate), 4.5 (0.1 M acetate), or 6.8 (0.03 M phosphate).

The acetic acid solution was concentrated to dryness at 35°. The syrup was dissolved in a minimum of water, the pH was adjusted to 8.0 with NH_4OH, and the solution was put on a column of analytical grade Dowex 1-X2 (formate form), measuring 2.7 × 40 cm, which had previously been washed well with water. Separation was effected by gradient elution taking 18-ml fractions at a flow rate of 1 ml/min, 1 l. of 0.02 M to 1 l. of 0.06 M ammonium formate (pH 5), 500 ml of 0.06 M to 500 ml of 0.08 M ammonium formate (pH 5), 2 l. of 0.08 M to 2 l. of 0.10 M ammonium formate (pH 5), and 4 l. of 0.15 M ammonium formate (pH 5).

Fractions 360–470 (*ca.* 10,000$A_{270}^{pH\ 7.5}$ units) contained 3.5 g (60%). This fraction appeared to be pure by papergram (R_F 0.54 in system A) and by paper electrophoresis at pH 3.6 for 45 min (4000 V, 90 mA) when detected by ultraviolet light. When, however, the paper chromatogram was dipped in a phosphate detection solution containing 250 mg of sulfosalicylic acid and 25 mg of ferric chloride hexahydrate in 80 ml of alcohol and 15 ml of water, and allowed to dry, a fast-moving spot, which was nonultraviolet absorbing, showed up as a yellow spot on a blue background whereas MepTu showed up as a white spot.

For the initial preparation of crystalline MepTu, 50 mg of the above material was dissolved in a minimum of water, and a small amount of methanol was added to facilitate application of the solution to paper. The solution was applied to two sheets of Whatman No. 3MM paper and developed in a solvent system of 1-butanol, acetic acid, and water (2:1:1) for 18–20 hr. The band of MepTu, detected by ultraviolet light, was cut out and eluted with distilled water. The solution was lyophilized to give 46.7 mg of a white solid which was dissolved in a minimum of warm methanol. The solution was brought to opalescence with 2-propanol and crystallization induced by scratching the wall of the flask. Using these crystals as seed, the column fraction containing MepTu was crystallized from methyl alcohol–isopropyl alcohol to give material melting at 236.5–238°. Several triturations with warm methanol removed a small amount of the nonultraviolet-absorbing impurity still present to give material melting at 239.5°: $\lambda_{max}^{H_2O}$ 202 (ϵ 21,850), 224 (18,500), and 269 mμ (11,350); $[\alpha]_D^{H_2O}$ −50°; nuclear magnetic resonance (D_2O): doublet centered at 3.72 ppm, J_{PH} = 11 cycles/sec (methyl group); doublet at 6.3 ppm, J = 5 cycles/sec (anomeric proton); doublets at 6.8 (C-7 proton) and 7.70 ppm (C-8 proton), J = 4 cycles/sec MepTu is split quantitatively by purified snake venom phosphodiesterase to give exclusively pTu. It is unaffected on incubation with spleen phosphodiesterase for 16 hr.

Anal. Calcd for $C_{12}H_{17}N_4O_7P$: C, 40.00; H, 4.76; N, 15.55; P, 8.60; equiv wt, 360.27. Found: C, 40.30; H, 4.78; N, 15.16; P, 8.64; equiv wt, 361.

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