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Nucleoside Antibiotics. VI. Biosynthesis of the Pyrrolopyrimidine Nucleoside Antibiotic Toyocamycin by *Streptomyces rimosus**

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ABSTRACT: The biosynthesis of the pyrrolopyrimidine nucleoside antibiotic, toyocamycin elaborated by *Streptomyces rimosus*, has been studied. Adenine-2-^{14}C, but not adenine-8-^{14}C, is incorporated into toyocamycin. All of the ^{14}C in the toyocamycin from the adenine-2-^{14}C experiments resides in C-2 of toyocamycin. This was shown by the conversion of toyocamycin (**1**) into the 3-carboxyethyl derivative (**5**). C-2 of toyocamycin was released as formic acid by heating **5** in alkali. 5-Carboxy-4-hydroxy-7-(β-D-ribofuranosyl)pyrrolo-[2,3-*d*]pyrimidine (**8**), synthesized from the intermediate 2-amino-4-carboxy-3-carbonyl-[*N*-(2-carboxyethyl)]-1-β-D-ribofuranosylpyrrole (**7**), by treatment with formic acid and acetic anhydride was not radioactive. Compounds **2**, **3**, **4**, **5**, and **8** were all isolated and crystallized, and their structures were rigorously characterized. The isolation of formic acid (**6**) from **5** was unequivocally established. These data provide evidence that N-7 and C-8 of the imidazole ring of a purine are lost during the biosynthesis of the pyrrole ring of toyocamycin. 5-Carboxamido-4-hydroxy-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine (**3**) has been alkylated in good yields with β-propiolactone at N-3 of the pyrrolopyrimidine ring to form the carboxyethyl nucleoside (**5**). This procedure affords an excellent method for opening of the pyrimidine ring and the isolation of C-2 as formic acid. The studies reported here add another role of purines in the biosynthesis of naturally occurring compounds.

During the past 18 years 34 nucleoside antibiotics, having a wide spectrum of chemical structures and biological activity against viruses, bacterial, and tumor cells, have been isolated from the bacteria and fungi (Suhadolnik, 1970). The importance of adenine and/or adenosine in the biosynthesis of the nucleoside antibiotics, cordycepin (3′-deoxyadenosine), 3′-amino-3′-deoxyadenosine, 3′-acetamido-3′-deoxyadenosine, psicofuranine, decoyinine, tubercidin, and toyocamycin have already been reported from this laboratory (Suhadolnik, 1970).

The three known pyrrolopyrimidine nucleoside antibiotics, tubercidin, toyocamycin (**1**), and sangivamycin (**2**) have been isolated from the culture filtrates of *Streptomyces* in eight independent laboratories. Tolman *et al.* (1968) established the chemical structure of tubercidin, toyocamycin, and sangivamycin. The synthesis of toyocamycin by these investigators proved that toyocamycin, unamycin B, vengicide, and antibiotic E-212 have the same structures.

This laboratory had reported earlier that ^{14}C from adenine-2-^{14}C, but not adenine-8-^{14}C, was incorporated into the pyrrolopyrimidine ring of tubercidin and toyocamycin (Smulson and Suhadolnik, 1967; Suhadolnik and Uematsu, 1968). These isotope-labeling patterns suggest that the pyrimidine ring of a purine was used directly in the biosynthesis of the pyrimidine

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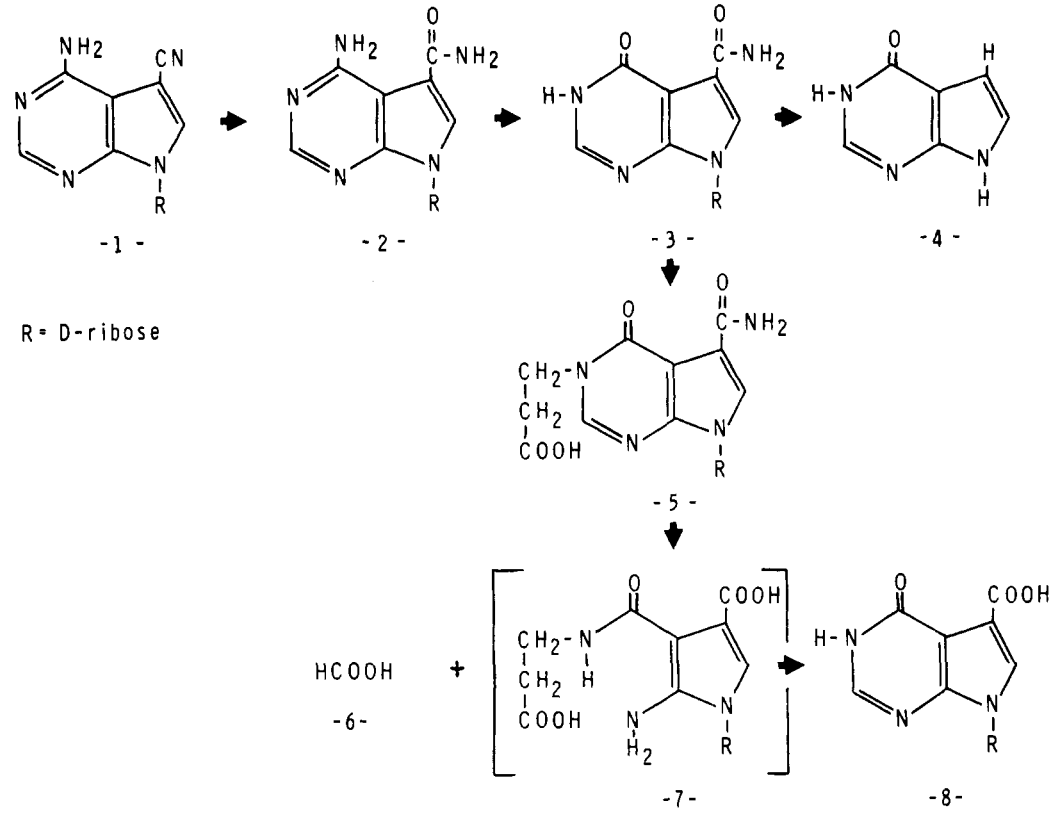
SCHEME I: Degradation of Toyocamycin and Isolation of C-2 as Formic Acid.



ring of tubercidin and toyocamycin by *S. tubercidicus* and *S. rimosus*. Chemical degradations of the ^{14}C-labeled tubercidin and toyocamycin from the adenine-2-^{14}C experiments, to determine the location of the ^{14}C, had not been done previously since a satisfactory degradation method was not available. By using a degradation procedure similar to that described by Baugh and Shaw (1966), the unusual reactivity of inosinic acid to N-1 alkylation and opening of the pyrimidine ring of 1-(2-carboxyethyl)inosine 5′-phosphate has now been used to isolate C-2 of the pyrrolopyrimidine ring. The work reported here describes (1) experiments that strongly suggest that the pyrimidine ring of a purine serves as the direct precursor for the pyrimidine ring of toyocamycin; (2) the isolation of C-2 of toyocamycin as formic acid; (3) the synthesis of 5-carboxy-4-hydroxy-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine (**8**) from the proposed intermediate, 2-amino-4-carboxy-3-carbonyl-[*N*-(2-carboxyethyl)]-1-β-D-ribofuranosylpyrrole (**7**) (Scheme I).

Experimental Section

Melting points were taken with a Thomas-Hoover silicone bath apparatus and are uncorrected. The ultraviolet spectra were recorded on a Beckman Model DB spectrophotometer. Samples were dissolved in 0.1 M potassium phosphate buffer (pH 7.4), 0.1 N hydrochloric acid, and 0.1 N sodium hydroxide. Elemental analyses were performed by Huffman Laboratory, Wheatridge, Colo. The mass spectrum of **5** and **8** were obtained with a Perkin-Elmer Model 270 double-focusing mass spectrometer. The samples were introduced by solid probe inlet at a temperature of 279°. Infrared and nmr spectra were obtained by a Perkin-Elmer-437 grating spectrophometer with KBr pellets and a Varian A-60A instrument using DSS[1] as internal standard (compounds were dissolved in D_2O–NaOD). Culture flasks for toyocamycin production were shaken on a New Brunswick Gyrotort (Model G-25); speed setting 6. ^{14}C measurements were made in a Packard spectrometer, series 314E. Radioactivity was measured in Bray's (1960) scintillation fluid. Paper electrophoresis was carried out on a Gelman Model 51170 chamber. DEAE-cellulose (exchange capacity 1.0 mequiv/g) was obtained from the Whatman Co. The resin was washed with 1 N sodium hydroxide, water, 1 N hydrochloric acid, water, 0.5 M potassium bicarbonate, and water. AG 1-X8 (200–400 mesh, formate and hydroxide) and AG 50 (H^+)-X4 (100–200 mesh) were purchased from Bio-Rad Laboratories, Calif. Whatman No. 1 and 3MM papers were used for paper chromotography and electrophoresis. Adenine-2-^{14}C, adenine-8-^{14}C, and guanine-2-^{14}C were purchased from International Nuclear Corp. and from Calbiochem. The solvents used for paper chromotography were: solvent A, ammonia–water, pH 10.3; solvent B, ethyl alcohol–acetic acid–water (85:5:10); solvent C, 1-butanol–1 N ammonium hydroxide (86:14); solvent D, 2-propanol–6 N hydrochloric acid–water (63:33.5:35); solvent E, methyl alcohol–ethyl alcohol–concentrated HCl–water (50:25:10:15); and solvent F, ethyl alcohol–water–concen-

[1] The abbreviation used is: DSS, sodium 2,2-dimethyl-2-silapentanesulfonate (also referred to as Tier's salt).

trated ammonium hydroxide (80:60:4). Paper electrophoresis was done in 0.05 M potassium phosphate buffer (pH 8.0) at 500 V and 24 mA for 2 hr. Formic acid was isolated and determined by steam distillation. The formic acid was reduced to formaldehyde (Wood and Gest, 1957). The formaldehyde was then determined quantitatively by the chromotropic acid procedure. 4-Hydroxypyrrolo[2,3-*d*]pyrimidine (**4**) was supplied by Dr. G. Hitchings. Infrared, nuclear magnetic resonance, and mass spectra were run by Drs. P. Porcoro, R. Ramer, and P. Vallin, Research Laboratories, Givaudan Corp.

Biosynthesis of ^{14}C-Labeled Toyocamycin from Adenine-2-^{14}C and Guanine-2-^{14}C by S. rimosus and Hydrolysis of Toyocamycin (**1**) *to Sangivamycin* (**2**). *S. rimosus* (ATCC No. 14500) was maintained on agar slants (0.2% D-glucose, 0.02% asparagine, 0.025% beef extract, 0.05% dipotassium hydrogen phosphate, and 1.5% agar). The culture medium for the production of the nucleoside antibiotic was as follows: 1.0% D-glucose, 1.5% soy bean meal, 0.25% distiller's solubles, 0.25% sodium chloride, 0.25% dipotassium hydrogen phosphate, 0.2% calcium carbonate, and tap water. A flask was inoculated with one agar slant of *S. rimosus* and maintained at 27° on the rotary shaker; 48 hr after inoculation 1 ml from this seed flask was added to three 2-l. baffled flasks containing 300 ml of medium. A sterile solution of adenine-2-^{14}C, adenine-8-^{14}C, or guanine-2-^{14}C was added to each of three flasks 45 hr after inoculation. The cultures were stopped either 20 min or 3 hr later. Toyocamycin was isolated by adjusting the medium to pH 2.0 with hydrochloric acid and filtered. The filtrate was evaporated *in vacuo* to about one-third the volume. The concentrated solution was extracted with 1-butanol (three times, 300 ml each time). The butanol extract was evaporated to dryness and the residue was dissolved in 100 ml of water at 70°. The insoluble material was removed by filtration and the filtrate was concentrated to about 30 ml. The anion column chromotography technique of Dekker (1965) was then used to convert toyocamycin (**1**) quantitatively into sangivamycin (**2**) as follows. The filtrate containing the toyocamycin was added to a column of AG 1-X8 (OH^-) (6 g) in water–methanol (85:15). The column was washed with 200 ml of water–methanol (70:30). Sangivamycin was eluted with 500 ml of water–methanol (30:70) and evaporated to 1 ml. Sangivamycin crystallized overnight at 0°; yield 47 mg. The physical and chemical properties of this sangivamycin were identical with those properties for the chemically synthesized sangivamycin (Tolman *et al.*, 1968) as well as sangivamycin obtained from the John L. Smith Memorial for Cancer Research, Charles Pfizer and Co., Inc.

5-Carboxamido-4-hydroxy-7-(β-D-ribofuranosyl)pyrrolo-[2,3-d]pyrimidine (**3**). A. NONRADIOACTIVE SYNTHESIS OF **3**. Sangivamycin (70 mg) was dissolved in 0.6 ml of glacial acetic acid and 7.0 ml of water. The mixture was heated to 70–80° with stirring for 3 hr while sodium nitrite (305 mg in 6 ml of water) was added every 15 min. The solution was cooled and added to an AG 50 (H^+) column (10 × 1 cm). Compound **3** was collected in the first 100 ml of water from the column. The solution was evaporated to dryness *in vacuo* and the residue was dissolved in 4 ml of water, crystallized overnight at 4°, and recrystallized from water as colorless needles, mp 285–286°. The melting point agreed with that reported by Rao (1968). The yield was 44 mg, 63%; the ultraviolet absorption properties were as follows: λ_{max} (pH 7.4) 269 mμ (ε 12,300), λ_{max} (0.1 N HCl) 269 mμ (ε 12,600), and λ_{max} (0.1 N NaOH) 276 mμ (ε 14,300). The ultraviolet spectra agreed with that reported by Rao (1968). *Anal.* Calcd for $C_{12}H_{14}N_4O_6$: C, 46.45; H, 4.51; N, 18.06. Found: C, 46.70; H, 4.60; N, 17.90.

B. RADIOACTIVE SYNTHESIS OF COMPOUND **3**. ^{14}C-Labeled sangivamycin (29 mg, 91.7 μmoles, 350 mμCi, 3.85 mμCi/μmole) was converted into **3** as described above for the synthesis of unlabeled **3**. The yield of radioactive **3** was 12 mg (41.4%, 38.6 μmoles); the specific activity was 3.80 mμCi/μmole. This compares with a specific activity of 3.85 mμCi/μmole for the sangivamycin (**2**). The physical and chemical properties of ^{14}C-labeled **3** were the same as **3** synthesized above.

4-Hydroxypyrrolo[2,3-d]pyrimidine (**4**). The ^{14}C-labeled **3** (5.5 μmoles, 21 mμCi, 3.80 mμCi/μmole was heated in a sealed tube with 10 mg of red phosphorus and 0.3 ml of hydroiodic acid (sp gr 1.7) at 150° for 60 min, and the reaction mixture was made alkaline with concentrated ammonium hydroxide and centrifuged (Ohkuma, 1961). The supernatant was concentrated and applied to the origin of a Whatman No. 3MM paper chromatogram and developed in solvent A. One ultraviolet-absorbing spot, with a R_F corresponding to authentic, chemically synthesized 4-hydroxypyrrolo[2,3-*d*]pyrimidine (Davoll, 1960) was observed on the paper chromatogram. The 4-hydroxypyrrolo[2,3-*d*]pyrimidine was cut out, eluted with water, and rechromatographed on Whatman No. 3MM paper in solvent B. The area on the paper chromatogram corresponding to a R_F of authentic 4-hydroxypyrrolo-[2,3-*d*]pyrimidine was cut out and eluted with water. The yield was 1.17 μmoles, 21%, 4.35 mμCi, 3.7 mμCi/μmole. Ninety-eight per cent of the radioactivity resided in the aglycone. The ultraviolet absorption spectra were as follows: λ_{max} (pH 7.4) 262 mμ, λ_{max} (0.1 N HCl) 263 mμ, and λ_{max} (0.1 N NaOH) 265 mμ. Authentic 4-hydroxypyrrolo[2,3-*d*]-pyrimidine had the same ultraviolet spectra (Uematsu and Suhadolnik, 1968). Further proof for the isolation of **4** from **3** was obtained by the addition of authentic, chemically synthesized **4** to radioactive **4**, and the isolation of crystalline, ^{14}C-labeled **4**.

5-Carboxamido-3-(2-carboxyethyl)-4-hydroxy-7-β-D-ribofuranosylpyrrolo[2,3-d]pyrimidine (**5**). A. NONRADIOACTIVE SYNTHESIS OF **5**. The alkylation of **3** was performed according to the procedure of Baugh and Shaw (1966). Compound **3** (77 mg, 0.237 mmole) was dissolved in 3.7 ml of 2 M potassium carbonate, 3.7 ml of *p*-dioxane followed by the addition of 0.37 ml of β-propiolactone. This solution was stirred at room temperature for 24 hr and diluted with water to 100 ml, adjusted to pH 5.0 (with hydrochloric acid), followed by the addition of 2.5 g of Norit A. After filtering, the Norit A was washed with water (three times, 100 ml each time) and ammonia–water (pH 10) (two times, 100 ml each time). The charcoal was then treated with 50-ml portions (ten times) of ethanol–chloroform–water–concentrated ammonium hydroxide (60:40:5:5, v/v) until the absorption of the filtrate at 270 mμ was less than 0.8. The filtrates were combined and taken to dryness *in vacuo*. Water (20 ml) was added to the dried residue. This solution was added to an AG 1 (formate) column (12.5 × 1.5 cm). This column was developed by a linear gradient from water to 0.5 M formic acid, 200 ml each. Fractions of 10 ml were collected. Compound 5 was eluted in tubes 15–34. The contents of these tubes were combined and evaporated *in vacuo*. The residue was dissolved in methanol

and **5** was crystallized by dropwise addition of chloroform; yield 61.1 mg (64.5%), mp 197–198°. *Anal.* Calcd for $C_{15}H_{18}N_4O_8 \cdot H_2O$; C, 45.00; H, 5.00; N, 14.00. Found: C 45.16; H, 5.12; N, 14.11.

The nuclear magnetic resonance of **5** show the aromatic protons with singlets at δ 8.05 (1 proton singlet, H 2) and 7.5 (1 proton singlet, H 6). The five protons of the ribofuranosyl ($C_{5'}$, 2 H; $C_{4'}$, H; $C_{3'}$, H; $C_{2'}$, H) and two protons of the methylene group (CH_2N) occur as a broad multiplet in the region δ 3.90–4.60. The methylene group (CH_2COO^-) appears as a triplet centered at δ 2.8. The anomeric proton at $C_{1'}$ of **5** is split with a sharp doublet centered at δ 5.95 (J = 6 cps); infrared spectrum, 1710 cm^{-1} (carboxyl group), 1645 cm^{-1} (C=O, C=C, and C=N); partial mass spectrum was as follows: *m/e* 310, 267, 250, 207, 178, 162, 135, 60, and 44. The peak at *m/e* 60 would arise by fragmentation of acetic acid from the propionic acid side chain of 5. This peak (*m/e* 60) is not present in the mass spectral pattern observed for the aglycone of tubercidin (Smulson and Suhadolnik, 1967); ultraviolet spectral properties were as follows: λ_{max} (pH 7.4) 270 mμ (ε 10,500), λ_{max} (0.1 N HCl) 269 mμ (ε 10,400), and λ_{max} (0.1 N NaOH) 271 mμ (ε 11,000). Proof that the carboxyethyl group was substituted on N-3 was obtained by comparing the shifts in the ultraviolet spectra in acid and alkali. Nucleosides with no substituent on N-3 (**3** and **8**) show a greater bathochromic shift in alkali than those compounds with the N-3 carboxyethyl group (**5**). This observation is in agreement with the earlier studies of N-alkylated 6-oxopurine, nucleosides, and nucleotides (Broom *et al.*, 1964; Jones and Robins, 1963; Baugh and Shaw, 1966). The data reported (infrared, nuclear magnetic resonance, elemental analysis, ultraviolet, and mass spectra) on the crystalline compound isolated are taken as unequivocal evidence for the structure of 5 as proposed in Scheme I.

B. RADIOACTIVE SYNTHESIS OF **5**. The alkylation of ^{14}C-labeled **3** (12 mg, 38.7 μmoles, 147 mμCi, 3.80 mμCi/μmole) was done as described above for the nonradioactive synthesis of **5**. The reaction mixture was evaporated to dryness under a stream of air at room temperature, dissolved in 8 ml of water, and neutralized to pH 7.0 with perchloric acid at 0°. The potassium perchlorate was removed by filtration. The salt was washed with 5 ml of 95% ethanol. The filtrates were combined and added to an AG-1-X8 (formate) column (9.0 × 1.4 cm). The column was washed with 150 ml of 0.01 M formic acid. Compound **5** was then eluted in tubes 9–62 (10 ml/fraction) with 0.1 M formic acid and evaporated to dryness *in vacuo* to a colorless syrup (120 mμCi; 82% yield). The ultraviolet spectral properties and R_F values of 5 (paper chromatography in solvents A, B, C, and E and paper electrophoresis) were identical with those described above for authentic **5**.

Formic Acid (**6**) *and 2-Amino-4-carboxy-3-carbonyl-[N-(2-carboxyethyl)]-1-β-D-ribofuranosylpyrrole* (**7**). A. NONRADIOACTIVE SYNTHESIS. Compound **5** (60 mg, 150 μmoles) was dissolved in 2 ml of 1 N NaOH and heated in a sealed tube at 130° for 60 min. The reaction mixture was cooled and adjusted to pH 7.8 with Dowex 50 (H^+). The resin was removed by centrifugation and washed with water (1.5 ml, three times). A 1.0-ml aliquot of the combined supernatants (10 ml) was removed to determine the amount of formic acid prior to column chromatography. The yield of formic acid was 34% (52.1 μmoles).

The remaining 9 ml was added to a DEAE-cellulose column (bicarbonate form, 1.7 × 18 cm). Water was added followed by a linear gradient from water to 1 N ammonium bicarbonate (pH 8.85), 500 ml of each. An aliquot of each tube was assayed for formic acid. Four fractions were collected: fraction I (tubes 14–18), formic acid; fraction II (tubes 12–20), **8**; fraction III (tubes 21–25), **3**; fraction IV (tubes 26–50), **7**. Compound **8** (fraction II) was further purified by rechromatography on an AG 1 (formate) column (15-ml fractions). Compound **8** was displaced with 0.1 M formic acid and collected in fractions 18–40 (10 ml each). The fractions were collected, evaporated to dryness *in vacuo*, and crystallized from water: yield 34.5 mg, mp 275° (decomposition). There was no depression of the melting point when **8**, isolated by this procedure, was mixed with authentic **8** and synthesized as described below by either methods A, B, C, or D. In addition, the infrared, nuclear magnetic resonance, and ultraviolet spectra were identical with that of authentic **8**. Compound **3** (fraction III) had the same ultraviolet spectra and R_F values (paper chromatography, solvents A, B, and E; paper electrophoresis) as authentic **3** synthesized above. Compound **7** (fraction IV) was concentrated *in vacuo* to 10 ml and rechromatographed on a DEAE-cellulose bicarbonate column (1.7 × 18 cm). Fraction IV was added to the column, followed by 50 ml of water and a linear gradient from water to 1 N ammonium bicarbonate (pH 8.85), 500 ml each. Fraction IV was shown to be one compound on paper chromatography (solvents A, B, E) and paper electrophoresis (R_F 7.8 cm toward anode). The compound in fraction IV moved 1.73 times faster than 5, suggesting two carboxyl groups. It gave a positive Braton–Marshall reaction for a diazotizable amino group (λ_{max} 618 and 573 mμ) (Lukens and Buchanan, 1959). The ultraviolet spectra were as follows: λ_{max} (pH 7.4) 286 mμ, λ_{max} (0.1 N HCl) 295 mμ, and λ_{max} (0.1 N NaOH) 286 mμ.

The ultraviolet absorption ratios at pH 7.4: 0.1 N HCl–0.1 N NaOH were 1.29:1.0:1.25. The estimated molar extinction coefficient of **7** of pH 7.4 is 5600. This compound also gave a positive Bial test for ribose (Fernell and King, 1953). The compound in fraction IV is stable in alkali but unstable in neutral or acid as shown by the rapid formation of a violet color. This instability in acid is not due to the hydrolysis of the riboside bond since the pyrrole–riboside bond is very stable in acid which is unlike the imidazole riboside bond of purine ribosides. It is suggested that the structure of the compound in fraction IV is as shown in Scheme I (**7**; 2-amino-4-carboxy-3-carbonyl-[*N*-(2-carboxyethyl)]-1-β-D-ribofuranosylpyrrole).

B. RADIOACTIVE SYNTHESIS OF FORMIC ACID (**6**) AND COMPOUND **7**. Compound **5** (16 μmoles, 61 mμCi, 3.80 mμCi/μmole) was heated in a sealed tube with 1 ml of 1 N sodium hydroxide for 60 min at 130° and cooled. The reaction mixture was adjusted to pH 9.0 with a suspension of AG 50 (H^+) resin and centrifuged. The resin was washed twice with 2 ml of water. The supernatant solutions were combined (7.7 ml, 61 mμCi, 100% yield). The solution was added to a DEAE-cellulose column (bicarbonate form, 18 × 1.5 cm). After washing the column with 50 ml of water, a linear gradient from water to 1 N ammonium bicarbonate (pH 8.85, 500 ml each) was used to elute formic acid (**6**) (fraction I, tubes 14–16) and **7** (fraction IV, tubes 29–33) (Figure 1). Fractions of 10 ml were collected. The yield of formic acid was 51.3% (8.23 μmoles, 31.3 mμ). The radioactive formic acid-^{14}C eluted from the column was then steam distilled. The formic acid in the

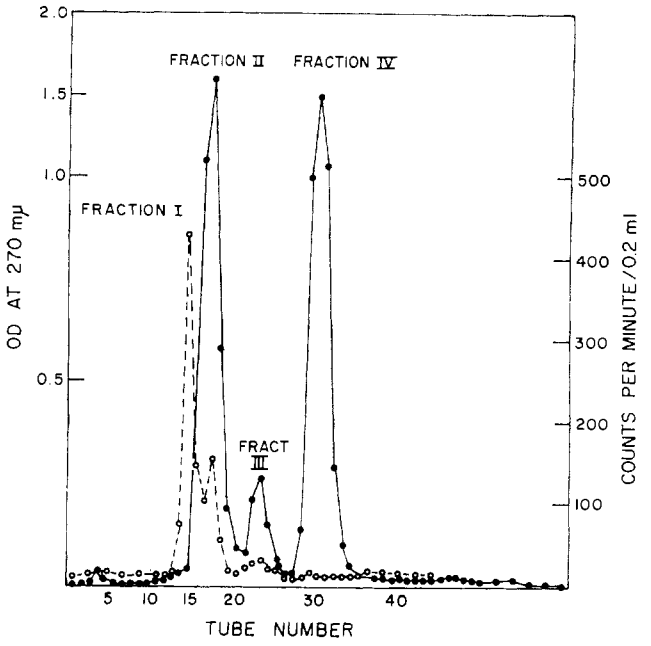


FIGURE 1: DEAE-cellulose chromatography of products following alkaline treatment of ^{14}C-labeled **5**. Ten-milliliter fractions; (●—●) $OD_{270m\mu}$; (○---○) radioactivity. Formic acid, fraction I, tubes 14–16; **8**, fraction II, tubes 17–20; **3**, fraction III, tubes 22–26; **7**, fraction IV, tubes 29–33.

distillate was then reduced to formaldehyde. The formaldehyde was measured quantitatively by the chromotropic acid method. Additional proof for the isolation of formic acid-^{14}C was obtained by showing that the R_F was the same as that of authentic formic acid (paper chromatography and paper electrophoresis). As reported above in the nonradioactive synthesis of formic acid and **7**, **3**, and **8** were also isolated (Figure 1). Compound **7** had the same chemical and physical properties as that described above for nonradioactive synthesis of **7**. In addition, no ^{14}C was present in **7** from the degradation of the radioactive toyocamycin. This is further evidence that all of the ^{14}C resides in C-2 of the pyrimidine ring of toyocamycin following the incorporation of adenine-2-^{14}C by *S. rimosus*.

5-Carboxy-4-hydroxy-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (**8**). The synthesis, isolation, crystallization, and characterization of 5-carboxy-4-hydroxy-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**8**) has been accomplished by four independent procedures. The four methods for synthesis of **8** are as follows: method A—the ring closure of **7** to **8** (Scheme I); methods B, C, and D—the conversion of sangivamycin (**2**) into **8** by three different procedures.

METHOD A. Ring closure of **7** (Scheme I). Purified fraction IV (**7**) (288 units, OD_{286}, pH 7.4) was evaporated to dryness by heating on a steam bath with a stream of nitrogen. After drying overnight in a desiccator, the residue was taken up in 0.66 ml of 98% formic acid and 1 ml of acetic anhydride. The mixture was heated on a steam bath, equipped with drying tube for 4 hr and then evaporated to dryness *in vacuo*. Formamide (2 ml) was added and the solution was heated 150–160° on an oil bath for 90 min. After cooling, 2 ml of concentrated ammonium hydroxide was added to the solution and allowed to stand overnight at 2°. The excess ammonia was removed *in vacuo* and the solution was poured onto an AG 1-X8 (formate) column (10 ml, 1.2 × 15 cm). After washing with 50 ml of water, 0.10 M formic acid was added. A minor peak (tubes 9–19) and a major peak (tubes 21–40) were collected. The formic acid and water in the major peak was evaporated *in vacuo*. The residue was dissolved in water and crystallized to give 5.1 mg (16.5 μmoles) colorless needles; mp 275° (decomposition). The melting point reported by Okhuma (1961) for **8** was 278°. The ultraviolet spectral properties are as follows: λ_{max} (pH 7.4) 266 mμ, λ_{max} (0.1 N HCl) 270 mμ, and λ_{max} (0.1 N NaOH) 274 mμ; nuclear magnetic resonance spectra (δ) 8.05 (1 proton singlet, H 2), 7.50 (1 proton singlet, H 6), a broad multiplet for D-ribofuranosyl (five protons in the region 3.90–4.50). The anomeric proton at $C_{1'}$ of **8** is split with a sharp doublet centered at δ 5.95 (J = 6 cps); the nuclear magnetic resonance spectra for **8** is similar to that reported for pyrrolopyrimidine nucleosides and adenosine (Hinshaw *et al.*, 1969; Robins *et al.*, 1966; Jardetzky, 1960); infrared, 1705 cm^{-1} (carboxyl group). The ultraviolet spectrum of the compound in the minor peak was as follows: λ_{max} 266 (pH 7.4), 270.5 (0.1 N HCl), and 267 mμ (0.1 N NaOH). This compound was not clarified further.

METHOD B. Compound **8** was synthesized by the method according to Okhuma (1961).

METHOD C. Sangivamycin (80 mg) was hydrolyzed with 1.5 ml of 1 N NaOH in a sealed tube at 130° for 60 min. The reaction mixture was added to a Dowex 1 (formate) column (10 ml, 200–400 mesh). The column was washed with 200 ml of 0.01 M formic acid followed by 0.1 M formic acid. Desamidosangivamycin was eluted in tubes 23–33 (10-ml fractions). The fractions were combined and evaporated to dryness *in vacuo*. HCl (5 ml of 1 N) was added to the dry residue and the solution was taken to dryness. HCl (0.5 ml of 6 N) was added and the residue was dissolved by heating. Methanol (1.5 ml) was added and the solution was allowed to stand at room temperature: yield 60 mg, 173 μmoles, 71%, mp 235–240° (decomposition at 245°); melting point and ultraviolet spectra were the same as reported by Rao (1968). Desamidosangivamycin (55 mg) was deaminated by the addition of a solution of acetic acid (0.45 ml), water (5 ml), and sodium nitrite (220 mg in 10 ml of water) over a 3-hr period at 70–80° with stirring. The reaction mixture was cooled to room temperature and passed through a Dowex 50 (H^+) (10 ml, 20–40 mesh) column and crystallized at room temperature; yield 42 mg, melting points, mixture melting points, ultraviolet, nuclear magnetic resonance, and infrared spectra were identical with **8** synthesized by ring closure from **7** as described above.

METHOD D. Compound **3** (10 mg) was heated for 60 min, 130°, in a sealed tube with 1 ml of 1 N NaOH. The reaction mixture was cooled, and adjusted to pH 9.0 with a suspension of AG 50 (H^+). The mixture was centrifuged, the supernatant was decanted, and the resin was washed with 2 ml of water. This process was repeated twice. The combined supernatants were added to a DEAE-cellulose column (18 × 1.5 cm). Compound **8** was eluted by a linear gradient from water to 1 N ammonium bicarbonate, 500 ml of each. The product, **8** was eluted in tubes 14–20. The fractions containing **8** were collected, evaporated to dryness, taken up in 5 ml of water, and added to an AG 1 (formate) column (10 × 1.2 cm). Compound **8** was eluted in fractions 6–20 (10 ml of each fraction) with 0.1 N formic acid. The fractions were combined,

TABLE I: Incorporation of Adenine-2-^{14}C, Adenine-8-^{14}C, and Guanine-2-^{14}C into Toyocamycin by *S. rimosus*.[a]

Purine Added	Toyocamycin Isolated		
	μmoles	mμCi/μmole	Incorp Efficiency
Adenine-2-^{14}C	12.0	2.2	0.25
Adenine-8-^{14}C	14.0	0	0
Guanine-2-^{14}C	5.6	3.16	0.54

[a] The amount of adenine-2-^{14}C added to two flasks—4.0 μCi, 4.6 μmoles, 874 mμCi/μmole; amount of adenine-8-^{14}C added to two flasks–4.0 μCi; 4 μmoles, 1000 mμCi/μmole; amount of guanine-2-^{14}C added to two flasks—3.2 μCi, 5.6 μmoles; 570 mμCi/μmole. Toyocamycin was isolated 20 min after the addition of ^{14}C-labeled adenine or guanine.

TABLE II: Distribution of ^{14}C in Toyocamycin Isolated from the Adenine-2-^{14}C Experiment.[a]

Compound	Act. (mμCi/μmole)
Sangivamycin (**2**)	3.85
5-Carboxamido-4-hydroxy-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**3**)	3.80
4-Hydroxypyrrolo[2,3-*d*]pyrimidine (**4**)	3.71
Formic acid (**6**)	3.80
2-Amino-4-carboxy-3-carbonyl-[*N*-(2-carboxyethyl)]-1-β-D-ribofuranosylpyrrole (**7**)	0
5-Carboxy-4-hydroxy-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine (**8**)	0

[a] Seven micromoles of adenine-2-^{14}C (570 mμCi/μmole) was added to two flasks of *S. rimosus*. Toyocamycin was isolated after 3 hr. The addition of adenine-2-^{14}C and degradation is described under Experimental Section.

concentrated to dryness *in vacuo*, crystallized from 3 ml of water at 4° overnight, and filtered to give 8.1 mg (81%) of **8** as colorless needles, mp 280–281°; the ultraviolet spectral properties were identical with **8** synthesized by either of methods A, B, or C above. There was no melting point depression when **8**, prepared by this method was mixed with the product isolated by methods A, B, or C above. The molecular weight of **8** (as determined with the aid of perfluorokerosene) is 311. The mass spectrum of **8** had a prominent peak at *m/e* 267, due to the loss of CO_2. This peak at 267 is in agreement with the empirical formula, $C_{11}H_{13}N_3O_5$. Compound **8** gave a positive Bial test for ribose (Fernell and King, 1953).

Results and Discussion

Incorporation of Adenine-2-^{14}C, Adenine-8-^{14}C, and Guanine-2-^{14}C into Toyocamycin by S. rimosus. The data in Table I showed that ^{14}C from adenine-2-^{14}C is incorporated into toyocamycin while the ^{14}C-from adenine-8-^{14}C was not incorporated. The incorporation efficiency of toyocamycin from the guanine-2-^{14}C experiment is double that from the incorporation efficiency of the toyocamycin from the adenine-2-^{14}C experiments. From these *in vivo* studies it is not clear whether the adenine or the guanine ring is the precursor of the aglycone moiety of toyocamycin.

Distribution of ^{14}C in Toyocamycin Isolated from Adenine-2-^{14}C. Scheme I shows the experimental procedure employed to (1) open the pyrimidine ring of the ^{14}C-labeled carboxyethyl derivative (**5**) prepared from radioactive toyocamycin (**1**) from the adenine-2-^{14}C experiments, (2) the isolation of C-2 as formic acid (**6**), and (3) the synthesis of compound **8** from the aminopyrrole riboside (**7**); 98% of the ^{14}C in toyocamycin resided in the pyrrolopyrimidine base (**4**) (Table II). Degradation of **5** to **7** and formic acid (**6**) (C-2) showed that all of the ^{14}C resided in C-2. Finally, **8** synthesized from **7** was not radioactive (Table II). Compounds **2**, **3**, **4**, **5**, and **8** were synthesized, purified, and crystallized according to degradation Scheme I. Their physical and chemical properties firmly established the structures assigned. The physical and chemical properties of compounds **2**, **3**, **4**, **5**, and **8**, synthesized from radioactive toyocamycin, were identical with the same compounds from the nonradioactive syntheses. Although the structure of **7** has not been as rigorously established, the intermediate compound proposed (Scheme I) is based on the analytical data and structures assigned to compounds **2**, **3**, **4**, **5**, and **8**. Compound **7**, isolated from radioactive **5** was readily converted into **8**. This latter compound was not radioactive. Formic acid-^{14}C (**6**) was isolated and identified following alkaline treatment of **5**. The melting points, mixture melting points, nuclear magnetic resonance, ultraviolet, infrared, and mass spectra of **8** synthesized, purified, and crystallized by methods A, B, C, or D are identical.

The chemical degradation of toyocamycin and isolation of formic acid-^{14}C from C-2 provides experimental evidence that the pyrimidine ring of a purine serves as the precursor for the pyrimidine ring of this pyrrolopyrimidine nucleoside antibiotic. Smulson and Suhadolnik (1967) reported similar findings on the biosynthesis of the pyrrolopyrimidine nucleoside antibiotic, tubercidin. Adenine-2-^{14}C was incorporated into the pyrrolopyrimidine ring of tubercidin. When adenine-8-^{14}C was added to culture filtrates of *S. tubercidicus*, the ^{14}C was released as CO_2-^{14}C and there was no incorporation of ^{14}C into tubercidin. Apparently, the pyrimidine ring of a purine is the precursor for the pyrimidine ring of the pyrrolopyrimidine nucleosides. The difference of the incorporation of adenine-2-^{14}C compared with adenine-8-^{14}C into toyocamycin can be explained by the loss of N-7 and C-8 of a purine during the biosynthesis of toyocamycin. The elimination of C-8 of a purine by *S. rimosus* may be similar to the biosynthesis of the pteridine ring in which C-8 of GTP is eliminated as formic acid (Burg and Brown, 1966; Levenberg and Kaczmarek, 1966; Krumdieck *et al.*, 1966). Similarly, Shiota *et al.* (1967) reported on the chemical elimination of C-8 of GTP and ATP as formic acid.

Toyocamycin biosynthesis differs from folic acid biosynthesis in that N-7 and C-8 of the imidazole ring must be eliminated prior to pyrrole ring formation. The manner in which the expulsion of N-7 and C-8 and the biosynthetic

origin pyrrole carbon atoms and the cyano group of toyocamycin has been studies in this laboratory. The pyrrole carbons and the cyano group of toyocamycin have been reported to arise from C-1, -2, and -3 of D-ribose (Suhadolnik and Uematsu, 1968). Since the incorporation of adenine-2-^{14}C and guanine-2-^{14}C into toyocamycin was essentially the same, it is not known which purine is involved in pyrrolopyrimidine nucleoside biosynthesis.

N-3 alkylation of **3** with β-propiolactone emphasizes the similarity in the anionic behavior of the pyrrolopyrimidine ring and the purine ring in alkaline solution. Once N alkylation of the pyrrolopyrimidine ring occurs, the pyrimidine ring is readily opened in alkali as evidenced by formation of formic acid (**6**) and **7** with a diazotizable amino group. The procedure here describes a convenient method for the synthesis of the aminopyrrole riboside (**7**) and the isolation of C-2 as formic acid. The experimental conditions needed to form **7** from **5** are more drastic than the analogous reaction in the synthesis of the imidazole riboside from inosinic acid as reported by Baugh and Shaw (1966). The N-3 position of the pyrrolopyrimidine nucleoside must be alkylated to open the pyrimidine ring since treatment of **3** in the same manner as **5** did not result in ring opening. Since Baugh and Shaw (1966) reported on ring closure of the diazonium salt of the aminoimidazole ribotide and the subsequent synthesis of the 2-aza derivative of inosinic acid, the experimental procedure described here may also provide a convenient synthesis of a pyrrolotriazine nucleoside. No information is available concerning the biological properties of 2-azapyrrolopyrimidine nucleosides. Montgomery and Hewson (1967) have reported their studies on the biological properties of analogs of tubercidin in which the ribose moiety attached to N-7 of the pyrrolopyrimidine ring was changed.

The resynthesis of the pyrrolopyrimidine ring (**8**) from the proposed intermediate, **7**, by refluxing with formic acid and acetic anhydride resulted in the loss of the carboxyethyl group. This procedure is a suitable method for the synthesis of 5-carboxy-4-hydroxy-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]-pyrimidine (**8**) from the 2-amino-4-carboxy-3-carbonyl-[*N*-(2-carboxyethyl)]-1-β-D-ribofuranosylpyrrole. By using the method described here, it is now possible to synthesize 4-hydroxypyrrolopyrimidine nucleosides labeled with ^{14}C in C-2.

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