Research Laboratories, The Upjohn Company

Tubercidin and Related Compounds

J. E. Pike, L. Slechta and P. F. Wiley

Chemical studies with the nucleoside-type antibiotic tubercidin isolated from *Streptomyces tubercidicus* (1) have led to its structural formulation as 4-amino-7β-D-ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidine (I), (1,2,3). During these studies it was noted that the base-sugar bond in tubercidin was much more resistant to acid hydrolysis than the corresponding bond in purine nucleosides. Subsequent work with *E. coli* revealed a complete resistance of tubercidin to the action of the enzyme nucleoside phophorylase (5) which cleaves a nucleoside in the presence of inorganic phosphate to the free base and ribose-1-phosphate. It was considered that this nucleoside stability would offer some special value in cancer chemotherapy. In particular the 4-mercapto analogue (III, R = H) which has a clear resemblance to 6-mercaptopurine riboside was felt to be an important specific objective.

In mammalian as well as microbial cells, 6-mercaptopurine is converted to the active form, 6-mercaptopurine ribonucleotide, by the action of pyrophosphorylase (6, 7, 8, 9, 10).

Cells resistant to the inhibitory action of 6-mercaptopurine exhibit an impaired capacity in this conversion (7, 11). The substitution of 6-mercaptopurine by 6-mercaptopurine riboside does not offer any advantage, since in sensitive cells, both are equally active and in resistant cells both are inactive (12). The explanation for this lies in the fact that 6-mercaptopurine riboside is cleaved by nucleoside phosphorylase to the free base which in turn is converted to the nucleotide by the pyrophosporylase.

It was the stability of tubercidin to the nucleoside phosphorylase which prompted the described synthesis of the 4-mercapto analogue, especially since it was found that tubercidin is converted in *E. coli* and Ehrlich ascites tumor cells *in vivo* to moni, di and triphosphate (5). The synthesis of tubercidin-5'-phosphate was also considered to be of interest since such a derivative might enhance activity by changing the distribution of active material within the cell.

Deamination of tubercidin (I) with nitrous acid gave the inosine analog (II) [R = H, 7β-D-ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidone-4]. The infra red spectrum of this compound was consistent with the amide structure; this compound has been reported by Mizuno, Ikehara, Watanabe and Suzaki (3, 13) who prepared it both by total synthesis and from tubercidin. Since the experimental details for the deamination were somewhat different from those of Mizuno, *et al.*, we have reported our procedure. It is interesting that attempts to deaminate the free base were not successful (14). Protection of the hydroxy groups of the sugar in (II, R = H) as acetates, was followed by reaction with phosphorus pentasulfide (15) to give the 4-mercapto derivative (III, R = Ac). Removal of the acetate protecting groups with base gave 7β-D-ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidine-4-thiol (III, R = H).

Tubercidin-5'-phosphate was synthesized by a modification of the procedure of Tener (16). Tubercidin was converted to its 2',3'-isopropylidene derivative (IV) by the procedure of Cramer and Weimann (17). This ketal has been reported by Mizuno *et al.*, (3, 4) but the compound was not crystallized and very few physical properties were reported. The ketal (IV) was treated with 2-cyanoethylphosphate in the presence of N,N'-dicyclohexylcarbodiimide followed by base elimination of acrylonitrile with subsequent acid hydrolysis of the ketal. Tubercidin-5'-phosphate was isolated as a salt which was chromatographed on Dowex-1 (formate cycle) to give the pure free acid.

Biological studies will be reported at a later date.

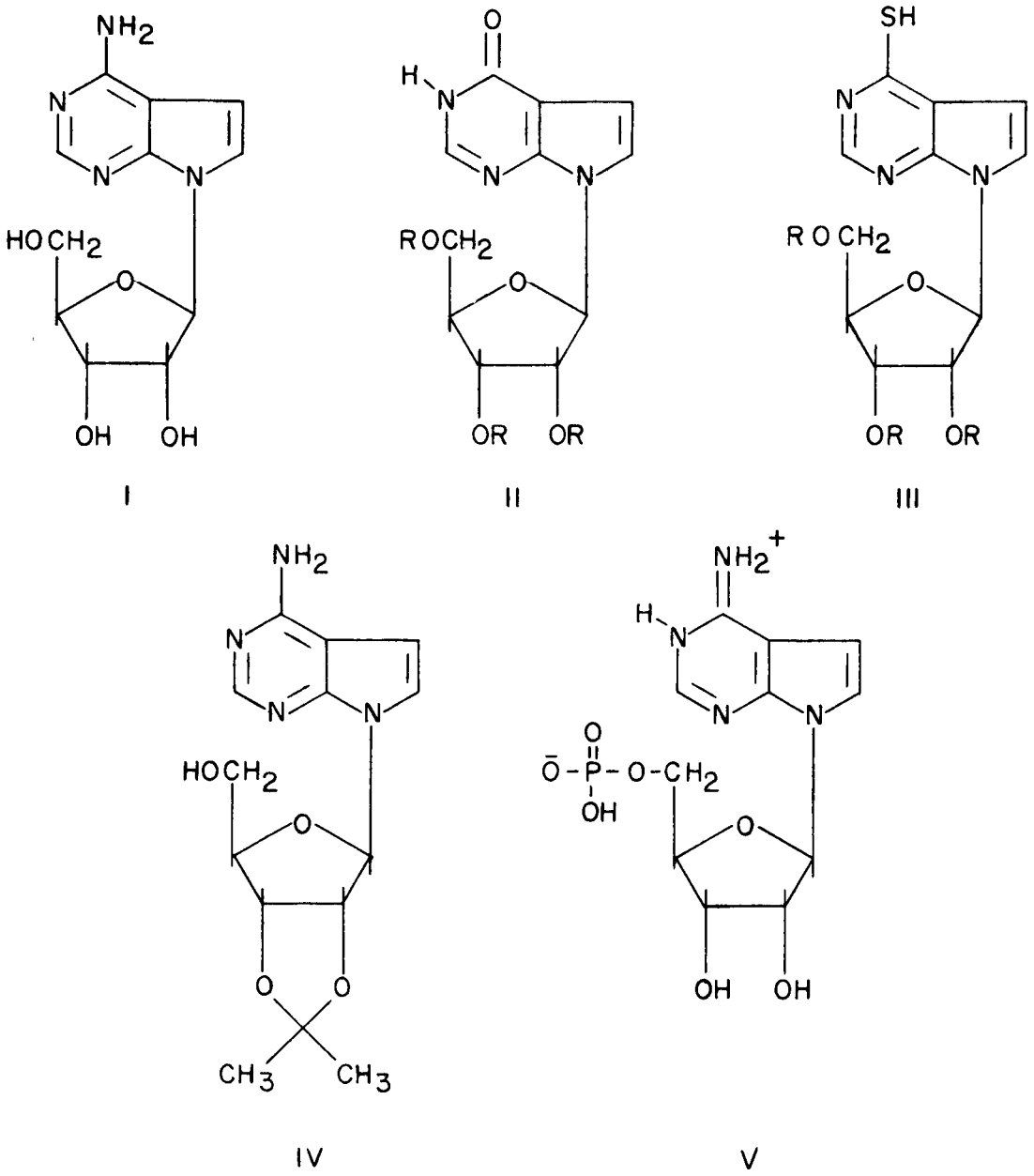
EXPERIMENTAL (18)

7β-D-Ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidone-4 (II); R = H.

A solution of tubercidin (I), (8.15 g.) was prepared in 450 ml. of water by boiling. Barium nitrite (36 g.) was added to the hot solution, and the reaction mixture was allowed to cool. When the temperature was *ca.* 65°, acetic acid (18 ml.) was added dropwise and the cooling allowed to continue to room temperature. The reaction was allowed to proceed overnight (18 hrs.) at room temperature. Sodium sulphate (50 g.) was added to the stirred solution and the barium sulphate which precipitated was collected by filtration (Supercel). Then lead acetate (60 g.) was added to the filtrate and the insoluble material was again removed by filtration (Supercel). After making the aqueous filtrate alkaline with excess aqueous ammonia, the lead salt was collected by filtration, washed carefully with water and dried *in vacuo*. This salt was dissolved in 200 ml. of 20% aqueous acetic acid and hydrogen sulphide was passed into this solution; the insoluble lead sulphide was removed by filtration (Supercel) and the precipitate washed with water. The acetic acid:water was then removed from the filtrate by freeze-drying; a first crop of II (R = H), was obtained by trituration of the residue with water to give after washing with water, and drying *in vacuo*, 2.13 g.; a second crop was obtained from the mother liquors, 1.76 g. Further crystallization of these 2 crops from water gave crop 3, 1.21 g.; m.p. 240-242° and crop 4, 1.41 g.; m.p. 242-247° (19). The infrared spectrum supports the assigned structure II, R = H. ν max (Nujol), 3430, 3350, 3190, 3120, 3065, 3020, 1680, 1665, 1596, 1534, 1510, 1228, 1128, 1055, 1005, 862, 746 cm^{-1}. λ max (H_2O) 259 mμ, (ϵ, 9,600); λ max (0.01N acid) 259 mμ, (ϵ, 9,720); λ max (0.01N base) 263 mμ (ϵ, 10,650). Tubercidin shows λ max (H_2O) 270 mμ (ϵ, 12,000); λ max (0.01N acid) 270 mμ (ϵ, 11,400); λ max (0.01N base) 270 mμ (ϵ, 11,800). NMR spectrum in D_6 DMSO shows peaks at 474 cps. (2-H); 393 cps. (5-H); 442 cps. (6H), $J_{5,6}$ = 3.5 cps.; 364 cps. (1'H), $J_{1'-2'}$ = 5.5 cps.; 219 cps. (5'-H).

Anal. Calcd. for $C_{11}H_{13}O_5N_3$: C, 49.43; H, 4.90; N, 15.73. Found: C, 49.47; H, 5.09; N, 15.67.

7β-D-Ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidine-4-thiol, (III), R = H.



A solution of 1.74 g. of II; R = H was prepared in 45 ml. of pyridine by warming; after cooling in an ice:salt bath, acetic anhydride (15 ml.) was added dropwise. After standing for 1.5 hrs. at 0° to +5° C. the reaction was allowed to proceed for 18 hrs. at +5° C. Then 9 ml. of water was added to the reaction mixture, and the solution stirred for 4 hrs. at room temperature. The solvents were removed in a stream of nitrogen at *ca.* 50° C.; and the residue was partitioned between methylene chloride and water. The methylene chloride extracts were washed with water, dilute sulphuric acid, water and dried with sodium sulphate. Removal of the solvent gave an oil. The infrared spectrum was in agreement with the structure (II), R = Ac. Thin layer chromatography [5% methanol:ethyl acetate; Florisil (20)] showed one spot moving rapidly relative to the starting alcohol.

To a solution of II, R = Ac; (1.599 g.) in 75 ml. pyridine was added 6.0 g. of phosphorus pentasulphide, and the reaction mixture stirred at 30° C. for 2 hrs. Then the mixture was heated to 85-90° for 18 hrs. under nitrogen. After cooling 15 ml. water was added, and the mixture stirred 1 hr. at room temperature. Then the solvent was removed in a nitrogen stream (at *ca.* 50° C.). The residue was partitioned between methylene chloride and water. The organic extracts were washed with water, dilute aqueous acid, water and dried (sodium sulphate). Removal of the solvent gave an oil (2.87 g.). This material was dissolved in methylene chloride and chromatographed on 300 g. Florisil (Gradient elution; Benzene ⟶ Ethyl acetate ⟶ 5% methanol: ethyl acetate). One main peak (non-crystalline) was obtained (2.103 g.) which showed one spot on thin layer chromatography (5% methanol: ethyl acetate; Florisil) and had λ max (EtOH) 325 mμ. The infrared spectrum is in agreement with structure (III), R = Ac.

A solution of the triacetate (III), R = Ac; (1.0 g.) in 30 ml. methanol was allowed to stand 18 hrs. at room temperature with 6.0 ml. of 27% methanolic sodium methoxide. At the end of the reaction period, acetic acid was added to *p*H 7, the methanol was then removed *in vacuo*, and the residue was crystallized from water to give 7β-D-ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidine-4-thiol; 0.46 g., m.p. 203-207°. Further

crystallization (2x) from water gave material, m.p. 204-207°. ν max (Nujol) 3500, 3460, 3370, 3220, 3120, 1600, 1570, 1550, 1530, 1500, 1200, 1190, 1095, 1040, 1030 cm^{-1} λ max (H_2O) 322 mμ (ϵ, 23,800); λ max (0.1N H_2SO_4) 322 mμ (ϵ, 23,800); λ max (0.1N KOH) 309 mμ, (ϵ, 20,500). NMR in D_6DMSO shows peaks at 483 cps. (2-H); 404 cps. (5-H), 455 cps., 6-H, $J_{5,6}$ = 3.5 cps. 367.5 cps. (1'H). $J_{1,2}$ = 5 cps., 803 cps. (3-H), broad line (21).

Anal. Calcd. for $C_{11}H_{13}O_4N_3S$: C, 46.65; H, 4.59; N, 14.84; S, 11.31. Found: C, 46.65; H, 4.70; N, 14.65; S, 11.46.

4-Amino-7-[2',5'-O-Isopropylidene-β-D-ribofuranosyl]-7H-pyrrolo[2,3-d]-pyrimidine (IV).

Tubercidin (1 g.) was stirred with well-dried acetone (50 ml.) while *p*-toluene sulfonic acid monohydrate (7.5 g.) was added. A clear solution resulted in a few minutes. The reaction mixture was stirred at room temperature for two hrs. followed by cooling to 3° and adding to a solution of 0.5M sodium bicarbonate (200 ml.) at 3°. The resulting solution was evaporated to dryness under reduced pressure. The residue was extracted with two 100 ml. portions of boiling chloroform and two 100 ml. portions of chloroform at room temperature, filtering each extract. The combined extracts were evaporated to dryness under reduced pressure. The residue was recrystallized from water to give 0.75 g. of crystalline product melting at 170-173°.

A small amount of this product was recrystallized twice more from water for an analytical sample, m.p. 174-177°, λ max (H_2O) 268 mμ (ϵ, 11,016). NMR in D_7 DMF shows peaks at 78 cps. and 90 cps. attributable to the two CH_3C groups.

Anal. Calcd. for $C_{14}H_{18}N_4O_4$: C, 54.89; H, 5.92; N, 18.29; O, 20.90; CH_3C (1), 4.92. Found: C, 54.72; H, 5.92; N, 18.51; O, 21.2; CH_3C, 4.3.

4-Amino-7-β-D-Ribofuranosyl-5'-phosphate-7H-pyrrolo-[2,3-d]pyrimidine (V).

A mixture of 4-amino-7-[2',3'-O-isopropylidene-β-D-ribofuranosyl]-7H-pyrrolo-[2,3-d]pyrimidine (IV) (2.78 g.), 2-cyanoethylphosphate reagent (16) (50 mmoles) and dry pyridine (120 ml.) was evaporated to dryness under reduced pressure The residue was dissolved in dry pyridine (120 ml.), and the solution was again evaporated to dryness under reduced pressure. This procedure was repeated twice. The final residue was dissolved in dry pyridine (140 ml.) and N,N'-dicyclohexylcarbodiimide (24 g.) was added. The solution was allowed to stand at room temperature for 18 hrs. Water (12 ml.) was added, and the reaction mixture was allowed to stand at room temperature for 45 min. followed by filtration. The filtrate was extracted with two 100 ml. portions of petroleum ether. The aqueous layer was evaporated to dryness under reduced pressure. The residue was dissolved in 0.4N lithium hydroxide (480 ml.), and the solution was boiled for one hr. The cooled mixture was filtered, and the filtrate was extracted with two 100 ml. portions of ether. The aqueous layer was passed over 200 ml. of IRC-50 (H cycle), and the column was washed with 400 ml. of water. The effluent was adjusted to *p*H 2.5 with 1.0 N sulfuric acid and boiled for one and one-half hrs. The hydrolysate was concentrated to 250-300 ml. under reduced pressure. The residue was adjusted to *p*H 7.5 with saturated barium hydroxide solution. The precipitate was removed by centrifugation. The supernatant was diluted with two volumes of alcohol. Refrigeration usually gave a precipitate, but if none occurred, the alcohol and about half of the water were removed by evaporation under reduced pressure, and the residue was again diluted with two volumes of alcohol. The precipitate was removed by centrifugation, washed with ethanol, ether and dried. The yield was 1.62 g. The product at this stage was of variable composition apparently consisting of a mixture of barium, lithium and sodium salts.

Two grams of such material was heated in water (50 ml.) and the mixture was filtered. The filtrate was applied to 100 ml. of Dowex-1 x 8 (formate cycle). The column was developed with 500 ml. portions of 0.01N, 0.02N, 0.04N and 0.08N formic acid collecting 10 ml. portions. Fractions 31-60 were combined and evaporated to dryness under reduced pressure. The residue was triturated with water (10 ml.), and the mixture was refrigerated. The crystals were collected by filtration; wt. 236 mg.; m.p. 255-265° dec. λ max (0.1N acid) 228 (ϵ, 20,964), 271 mμ (ϵ, 10,553) (21).

Anal. Calcd. for $C_{11}H_{15}N_4O_7P$: C, 38.16; H, 4.37; N, 16.18; P, 8.95. Found: C, 38.25; H, 4.57; N, 16.17; P, 9.06.

Acknowledgment.

The authors are indebted to Dr. W. A. Struck and associates for the analyses, ultraviolet and infrared spectra, and to Dr. G. Slomp and F. MacKellar for the NMR curves and their interpretation.

REFERENCES

(1) K. Anzai and S. Marumo, *J. Antibiotics, Ser., A.*, **10**, 20(1957); *Cancer Chemotherapy Reports*, **36**, 19 (1964).

(2) S. Suzuki and S. Marumo, *J. Antibiotics, Ser., A.*, **14**, 34 (1961).

(3) Y. Mizuno, M. Ikehara, K. Watanabe, S. Suzaki, *Chem. Pharm. Bull.*, **11**, 1091 (1963).

(4) Y. Mizuno, M. Ikehara, K. Watanabe, S. Suzaki and T. Itoh, *J. Org. Chem.*, 28, 3329 (1963).

(5) L. Slechta, unpublished data.

(6) R. W. Brockman, C. Sparks, M. S. Simpson, H. E. Skipper, *Biochem. Pharmacol.*, **2**, 77 (1959).

(7) R. W. Brockman, C. Sparks, D. T. Hutchinson, H. E. Skipper, *Cancer Research*, **19**, 172 (1959).

(8) R. W. Brockman, L. L. Bennett, M. S. Simpson, A. R. Wilson, T. R. Thomson, H. E. Skipper, *ibid.*, **19**, 856 (1959).

(9) R. W. Brockman, C. Sparks, M. S. Simpson, *Biochem. Biophys. Acta*, **26**, 671 (1957).

(10) A. R. P. Paterson, *Proc. Am. Assoc. Cancer Research*, 3, 50 (1959).

(11) M. E. Balis, V. Hylin, M. K. Coultos, D. T. Hutchinson, *Cancer Research*, 18, 220 (1958).

(12) H. E. Skipper, T. R. Thomson, D. T. Hutchinson, F. M. Schabel, J. J. Johnson, *Proc. Soc. Exptl. Biol. Med.*, **95**, 135 (1957).

(13) Y. Mizuno, M. Ikehara, K. Watanabe, and S. Suzaki, *J. Org. Chem.*, 28, 3331 (1963).

(14) J. Davoll, *J. Chem. Soc.*, 131 (1960).

(15) J. J. Fox, D. Van Proag, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich and G. B. Brown, *J. Am. Chem. Soc.*, **81**, 178 (1959); G. B. Elion, S. Mueller and G. H. Hitchings, *ibid.*, **81**, 3042 (1959); G. Levin, A. Kalmus and F. Bergmann, *J. Org. Chem.*, **25**, 1752 (1960).

(16) G. W. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961); J. C. Perry, R. V. Sunthanpar and C. Heidelberger, *J. Org. Chem.*, **27**, 2496 (1962); J. Zamlicka, J. Smrt, and F. Sorm, *Coll. Czech. Comm.*, 27, 1462 (1962).

(17) F. Cramer and G. Weimann, *Ber.*, **24**, 996 (1961).

(18) Melting points were taken in capillary tubes and are corrected. Infrared spectra were recorded on a Perkin-Elmer Model 221 spectrophotometer from Nujol mulls. Ultraviolet spectra were taken on a Carey Model 14 spectrophotometer. NMR spectra were obtained with a Varian DP-60 or A-60 spectrophotometer operating at 60 M.C. DP-60 internal tetramethylsilane using the audiofrequency side-band technique. Frequencies are reported in cycles per second downfield from tetramethylsilane. The A-60 spectra were run on 0.25M solutions.

(19) Reported m.p. (Ref. 3) is 242-243° (decomp.).

(20) Florisil is a synthetic magnesia-silica gel manufactured by the Floridin Co., Warren, Pennsylvania.

(21) As kindly pointed out by a referee the presence of a peak in the NMR spectrum of (III, R = H) at 803 cps. is evidence for the thioamide tautomer in D_6DMSO [see J. P. Kokko, J. H. Goldstein and L. Mandell, *J. Am. Chem. Soc.*, 83, 2909 (1961)]. The absence of a comparable NMR peak for (II, R = H) could be due to rapid proton exchange under the experimental conditions, or to the existence of this compound as the enol tautomer in this solvent.

(22) Mr. E. Louis Caron crystallized the phosphate (V) and obtained the analytical and physical data reported.

Received April 18, 1964 Kalamazoo, Michigan