Biosynthesis of 2′-Deoxycoformycin: Evidence for Ring Expansion of the Adenine Moiety of Adenosine to a Tetrahydroimidazo[4,5-*d*][1,3]diazepine System†

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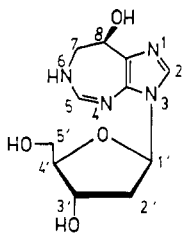
ABSTRACT: 2′-Deoxycoformycin (2′-dCF), a nucleoside antitumor agent produced in trace quantities by *Streptomyces antibioticus*, has been shown in earlier work to originate from the intact carbon–nitrogen framework of adenosine. Additional experiments using ^{13}C and two-dimensional Fourier transform NMR techniques, together with radiolabeling studies, identify the C-1 of D-ribose, and not the tetrahydrofolate "C-1 pool", as the source of the C-7 carbon in the aglycon of 2′-dCF. These results show that the adenine portion of adenosine (or a nucleotide thereof) undergoes a unique ring expansion, by insertion of a $-CH_2-$ unit between the N-1 and C-6 of the adenine ring, to furnish the 1,3-diazepine portion of 2′-dCF.

2′-Deoxycoformycin (2′-dCF),[1] an antibiotic of potential



2′-deoxycoformycin (2′-dCF)

utility in the treatment of certain hematological cancers [reviewed by Suhadolnik (1979), Glazer (1980), Poster et al. (1981), Goodchild (1982), and Buchanan and Wightman (1982)], is a 2′-deoxynucleoside that contains the unique tetrahydroimidazo[4,5-*d*][1,3]diazepine aglycon. The compound, which is produced in trace quantities by *Streptomyces antibioticus* (Woo et al., 1974) along with larger amounts of the antiviral nucleoside 9-(β-D-arabinofuranosyl)adenine (*ara*-A), has been the target of extensive structural (Woo et al., 1974), synthetic (Baker & Putt, 1979; Chan et al., 1982), biosynthetic (Hanvey et al., 1984c), and mechanistic (Schramm & Baker, 1985; Frick et al., 1986) studies in these laboratories. Both 2′-dCF and analogues (Hawkins et al., 1983; Schaumberg et al., 1985) are extremely tight binding inhibitors of adenosine deaminase (EC 3.5.4.4), a ubiquitous enzyme of significance in the chemotherapy of viruses and cancer (O'Dwyer & Marsoni, 1984; Grever et al., 1983).

In an earlier paper, using [U-^{14}C]adenosine, it was demonstrated that adenosine serves as the direct carbon–nitrogen precursor for the biosynthesis of both the aglycon and the carbohydrate moiety of 2′-dCF (Hanvey et al., 1984c). While these studies showed that five of the six carbon atoms of 2′-dCF arise from the five carbons of the adenine ring of adenosine, the origin of the C-7 methylene group (i.e., the "extra" or sixth carbon) in the 1,3-diazepine ring of 2′-dCF has heretofore remained an enigma. Results of experiments using both ^{13}C and two-dimensional Fourier transform (2DFT) NMR spectroscopy and radioisotope techniques that identify D-ribose, and not a contributor from the suspected tetrahydrofolate "1-carbon pool", as the carbon donor for the C-7 methylene of 2′-dCF are presented in this paper.

Experimental Procedures

General. Radioactivity measurements were made on a Beckman LS-100C liquid scintillation spectrometer with ACS (Amersham 196290) aqueous counting scintillant. Fermentations were carried out in a New Brunswick Gyrorotary shaker operating at 200 rpm. Gas chromatography/mass spectrometry (GC/MS) was conducted on a Hewlett-Packard 5985A instrument operating in the electron-impact mode at 70 eV and outfitted with an HP-5840A GC unit equipped with a DB-5 (Durabond 123-5032) fused-silica capillary column (film thickness 0.25 μm, 0.32-mm i.d., 30 m) operating at 80–220 °C (20 deg min^{-1} program) and at a helium carrier gas flow rate of 1 mL min^{-1}. Samples were trimethylsilylated for GC/MS analysis as follows: The dry nucleoside (1–2 mg) was dissolved in dry pyridine (0.2 mL/mg of nucleoside) in a Reacti-Vial (Pierce Chemical Co.) to which 0.2 mL of *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (Aldrich Chemical Co.) was added. After being allowed to stand for 24 h at ~25 °C, the sample was injected into the GC and chromatographed as described in the foregoing; $k = 33.2$ for per-Me_3Si-2′-dCF.

^{13}C NMR Experiments. ^{13}C NMR spectra for the ^{13}C-enriched samples of 2′-dCF were obtained on 0.03–0.12 M solutions in deuterium oxide contained in a 12-mm (0.9-mL) spherical coaxial tube (Wilmad 529-A-12) with a Nicolet NT-200 instrument operating at 50.3 MHz. The chemical shifts are reported in δ units downfield from tetramethylsilane,

† Aspects of this work have been presented in preliminary form. See Hanvey et al. (1984a,b, 1986). This work was supported, in part, by Grant AI-22296 from the National Institutes of Health.

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[1] Other names include covidarabine, pentostatin (USAN), and NSC-218321. The Chemical Abstracts name is (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol, CA Registry No. 53910-25-1. Abbreviations: AICAR ("aminoimidazole carboxamide ribotide"), 5-amino-1-(β-D-ribofuranosyl)-imidazole-4-carboxamide 5′-monophosphate; PRPP, 5-phosphoribose 1-pyrophosphate; *ara*-A, 9-(β-D-arabinofuranosyl)adenine; "2-deoxy-D-ribose", 2-deoxy-D-*erythro*-pentose; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate.

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Table I: Incorporation of DL-[3-^{13}C]Serine and D-[1-^{13}C]Ribose into 2′-Deoxycoformycin[a]

carbon no.	chemical shift of *R* isomer (*S* isomer) (δ)[b]	*x*-fold enrichment from DL-[3-^{13}C]serine[c]	*x*-fold enrichment from D-[1-^{13}C]ribose[c]
2	132.2 (132.6)	1.60	1.07
5	150.6 (150.6)	2.25	1.04
7	47.7 (47.8)	1.16	2.71
8	67.4 (67.6)	1.0	1.0
9[d]	135.9 (135.9)	1.0	1.0
10[d]	129.2 (129.5)	1.17	0.85
1′	84.1 (84.6)	1.18	2.82
2′	39.6 (39.6)	1.0	1.0
3′	72.0 (72.2)	1.18	1.17
4′	87.5 (87.6)	1.11	1.06
5′	62.4 (62.5)	1.89	1.30

[a] For details of the culturing and ^{13}C NMR experiments, see Experimental Procedures. [b] Values in parentheses are for the 8*S* isomer of 2′-dCF. [c] ^{13}C NMR peak heights were used. See Experimental Procedures for details. [d] These assignments may be reversed.

δ 0.0. 1,3-Dioxane (δ 67.2) was employed as an internal reference. The parameters were as follows: pulse angle 90°, pulse length 20 μs, delay time 15 s, acquisition time 582 ms, spectral width 7 kHz, and 16K data sets taken. A natural abundance ^{13}C NMR of 2′-dCF was obtained under identical parameters on a 0.2 M solution in a 12-mm tube (200 acquisitions), and all ^{13}C enrichments are reported relative to their respective peak heights in this spectrum (Figure 1 and Table I). The 2DFT NMR spectrum (Bax, 1982) was taken on a 0.6 M solution in deuterium oxide of 2′-dCF with the following parameters: pulse angle 90°, pulse length 42 μs, premixing pulse delay time 3.3 ms, preacquisition delay time 1.7 ms, acquisition increment 333 μs, number of acquisitions 256, sweep window in ^{1}H dimension ±1500 Hz, and sweep window in ^{13}C dimension ±3200 Hz. Details of this experiment are contained in the dissertation of Smal (1985).

Materials. D-[1-^{14}C]Ribose (53 mCi/mmol), L-[3-^{14}C]-serine (53 mCi/mmol), and [8-^{14}C]adenosine (59 mCi/mmol) were obtained from Amersham. D-[1-^{13}C]Ribose (90 atom %) and DL-[3-^{13}C]serine (90 atom %) were obtained from Merck and Co. [^{18}O]Water (95 atom %) was purchased from Monsanto Research Corp. Sodium hadacidin was kindly supplied by H. T. Shiguera of Merck and Co., Rahway, NJ. Ion-exchange chromatography was performed on Dowex 50 X2 (NH_4^+) (50–100 mesh). Adenosine deaminase was of calf mucosal origin (Sigma, type I).

Culture and Assay Techniques. S. antibioticus NRRL 3238 was obtained from Warner-Lambert/Parke-Davis stocks, and the cultures were maintained as previously described (Hanvey et al., 1984c). Inoculations into growth media were made directly from cultures stored in 10% glycerol at −20 °C. The concentration of 2′-dCF produced was determined by assay with adenosine deaminase (Hanvey et al., 1984c).

Incorporation of DL-[*3*-^{13}*C*]*Serine and* D-[*1*-^{13}*C*]*Ribose.* To two separate 100-mL cultures of *S. antibioticus* maintained at 37 °C in 1-L wide-mouth, Erlenmeyer flasks at 200 rpm was added filter-sterilized solutions of 200 mg of DL-[3-^{13}C]serine and 300 mg of D-[1-^{13}C]ribose, respectively, 72-h postinoculation (when the concentration of 2′-dCF was 1–10 μg/mL). The cultures were maintained as above for another 4 days, at which time production of 2′-dCF had ceased. (Assays for 2′-dCF indicated 263 and 268 μg/mL of 2′dCF for the serine and ribose experiments, respectively.) Celite was then added, the mixture was filtered with suction, and the Celite pad was washed with 40 mL of water. The dark-pigmented filtrates were combined and added to a 50-mL column of Dowex 50 X2 (NH_4^+), the dark pigments were eluted with

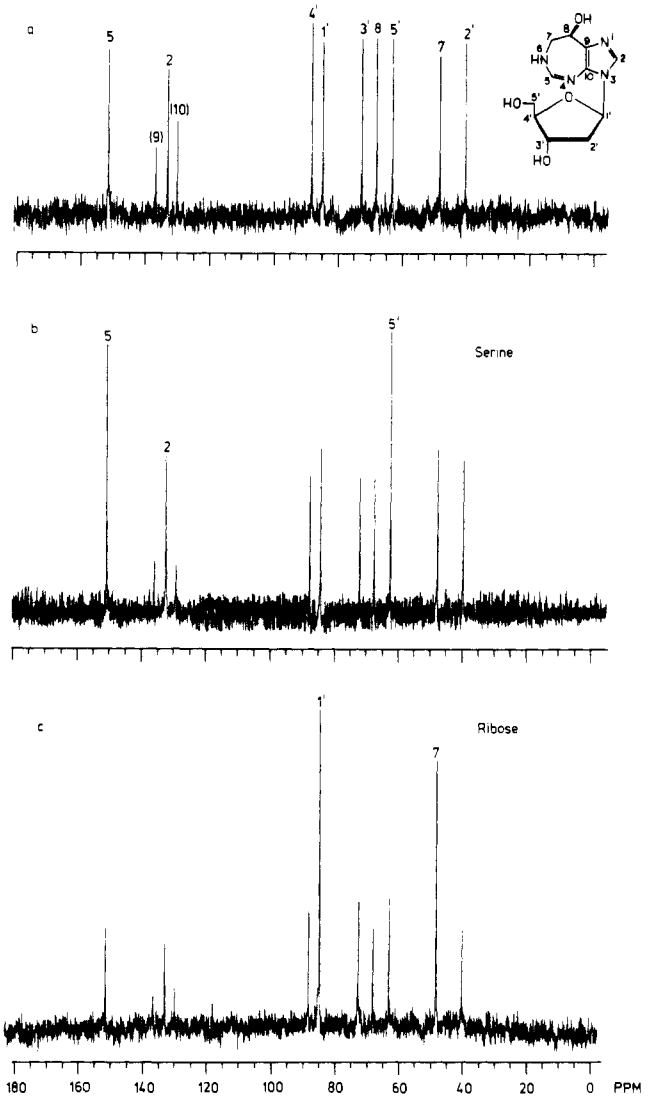


FIGURE 1: (a) Natural abundance ^{13}C NMR spectrum of (8*R*)-2′-deoxycoformycin (2′-dCF). (b) ^{13}C enrichment from experiment using DL-[3-^{13}C]serine. (c) ^{13}C enrichment from experiment using D-[1-^{13}C]ribose. For details of experiments, see Experimental Procedures.

100 mL of water, and the nucleosides 2′-dCF and *ara*-A were displaced with 200 mL of 1 N ammonium hydroxide. The ammonia fraction was freeze-dried, and the residue was reconstituted in 10 mL of methanol, filtered from insolubles (*ara*-A), and subjected to preparative reverse-phase liquid chromatography (LC) with 97.5:2.5 water–methanol (flow rate 3 mL/min) on a 2.25 × 45 cm column of 40-μm octadecylsilyl-dervatized silica gel (280-nm UV detection). The appropriate zones were collected and freeze-dried, yield 12 and 37 mg of 2′-dCF from the DL-serine and D-ribose experiments, respectively. ^{13}C NMR analyses of the ^{13}C-enriched 2′-dCF are summarized in Table I and Figure 1b,c.

Incorporation of L-[*3*-^{14}*C*]*Serine and* D-[*1*-^{14}*C*]*Ribose into 2′-dCF.* A total of 7.2 μCi of L-[3-^{14}C]serine and 10.0 μCi of D-[1-^{14}C]ribose were each added to two 200-mL cultures of *S. antibioticus*, and the products were isolated exactly as in the foregoing experiment with the following exceptions: (a) Cultures were maintained at 30 °C. (b) Additions of radiolabeled precursors were made 48-h postinoculation. (c) Cultures were harvested 72 h after addition of precursors. (Concentration of 2′-dCF was ~20 μg/mL.) (d) Purification was by reverse-phase semipreparative HPLC on a Whatman Magnum-9 octadecylsilyl column (0.9 × 60 cm) with 95:5 0.02 M phosphate buffer, pH 7.5–methanol. The appropriate peaks

Table II: Incorporation of L-[3-^{14}C]Serine and D-[1-^{14}C]Ribose into Deoxycoformycin and *ara*-A by *S. antibioticus*

	L-[3-^{14}C]serine		D-[1-^{14}C]ribose	
	2′-deoxy-coformy-cin	*ara*-A	2′-deoxy-coformy-cin	*ara*-A
sp act.[a]	0.34	0.54	5.8	2.8
percent ^{14}C in aglycon	87	86	53	6
percent ^{14}C in sugar moiety	13	14	47	94

[a] The specific activity is reported in mCi/μmol. For details, see Experimental Procedures.

for 2′-dCF (k = 4.5) and *ara*-A (k = 5.5) were collected, and the products were each hydrolyzed and assayed as previously described (Hanvey et al., 1984c). Data are collected in Table II.

[6-^{18}O]Inosine. A mixture of 250 mg (0.94 mmol) of adenosine and 1.0 mL of [^{18}O] water was heated until the adenosine was dissolved. Glacial acetic acid (130 μL, 2.3 mmol) was added, followed by the addition of 150 mg (2.2 mmol) of sodium nitrite over a period of 1 h (Shapiro & Pohl, 1968). After 3 h the reaction mixture was passed over a 2-mL column of Dowex 50 X8 (H^+). The inosine was collected by washing the column with 30 mL of water, and the appropriate fractions were combined and lyophilized to yield 150 mg (0.56 mmol) of inosine, which was 66% ^{18}O enriched as determined by GC/MS on a pertrimethylsilylated sample.

[8-^{14}C]Inosine. A mixture of 7 μCi (0.12 μmol) of [8-^{14}C]adenosine and 2 units of adenosine deaminase was incubated for 1 h at 25 °C. After the reaction was stopped by heating the mixture for 5 min at 90 °C, the [8-^{14}C]inosine, shown to be free of adenosine by HPLC (95:5 0.02 M phosphate buffer, pH 7.5–methanol, Waters μBondapak C-18 column, 4.5 × 30 cm), was used in the following experiment without purification.

Incorporation of [8-^{14}C]Inosine and [6-^{18}O]Inosine into 2′-dCF. To a 300-mL culture of *S. antibioticus* maintained as in the foregoing experiments at 30 °C was added 37.5 mg of [6-^{18}O]inosine and 7.2 μCi of [8-^{14}C]inosine when concentrations of 2′-dCF were 3–6 μg/mL. The cultures were harvested as in the foregoing experiment, and the results of the mass spectral assays for ^{18}O incorporation are as follows: m/e 556 (M, 100, 100), 557 (M + 1, 53, 51), and 558 (M + 2, 30, 30), where the first number in parentheses is the natural abundance for 2′-dCF and the second number is that from the [6-^{18}O]inosine experiment. As shown by the relative intensities of the M + 1 and M + 2 peaks, no incorporation of ^{18}O occurred in the experiment where the overall incorporation into 2′-dCF from [8-^{14}C]inosine was determined to be 25%.

Incorporation of [8-^{14}C]Inosine into 2′-dCF in the Presence of Hadacidin. Sodium hadacidin was added to a 6-mL culture of *S. antibioticus* maintained at 30 °C in 25-mL Erlenmeyer flasks to give concentrations of 5 and 10 mM when production of 2′-dCF was 2–3 μg mL^{-1}. One hour later, 0.9 μCi of [8-^{14}C]inosine was added, and 2′-dCF and *ara*-A were isolated as in the foregoing experiment after an additional 72 h.

Results and Discussion

In the earlier work wherein [U-^{14}C]adenosine was added to cultures of *S. antibioticus*, the ^{14}C ratio of the aglycon of 2′-dCF to the 2′-deoxy sugar moiety was essentially 1:1 (Hanvey et al., 1984c), reflecting precisely the ^{14}C ratio of the precursor adenosine (having five carbons in the aglycon and five carbons in the sugar moiety). It was therefore postulated that 10 of the 11 carbons of 2′-dCF are derived from a purine nucleoside or nucleotide. On the basis of these results, experiments were designed to determine the source of the eleventh carbon, likely to be the C-7 methylene unit of the 1,3-diazepine ring of 2′-dCF. This was accomplished by the addition of appropriate radiolabeled precursors to cultures of *S. antibioticus* in the stationary growth phase when concentrations of 2′-dCF were in the 1–10 μg/mL range as determined by adenosine deaminase assay. The product 2′-dCF was isolated 3–4 days later and analyzed for radioisotopic content (see Experimental Procedures).

Addition of L-[3-^{14}C]serine to a culture of *S. antibioticus*, followed by isolation and subsequent hydrolysis of 2′-dCF and analysis of the radioactivity in both the aglycon and sugar moieties, revealed that 87% of the ^{14}C resided in the aglycon and 13% in the sugar (Table II). On the basis of the known incorporation of carbon 3 of serine into carbons 2 and 8 of the purine ring via the tetrahydrofolate "C-1 pool" (Henderson, 1972), plus the earlier finding that adenosine is the direct precursor of 2′-dCF (Hanvey et al, 1984c), it was speculated that the ^{14}C from L-[3-^{14}C]serine would reside in carbons 2 and 5 of 2′-dCF (i.e., those most likely derived from the corresponding carbons 2 and 8 of the purine). If C-7 of 2′-dCF were dependent on the tetrahydrofolate "C-1 pool", then C-7 of 2′-dCF would also be derived from C-3 of serine (Huennekens, 1963). Furthermore, on the basis of the knowledge that serine is a gluconeogenic amino acid, it was speculated that the portion of ^{14}C from L-[3-^{14}C]serine would largely reside in C-5′ of the 2′-deoxy-D-pentose portion of 2′-dCF.[2] The observation of ^{14}C in both the aglycon and sugar is in accord with these predictions. It is noteworthy that both 2′-dCF and *ara*-A, the latter known to arise directly from adenosine by 2′-epimerization (Farmer & Suhadolnik, 1972; Farmer et al., 1973), show nearly identical aglycon:sugar ^{14}C ratios from the labeled serine, pointing to the likelihood that biogenetically identical atoms (i.e., C-2 and C-8 of the purine and the corresponding C-2 and C-5 of 2′-dCF) are involved in the biosynthesis of both nucleosides.

Having demonstrated the incorporation of the ^{14}C from L-[3-^{14}C]serine into 2′-dCF, the next problem was to determine the precise location of the labels. Since little is known concerning the degradation products of 2′-dCF,[3] ^{13}C NMR spectroscopic techniques were employed on an intact sample of 2′-dCF. By the addition of 200 mg of DL-[3-^{13}C]serine to cultures of *S. antibioticus*, followed by the isolation, purification, and ^{13}C NMR analysis of 2′-dCF, it was determined that C-2, C-5, and C-5′, but not C-7, of 2′-dCF showed substantial enrichment in comparison with the natural abundance ^{13}C spectrum (see Figure 1a,b and Table I). These results are in line with the aforementioned prediction. Thus, C-7 is not derived from the tetrahydrofolate C-1 pool to which serine is a contributor (Huennekens, 1963). In addition, the enrichment of C-2 and C-5 confirms the earlier findings (Hanvey et al., 1984c) that a purine nucleoside (or nucleotide) is the precursor of 2′-dCF.

As the above-mentioned ^{13}C NMR assignments for 2′-dCF could not be made in a definitive manner directly,[4] a 2DFT

[2] The conversion of L-serine to 2-deoxy-D-ribose could be based on the conversion of L-[3-^{14}C]serine → [3-^{14}C]pyruvate → [3-^{14}C]oxaloacetate → [3-^{14}C]phosphoenolpyruvate → [1,6-^{14}C]glucose 6-phosphate → [1,6-^{14}C]-6-phosphogluconate → D-[5-^{14}C]ribose → [5′-^{14}C]-2′-deoxycoformycin (2′-dCF).

[3] To date, identification of the ultimate (degradation) products from the acid hydrolysis of 2′-dCF has been difficult. Controlled acid hydrolysis of 2′-dCF leads to rapid glycosylic cleavage, wherein the chiral aglycon, a compound of limited stability, and the deoxy sugar can be isolated intact. See Hanvey (1983) and Smal (1985).

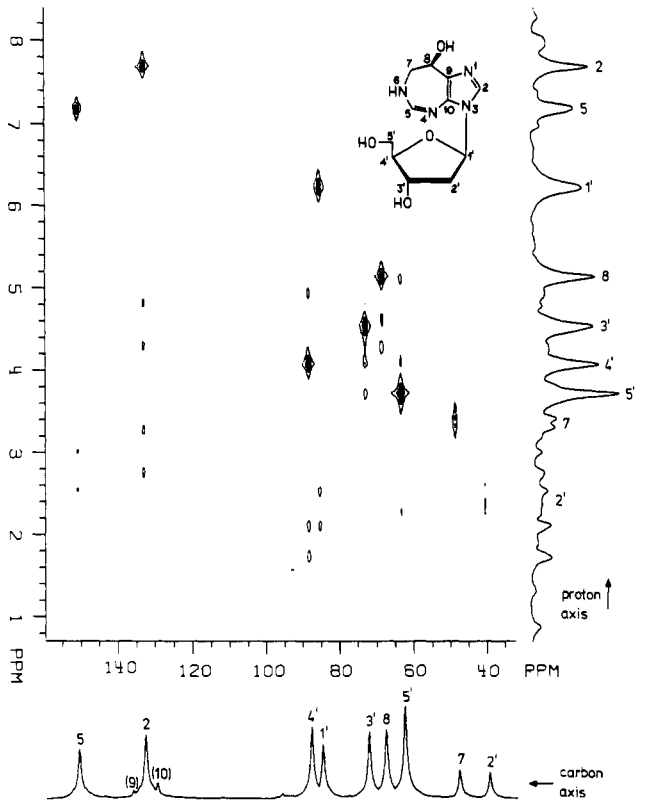


FIGURE 2: 2DFT NMR spectrum (Bax, 1982) of (8*S*)-2′-deoxycoformycin [(8*S*)-2′-dCF]. By use of firm assignments from the ^{1}H NMR spectrum (*y* axis), all ^{13}C resonances but those for C-9 and C-10 are assigned (*x* axis) through the correlation map. (8*S*)-2′-dCF is an acceptable substitute for (8*R*)-2′-dCF (see Table I).

NMR heteronuclear shift correlation study (Bax, 1982) was carried out as follows. The ^{1}H NMR spectrum of 2′-dCF in deuterium oxide is unambiguous, with the heterocyclic and sugar protons lending themselves to first-order analysis. The ^{1}H NMR assignments for H-2 and H-5, a possible source of error, were resolved by allowing the solution to stand, whereby the H-2 (imidazole) proton slowly exchanged with deuterium, a well-established phenomenon among imidazolo nucleosides.[5] A 2DFT NMR correlation was then determined (Figure 2) with (8*S*)-2′-deoxycoformycin (Chan et al., 1982), a synthetic diastereomer of 2′-dCF that was available in sufficient quantity for the 2DFT NMR experiment. The substitution of (8*S*)-2′-deoxycoformycin for 2′-dCF is without undue complications as the ^{1}H and ^{13}C NMR spectra for both compounds (see Table I for a comparison of ^{13}C NMR data.) are virtually indistinguishable (Chan et al., 1982). By these correlations, the assignments for all carbon atoms, except the internal C-9 and C-10 carbons, were unambiguously determined for 2′-dCF (Figure 1a, Table I).

Having shown that the C-7 methylene of 2′-dCF was not derived from serine via the C-1 tetrahydrofolate pathway, another source for the C-7 atom was sought. In reviewing known biosynthetic pathways involving adenosine, particularly those in which the N-1 and C-6 positions are sites for biochemical reactions, the possibility that D-ribose might be contributing the required carbon via the process of N-1-ribosylation (via PRPP), as in the early stages in the biosynthesis of histidine (Ames et al., 1961), was considered. Indeed radiolabeling studies using D-[1-^{14}C]ribose showed a 53:47 (i.e., ca. 1:1) incorporation of label into the aglycon and sugar portions of 2′-dCF, respectively, as revealed by analyzing the hydrolyzed nucleoside fragment (Table II). Interestingly, the label was greatly diminished (6%) in the adenine portion of *ara*-A, whereas 94% of the label appeared in the sugar moiety, indicating a relatively low level of involvement of D-ribose in the biosynthesis of the purine ring of *ara*-A as opposed to a profound involvement of D-ribose in the biosyntheis of the 1,3-diazepine ring of 2′-dCF. Thus, all indications are that D-ribose adds to an intact purine ring system for the biosynthesis of 2′-dCF.

In order to determine if the ^{14}C from the C-1 of D-[1-^{14}C]ribose was indeed at C-7, a ^{13}C labeling experiment was carried out by adding a substantial quantity (300 mg) of D-[1-^{13}C]ribose (90% atom enriched) to 100-mL cultures of *S. antibioticus*. The ^{13}C NMR spectrum (Figure 1c) of the purified, labeled product revealed enrichment at C-7 of the aglycon, as well as at C-1′ in the 2′-deoxy sugar portion of 2′-dCF. Futhermore, the ^{13}C enhancements of 2.71- and 2.82-fold over the natural abundances at C-7 and C-1′, respectively (Table I), are in near-perfect agreement with the ca. 1:1 ratios found in the ^{14}C labeling studies (Table II). Thus D-ribose provides, via donation of its C-1 atom, both the C-7 methylene group and the C-1′ of the sugar in 2′-dCF in a highly specific manner relative to the incorporation at the other atoms in the molecule.[6]

Another question concerns the origin of the 8-hydroxyl group of 2′-dCF. In one approach to the problem, [8-^{14}C]-inosine was added to a culture of *S. antibioticus* along with hadacidin (*N*-formylhydroxyaminoacetic acid), an antibiotic that is an inhibitor of adenylosuccinate synthetase (inosine 5′-phosphate–L-aspartate ligase, EC 6.3.4.4), the first of two enzymes that convert inosine monophosphate (IMP) to adenosine monophosphate (AMP) (Shiguera, 1967). A partial inhibition of labeled inosine incorporation into both 2′-dCF and *ara*-A, 9% and 5%, respectively, was observed with 5 mM hadacidin. By raising the hadacidin concentration to 10 mM, these values increased to 15% and 22%, respectively, for 2′-dCF and *ara*-A, whereas in a control experiment hadacidin was shown not to inhibit production of either 2′-dCF or *ara*-A in cultures of *S. antibioticus* at these levels. These results imply that inosine must be converted to adenosine before being converted to either 2′-dCF or *ara*-A. In a second, chemically direct approach to determine if inosine might be the direct precursor to 2′-dCF, a mixture of [6-^{18}O]inosine and [8-^{14}C]inosine was added to a culture of *S. antibioticus*, and the product 2′-dCF was examined by mass spectroscopy (MS) for ^{18}O content. In an experiment wherein 25% of 2′-dCF was shown to be derived from inosine added to the culture (based on the ^{14}C-labeled inosine), no ^{18}O-labeled product could be observed by mass spectrometry,[7] indicating that the O-6 atom

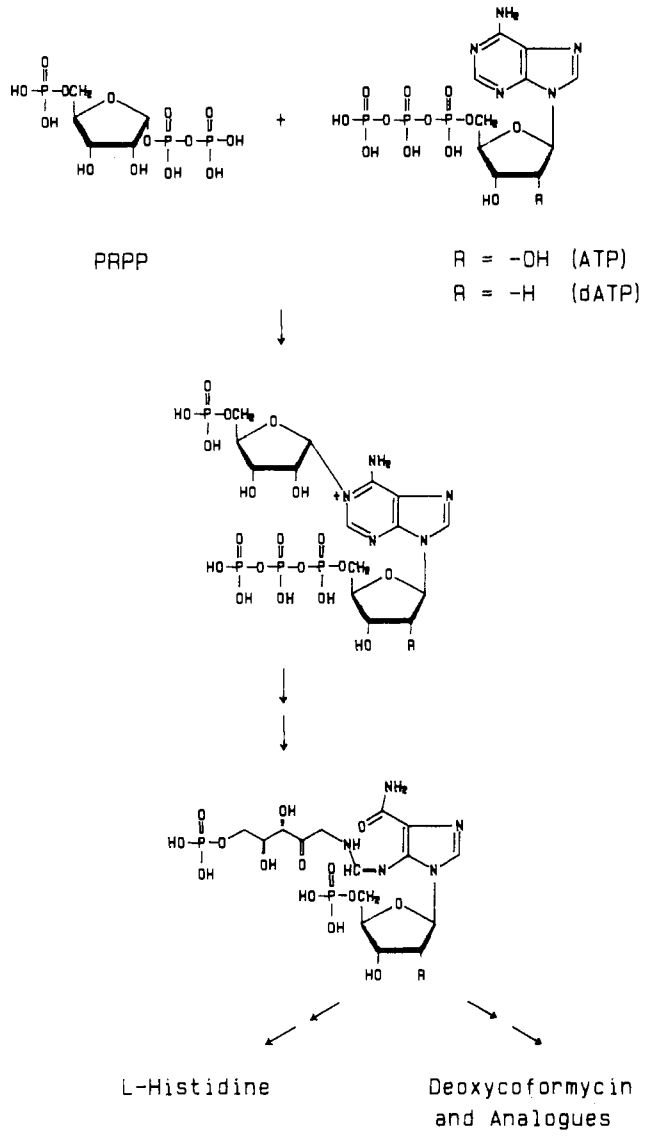
[4] For a partial assignment of the ^{13}C NMR spectrum of 2′-dCF, see Dion et al. (1977).

[5] H-2 in the ^{1}H NMR spectrum of 2′-dCF is considered firmly assigned on the basis of this slow ($t_{1/2} \sim 2$ weeks) exchange in neutral deuterium oxide. No reasonable mechanism can be postulated for a similar (H^+-mediated) exchange of the amidine H-5. Furthermore, this assignment is in agreement with that of Dion et al. (1977) wherein H-5 was observed as a doublet (split by N–*H*) in [$^2H_6$]dimethyl sulfoxide, upfield from the resonance of H-2 (s). For a theoretical discussion of these phenomena in purines, see Boerth and Harding (1985). For a discussion of similar assignments in a purine, see Schweizer et al. (1964).

[6] The possibility that ^{13}C from C-1 of D-[1-^{13}C]ribose is converted to formic acid (Trackman & Abeles, 1981) and subsequently incorporated via the C-1 pool into 2′-dCF is ruled out on the basis of the fact that C-2 and C-5 of 2′-dCF show no enrichment of ^{13}C label (Table I).

[7] A significant portion of ^{18}O-labeled 2′-dCF could be expected as [6-^{18}O]inosine was 66 atom %. With 25% derived from added, labeled inosine, the enrichment would be 0.25 × 66% = 16.5%.

Scheme I



of inosine does not provide the O-8 of 2′-dCF.

In conclusion, evidence from both the present and previous studies (Hanvey et al, 1984c) points to the possibility that adenosine (or one of its nucleotides) is the direct precursor for 2′-dCF. The process whereby the C-1 of D-ribose is incorporated into the 1,3-diazepine of 2′-dCF is likely related to that of the biosynthesis of histidine (Ames et al., 1961). It is envisioned that a process (Scheme I) which involves the initial ribosylation of adenosine triphosphate (ATP) by PRPP, followed by ring opening to the carbonyl sugar derivative via an Amadori-like rearrangement, could form a common branch point for the two processes, the biosynthesis of 2′-dCF and the biosynthesis of histidine.[8] Alternatively, an analogous process that would utilize 2′-deoxyadenosine triphosphate (dATP) could also be considered. The exact mechanism and determination of intermediate compounds are under study in these laboratories.

ACKNOWLEDGMENTS

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Registry No. 2′-dCF, 53910-25-1; D-ribose, 50-69-1; adenine, 73-24-5.

[8] The ca. 1:1 distribution of the ^{14}C into the algycon and sugar from D-[1-^{14}C]ribose would indicate that the labeled D-ribose is equilibrated into the "D-ribose pool" and, as PRPP, glycosylates adenine (available via adenosine nucleosidase) to give AMP. The latter, as ATP (or dATP), could then be N-1-ribosylated via PRPP to account for the dually labeled product 2′-dCF. This process is supported by earlier studies (Hanvey et al., 1984c) whereby [U-^{14}C]adenine is shown to be incorporated into 2′-dCF with an efficiency of 7%, compared with 1.6% for [U-^{14}C]-adenosine.

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NMR Studies on an Oligodeoxynucleotide Containing 2-Aminopurine opposite Adenine†

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ABSTRACT: A heteroduplex containing the mismatch 2-aminopurine (AP)–adenine has been synthesized and studied by proton NMR. The mismatch was incorporated into the seuquence d[CGG(AP)GGC]·d-(GCCACCG). One-dimensional nuclear Overhauser effect measurements in H_2O and two-dimensional nuclear Overhauser effect spectra in D_2O show AP·A base pairs in a wobble structure in which both bases are in the anti conformation. The adenine is stacked well in the helix, but the helix twist between the adenine and neighboring cytosine in the 3′ direction is unusually small. As a result, the aminopurine on the opposite strand is somewhat pushed out of the helix. From the measurements of the imino proton line widths, the two adjacent G·C base pairs are not found to be significantly destabilized by the presence of the purine–purine wobble pair.

2-Aminopurine (AP),[1] a base analogue of adenine, is a strong mutagen causing predominantly A·T ↔ G·C transitions in vivo (Ronen, 1979). AP preferentially forms base pairs with T during DNA synthesis (Bessman et al., 1974; Clayton et al., 1979; Watanabe & Goodman, 1981). The mutagenicity of AP occurs because it can form base mispairs with C (Freese, 1959; Rudner, 1960) when present either as a template base (A·T → G·C pathway) (Watanabe & Goodman, 1981) or as a substrate for DNA polymerase (G·C → A·T pathway) (Mhaskar & Goodman, 1984). We have recently demonstrated by proton NMR that AP and T pair with normal Watson–Crick geometry (Sowers et al., 1986); the preferred base pairing of AP and C involves protonation of the mispair also having normal Watson–Crick geometry (Sowers et al., 1986). In contrast with previously accepted models that invoke the presence of disfavored tautomeric forms to account for AP·C mispairs (Freese, 1959; Topal & Fresco, 1976,), we find that a second hydrogen bond is formed by acquisition of a proton from solvent water (Sowers et al., 1986).

We decided to investigate the possibility of additional hydrogen bonding interactions of AP with A because of the presence of higher T_m values observed for the case of oligomers containing an AP·A mispair in place of an AP·C mispair at the same location (Eritja et al., 1986). We were further interested in the AP·A interaction as we have previously shown that, to a small extent, DNA polymerase incorporates AP opposite template A residues (Mhaskar & Goodman, 1984). Also, weak transversion mutations have been reported to result following mutagenesis with AP in vivo (Persing et al., 1981).

If, as seems likely, base pairing occurs between aminopurine and adenine, two possibilities exist (Figure 1). In both cases, a wobble-type pairing is involved, but this may be Watson–Crick or Hoosteen. Unlike the G·T wobble (Brown et al., 1985) in which the two hydrogen bonds are formed with imino protons, for AP·A both would be amino protons. The G·A base pair has been shown to adopt a Watson–Crick wobble structure (Kan et al., 1983; Patel et al., 1984), to give Hoogsteen pairing with the A in a syn conformation (Kennard, 1985) or to provoke looped-out structures depending upon the DNA sequence (Fazakerley et al., 1986).

In order to investigate the possible base pairing of AP with A, we have synthesized the heteroduplex structure

	1	2	3	4	5	6	7
5′	C	G	G	AP	G	G	C
3′	G	C	C	A	C	C	G
	14	13	12	11	10	9	8

containing the AP·A pair which has been studied by proton NMR in H_2O and D_2O. The predominant structure of the AP·A pair is wobble in which both bases are in the anti conformation.

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; WC, Watson–Crick; nWC, non-Watson–Crick; 2-D, two dimensional; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; AP, 2-aminopurine.

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