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Evidence for the Conversion of Adenosine to 2′-Deoxycoformycin by *Streptomyces antibioticus*†

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ABSTRACT: The incorporation and distribution of ^{14}C in 2′-deoxycoformycin, elaborated by *Streptomyces antibioticus*, were studied with [U-^{14}C]glycine, [U-^{14}C]adenosine and [U-^{14}C]adenine. Similar ratios of ^{14}C in the aglycon and carbohydrate portions of 2′-deoxycoformycin, ara-A, and adenosine isolated from the RNA indicated that [U-^{14}C]adenosine was incorporated into 2′-deoxycoformycin without cleavage of the N-glycosylic bond. Following the addition of [U-^{14}C]adenine, 98% of the ^{14}C isolated from [^{14}C]-2′-deoxycoformycin resided in the aglycon. 2′-Deoxycoformycin biosynthesis may not require the de novo purine biosynthetic pathway as evidenced by the failure to detect incorporation of [U-^{14}C]glycine into 2′-deoxycoformycin. These data suggest that the biosynthesis of 2′-deoxycoformycin involves the incorporation of the carbon–nitrogen skeleton of an intact purine nucleoside or nucleotide, thereby implying that a purine ring is opened enzymatically between C-6 and N-1 and a one-carbon unit is added to form the 1,3-diazepine ring of 2′-deoxycoformycin.

2′-Deoxycoformycin,[1] the potent, tight-binding inhibitor of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) having a $K_i = 2.5 \times 10^{-12}$ M (Agarwal et al., 1977), has been isolated from the culture filtrates of *Streptomyces antibioticus* NRRL 3238 (Woo et al., 1974; Ryder et al., 1975; Dion et al., 1977). This nucleoside and its D-*ribo* analogue, coformycin, represent the two most potent inhibitors known for adenosine deaminase (Suhadolnik, 1979). The extremely tight-binding inhibitory properties are attributed, in part, to the unique heterocyclic ring system that structurally is very similar to the sp^3 transition state (Wolfenden, 1972) believed to be involved in the deamination process (Figure 1). The structure of 2′-deoxycoformycin was unequivocally established via single-crystal X-ray analysis by Woo et al. (1974) as (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol. Baker and co-workers have developed synthetic routes for this nucleoside and its congeners (Chan et al., 1982; D. C. Baker et al., unpublished results), establishing that the 8*R* isomers are the tight-binding entities among possible diastereomers. 2′-Deoxycoformycin has attracted considerable interest, as the inhibition of adenosine deaminase is of importance in the areas of both cancer and virus chemotherapy and in immunosuppression (Agarwal, 1982). 2′-Deoxycoformycin, both alone and in combination with ara-A, is at present under phase I and II clinical trials against a number of neoplasms, with dramatic results being observed in some patients with refractory T-cell disease (Prentice et al., 1980; Hershfield et al., 1983).

The object of the study reported herein is to investigate the biosynthesis of 2′-deoxycoformycin, particularly as it pertains to the origin of the fused 5,7-membered heterocyclic aglycon which contains an additional methylene group located between the N-1 and C-6 in a typical purine skeleton (see Figure 1). While 1,4-diazepines such as anthramycin have been established as being derived from L-tyrosine and L-tryptophan by Hurley & Gairola (1979), there is no information about the biosynthetic origins of the 1,3-diazepines such as 2′-deoxycoformycin. An additional attractive feature to these studies is the fact that another important nucleoside antibiotic, 9-β-D-arabinofuranosyladenine (ara-A), is produced concomitantly with 2′-deoxycoformycin and is in fact the major product of the *S. antibioticus* fermentation. Earlier studies on the epimerization of the C-2′ hydroxyl group of adenosine suggest that adenosine is directly converted to ara-A (Farmer & Suhadolnik, 1972), and more recently R. J. Suhadolnik et al. (R. J. Suhadolnik, J. M. Wu, M. M. Anderson, D. von Minden, and J. A. McCloskey, unpublished results) have reported the isolation and partial purification of an adenosine 2′-epimerase that catalyzes a 10% in vitro conversion of adenosine to ara-A. Perhaps the biosynthesis of 2′-deoxycoformycin might be a process closely related to the conversion of adenosine to ara-A or conceivably a process that could involve a common biosynthetic intermediate for the biosynthesis of both ara-A and 2′-deoxycoformycin. This paper provides evidence that adenosine and a one-carbon unit are the direct precursors of 2′-deoxycoformycin.

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[1] Abbreviations: ara-A, 9-β-D-arabinofuranosyladenine; AMP, adenosine 5′-monophosphate. Other names for 2′-deoxycoformycin (dCF) include co-vidarabine (CoV) and pentostatin (USAN). The chemical name is (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol.

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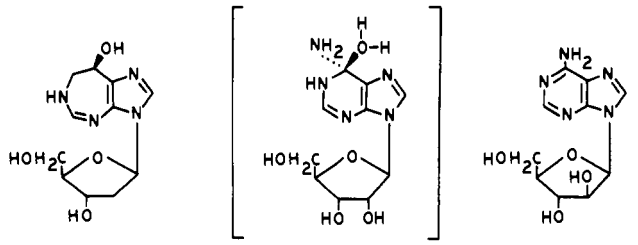

FIGURE 1: Structures of 2′-deoxycoformycin, the sp^3 transition state for the deamination of adenosine by adenosine deaminase, and ara-A.

Materials and Methods

Materials. Thin-layer chromatography (TLC) was carried out on E. Merck silica gel 60 F-254 aluminum-backed plates (20 μm; catalog no. 5539). Detection of the aglycon or nucleoside was by short-wave UV, and sugars were visualized by spraying the plates with 5% ethanolic phosphomolybdic acid, followed by charring at ca. 125 °C. The ^{14}C on the TLC plates was determined by soaking 0.5-cm slices of the TLC in 0.3 mL of 1% HCl for 1 h, followed by scintillation counting of the mixture. Analytical reverse-phase high-pressure liquid chromatography (HPLC) was performed on a 10-μm, 4 × 400 mm Spherisorb octadecylsilyl-derivatized column (Phase Separations, Ltd.; slurry-packed in-house at 9000 psi) at a flow rate of 2 mL/min. Semipreparative reverse-phase chromatography was conducted on a 10-μm, 0.9 × 60 cm octadecylsilyl-derivatized HPLC column (Whatman; Partisil M-9). The relative retention times are reported as k' values [$k' = (t_r - t_v)/t_v$]. Preparative ion-exchange chromatography was performed with 50 mL of Dowex 50 X2 (NH_4^+) (50–100 mesh) in an open column. Amberlite IR 120 (H^+) (50–100 mesh) was utilized for the hydrolysis reactions. Type I (calf mucosal) adenosine deaminase (in 3.2 M sodium sulfate) was purchased from Sigma Chemical Co. Cultures of *S. antibioticus* were shaken at 200 rpm in a Controlled Environment incubation shaker (New Brunswick Scientific Co.) at 36 °C in 1-L Erlenmeyer flasks containing 200 mL of media. Radioactive measurements were carried out in a Packard Tri-Carb liquid scintillation spectrometer, Model 3320, with Bray's scintillation solution (Bray, 1960).

Growth of S. antibioticus. Cultures of *S. antibioticus* NRRL 3238, supplied by Warner-Lambert Co., were maintained on agar slants or in culture media as described by Parke-Davis & Co. (Farmer & Suhadolnik, 1967). Inoculations into 200 mL of liquid media were made either directly from an agar slant or by a 1% inoculum from a 16-h culture.

Isolation of 2′-Deoxycoformycin and Ara-A. Both ara-A and 2′-deoxycoformycin were isolated essentially by procedures previously reported (Farmer & Suhadolnik, 1972; Dion et al., 1977; Ryder et al., 1975) followed by semipreparative reverse-phase HPLC (95:5 0.05 M, pH 6.86, phosphate buffer–methanol) separation of the two nucleosides.

Determination of 2′-Deoxycoformycin. Concentrations of 2′-deoxycoformycin in culture filtrates were directly determined by an adenosine deaminase assay procedure. The culture broth (100 μL) was diluted as needed, between 1:100 and 1:10000, with distilled water. Between 10 and 100 μL of the diluted sample was preincubated with adenosine deaminase for 5 min at 25 °C in 2.9 mL of 0.05 M phosphate buffer, pH 7.5. After the addition of 100 μL of 1×10^{-3} M adenosine, the rate of deamination was determined and converted to a molar per second rate which was subsequently converted to a 2′-deoxycoformycin concentration based on a previously prepared standard curve.

Addition of ^{14}C Compounds to Ara-A and 2′-Deoxycoformycin-Producing Cultures of S. antibioticus. A sterile solution of either [U-^{14}C]glycine (118 μCi/μmol), [U-^{14}C]-adenosine (550 μCi/μmol), or [U-^{14}C]adenine (250 μCi/μmol) was added to the culture medium when the 2′-deoxycoformycin concentration was 2–6 μg/mL. The cultures were filtered when production of deoxycoformycin had ceased approximately 48 h later (final concentration of ara-A was typically ~100 μg/mL and 2′-deoxycoformycin, ~20 μg/mL). 2′-Deoxycoformycin and ara-A were then isolated as described (see above).

Distribution of ^{14}C in 2′-Deoxycoformycin and Ara-A. Ara-A, recrystallized from hot water, and 2′-deoxycoformycin, purified by semipreparative HPLC, were purified to constant specific activity before hydrolysis. The physiochemical properties of ara-A and 2′-deoxycoformycin [i.e., TLC (adsorption-mode silica gel) R_f values and HPLC (reverse-phase) retention times] were identical with those for authentic ara-A (TLC, R_f 0.57, 70:30 acetonitrile–0.05 M ammonium chloride + 0.05 M ammonium bicarbonate; HPLC, $k' = 5.5$, 2 mL/min, 95:5 0.05 M, pH 6.86, phosphate buffer–methanol) and 2′-deoxycoformycin (R_f 0.33; $k' = 4.5$). Five milligrams of 2′-deoxycoformycin was hydrolyzed by 1 mL of Amberlite IR-120 (H^+) resin in 10 mL of water for 30 min at 25 °C to its aglycon[2] (R_f 0.17) and 2-deoxy-D-ribose (R_f 0.80) moieties. The resin, to which the aglycon was adsorbed, was filtered and washed 3 times (with 5 mL) of water. The aglycon was displaced from the resin with 10 mL of 1 N ammonium hydroxide. The filtrate contained the 2-deoxy-D-ribose; TLC (as above) was used to identify both the 1,3-diazepine and 2-deoxy-D-ribose. Ara-A was hydrolyzed to adenine and D-arabinose as above for 24 h at 100 °C.

Distribution of ^{14}C in Adenosine of RNA from S. antibioticus. The RNA of *S. antibioticus* was isolated from the culture mycelium (28 g wet weight) and hydrolyzed to the component nucleic acids as previously described (Farmer & Suhadolnik, 1972).

Results

Incorporation of [U-^{14}C]Glycine into 2′-Deoxycoformycin and Ara-A. Glycine, a substrate for de novo purine biosynthesis, when added to *S. antibioticus* when the production of ara-A and 2′-deoxycoformycin was initially detected was not incorporated into 2′-deoxycoformycin as evidenced by a failure to detect radioactivity in a zone determined to be 2′-deoxycoformycin on both TLC (silica gel) and HPLC (reverse phase). Also, no ^{14}C was detected in the ara-A that was isolated crystalline from the culture. Evidence that glycine was metabolized by *S. antibioticus* was the observation that ^{14}C was found in several, unidentified peaks from the HPLC elution (data not shown), indicating that [U-^{14}C]glycine was indeed taken up, metabolized, and released into the medium by *S. antibioticus.*

Incorporation of ^{14}C from [U-^{14}C]Adenosine and [U-^{14}C]-Adenine into 2′-Deoxycoformycin and Ara-A. To elucidate the pathway involved in the biosynthesis of 2′-deoxycoformycin, experiments were performed in which ^{14}C-labeled adenine and adenosine were added to the culture medium of *S. antibioticus.* ^{14}C from both [U-^{14}C]adenosine and [U-^{14}C]adenine, when separately added to the cultures as production of 2′-deoxycoformycin and ara-A began during the stationary phase of

[2] The heterocyclic base, (8*R*)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]-diazepin-8-ol, that was hydrolyzed from 2′-deoxycoformycin has been characterized by ^1H NMR, ^{13}C NMR, CD, and mass spectrometric methods (J. C. Hanvey, E. Smal and D. C. Baker, unpublished results).

Table I: Incorporation of [U-^{14}C]Glycine, [U-^{14}C]Adenine, and [U-^{14}C]Adenosine into 2′-Deoxycoformycin and Ara-A by *S. antibioticus*

compound added	amount/flask		2′-deoxycoformycin		ara-A	
	μCi	μCi/μmol	sp act.[a] (nCi/μmol)	incorpn efficiency (%)[b] (X10^3)	sp act.[a] (nCi/μmol)	incorpn efficiency (%)[b] (X10^3)
[U-^{14}C]glycine	10	118	0	0	0	0
[U-^{14}C]adenosine	10	550	8.6	1.6	8.2	1.5
[U-^{14}C]adenine	10	296	20.7	7.0	28.8	10

[a] 2′-Deoxycoformycin and ara-A were purified to constant specific activity. [b] The incorporation efficiency (%) is 100 times the specific activity of the compound divided by the specific activity of the isotope added to the flask.

Table II: Distribution of ^{14}C in 2′-Deoxycoformycin, Ara-A, and the Adenosine from the RNA of *S. antibioticus* following the Incorporation of either [U-^{14}C]Adenosine or [U-^{14}C]Adenine

fraction[a]	[U-^{14}C]adenosine (dpm) (%)	[U-^{14}C]adenine (dpm) (%)
2′-deoxycoformycin	1740	4320
2-deoxy-D-ribose	550 (45.5)	70 (2.0)
aglycon	660 (54.5)	3190 (98.0)
ara-A	4950	14190
D-arabinose	2210 (49.7)	140 (1.4)
adenine	2240 (50.3)	10200 (98.6)
adenosine (isolated from RNA)		
D-ribose	3788 (48)	
adenine	4102 (52)	

[a] 2′-Deoxycoformycin, ara-A, and adenosine isolated from RNA were shown to be pure by TLC. The values in parentheses are the percent (%) calculated by dpm/fraction divided by the sum of the dpm for both products.

cell growth, was incorporated into 2′-deoxycoformycin and ara-A (Table I). The 1.5% incorporation of [U-^{14}C]adenosine into ara-A is in agreement with the 2.3% described earlier (Farmer & Suhadolnik, 1972).

Distribution of ^{14}C in 2′-Deoxycoformycin, Ara-A, and the Adenosine in the RNA from the [U-^{14}C]Adenosine Experiment. 2′-Deoxycoformycin, ara-A, and the adenosine from the isolated RNA from the *S. antibioticus* mycelium were hydrolyzed to the aglycon–2-deoxy-D-ribose, adenine–D-arabinose, and adenine–D-ribose, respectively, as described under Materials and Methods. The radioactivity measured for each TLC slice corresponded to the UV-active zone indicative of the respective, authentic unlabeled product. The ratio of ^{14}C in the aglycon:2-deoxy-D-ribose of 2′-deoxycoformycin was 55:45. The ratio of ^{14}C in the adenine:D-arabinose of ara-A and the adenine:D-ribose of the AMP isolated from the RNA of *S. antibioticus* was 50:50 and 52:48, respectively (Table II). These findings are in agreement that adenosine is taken up intact by *S. antibioticus* (Farmer & Suhadolnik, 1972).

Distribution of the ^{14}C Label in 2′-Deoxycoformycin and Ara-A from the [U-^{14}C]Adenine Experiment. The possible incorporation of ^{14}C from adenine into the aglycon of 2′-deoxycoformycin, based on the incorporation of ^{14}C from the [U-^{14}C]adeosine experiment, was investigated by the addition of [U-^{14}C]adenine to *S. antibioticus*. 2′-Deoxycoformycin and ara-A were hydrolyzed to the aglycon–2-deoxy-D-ribose and adenine–D-arabinose, respectively. The ratio of ^{14}C in the aglycon:2-deoxy-D-ribose of 2-deoxycoformycin and in the adenine:D-arabinose of ara-A was 98:2 for both compounds (Table II).

Discussion

Three general pathways considered for the biosynthesis of 2′-deoxycoformycin by *S. antibioticus* were (i) utilization of the de novo purine biosynthetic pathway, (ii) conversion of a preformed purine nucleoside or nucleotide, or (iii) formation by a pathway totally independent of purine metabolism. The observed lack of incorporation of ^{14}C into 2′-deoxycoformycin and ara-A when [U-^{14}C]glycine was added to *S. antibioticus* implies that the formation of these nucleosides may not require a functional de novo purine biosynthetic process at the time of their biosyntheses. The major significance of this observation is that exogenously supplied [U-^{14}C]adenosine is incorporated into the AMP of RNA and into both nucleosides, 2′-deoxycoformycin and ara-A. Therefore, during the stationary phase of cell growth, there appears to be a sufficient source of adenosine either in the medium or in the cell to serve as the direct precursor for 2′-deoxycoformycin and ara-A and to supply AMP for mRNA synthesis without relying upon the established de novo purine biosynthetic pathway. The concentration of adenine nucleosides or nucleotides during the biosynthesis of 2′-deoxycoformycin and ara-A is estimated to be ~6 mM.

Since many naturally occurring purine analogues, including ara-A, 3′-deoxyadenosine (cordycepin), and the pyrrolopyrimidine antibiotics, are derived from purine nucleosides and nucleotides (Suhadolnik, 1979), it was reasoned that a purine nucleoside or nucleotide might be the direct precursor for the biosynthesis of 2′-deoxycoformycin. The data obtained by following the uptake, incorporation, and distribution of ^{14}C in the aglycon and 2′-deoxy-D-ribose of 2′-deoxycoformycin from the [U-^{14}C]adenosine and [U-^{14}C]adenine experiments strongly support this hypothesis. The data further imply that the entire carbon–nitrogen skeleton of adenosine is utilized, without cleavage of the N-glycosylic bond, for the biosynthesis of 2′-deoxycoformycin. This conclusion is based on the similarities of the ^{14}C ratios in the aglycon–2-deoxy-D-ribose of 2′-deoxycoformycin, the adenine–D-arabinose of ara-A, and the adenine–D-ribose of the AMP isolated from the RNA from the [U-^{14}C]adenosine experiment (Table II). Also, Farmer & Suhadolnik (1972) had previously demonstrated that [U-^{14}C]adenosine did not undergo *N*-glycosyl hydrolysis when taken up by *S. antibioticus*.

The data presented here, following the addition of [U-^{14}C]adenine to *S. antibioticus*, also support the hypothesis that adenosine is directly converted to 2′-deoxycoformycin (Table II). Furthermore, and perhaps more importantly, these data confirm that the biosynthesis of five of the six carbons of the aglycon of 2′-deoxycoformycin, with its fused imidazole-1,3-diazepine ring system, is formed from the five carbons of the purine ring of adenine. It is to be established whether the purine precursor is either adenosine, inosine, guanosine, and/or their corresponding nucleotides. Although the enzymatic reactions for the biosynthesis remain to be studied, it appears that the purine ring is cleaved between N-1 and C-6, followed by insertion of a one-carbon unit and ring closure (Figure 2). The presumed ring opening and ring closure at C-6 and N-1 of the pyrimidine ring are known to occur chemically in what

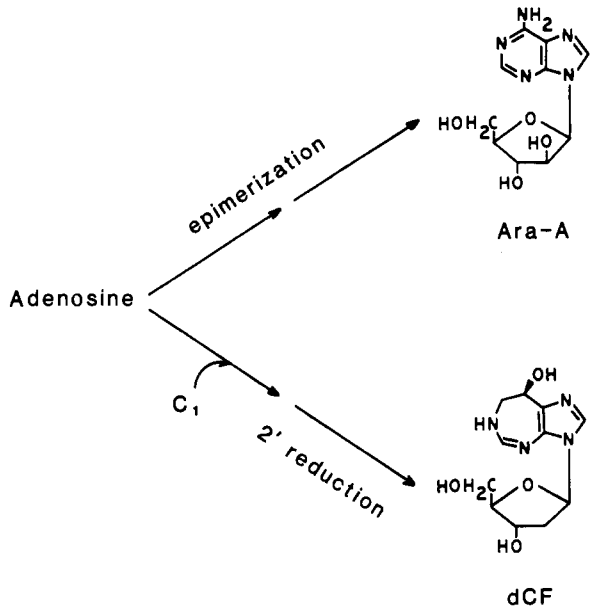

FIGURE 2: Proposed biosynthetic pathway for 2′-deoxycoformycin and ara-A from adenosine by *S. antibioticus*.

is generally referred to as the Dimroth rearrangement (Brown & Harper, 1963; Perrin, 1963). The inclusion of the one-carbon unit, i.e., carbon 7 of the 1,3-diazepine ring, might proceed via several known mechanisms. One mechanism involves the transfer of a one-carbon unit via tetrahydrofolic acid as occurs in the biosynthesis of IMP (Henderson, 1972). A second mechanism would utilize carbon 1 of D-ribose. Precedents for this mechanism include (1) the incorporation of C-1 of the D-ribityl moiety of riboflavin into C-2 of 5,6-dimethylbenzimidazole (Renz & Weyhenmeyer, 1972), (2) the utilization of D-ribose for the heterocyclic ring formation in the biosynthesis of the nucleoside antibiotics tubercidin and toyocamycin (Suhadolnik & Uematsu, 1970), and (3) the formation of *N*,5′-phosphoribosyl-AMP by the condensation of α-5-phosphoribosyl-1-pyrophosphate (PRPP) and ATP during histidine biosynthesis (Stryer, 1981). Studies are under way in this laboratory to determine the biosynthetic precursor for the insertion of the one-carbon unit into the purine ring to form the 1,3-diazepine ring of 2′-deoxycoformycin. The reduction of C-2′ of adenosine in the biosynthesis of 2′-deoxycoformycin is also under investigation. This reduction could proceed enzymatically via either the ribonucleoside diphosphate or the ribonucleoside triphosphates by using either the NADPH–ribonucleotide reductase, the B_{12}–ribonucleotide reductase, or an unknown reductase. In summary, the data presented strongly suggest that the biosynthesis of 2′-deoxycoformycin proceeds via the utilization of adenosine and a one-carbon unit of yet undefined origin.

Acknowledgments

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Registry No. 2′-Deoxycoformycin, 53910-25-1; 9-β-D-arabinofuranosyladenine, 5536-17-4; adenosine, 58-61-7; adenine, 73-24-5.

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