Pyrazolo[4,3-*d*]pyrimidine Nucleosides. 9. Studies on the Isomeric *N*-Methylformycins†

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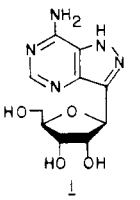
Abstract: The syntheses of 4-methylformycin (**7**) and 6-methylformycin (**8**) are described. Structural assignments of **7** and **8** were based on UV, ^{1}H NMR, and ^{13}C NMR data. *N*-7-Methylformycin (**9**) was resynthesized by an alternate route and comparisons of the physicochemical properties of all five of the mono-*N*-methylformycins are presented. 6-Methylformycin (**8**) was found to be unstable in aqueous solution yielding three products, formycin B (**2**), *N*-7-methylformycin (**9**), and 6-methylformycin B (**10**). 6-Methylformycin B (**10**), 4-methylformycin B (**11**), and 1-methylformycin B (**12**) were prepared by a reaction of nitrosyl chloride with **8**, **7**, and 1-methylformycin (**3**), respectively.

Formycin (**1**) was initially isolated[1] from the rice mold, *Norcardia interforma*, and identified[2,3] as a C-nucleoside isomeric with the natural nucleic acid constituent adenosine. Formycin has demonstrated antitumor, antibacterial, antifungal, and antiviral activity.[4] Formycin acts as a substrate for many adenosine specific enzymes including adenosine kinase.[5] Additionally, formycin is incorporated into RNA and DNA[6] and effectively replaces adenosine at the bindnig site of t-RNA to ribosomes.[7] Formycin 5′-triphosphate is a competitive in vitro inhibitor of the nucleoside triphosphate reductase of *Lactobacillus leichmanni* and an effective substrate when the positive effector dGTP is present.[8] Unfortunately, formycin is also a good substrate for the adenosine catabolic enzyme, adenosine deaminase,[9] and, thus, the activity of formycin is diminished by its ready conversion to the nearly inactive formycin B (**2**).



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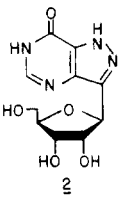

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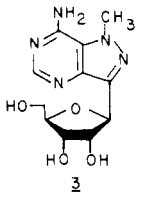
In order to increase the effectiveness of formycin as a chemotherapeutic agent, methods of limiting or eliminating the facile enzymatic deamination of formycin have been sought. A study[10] which suggested that adenosine derivatives in the syn rotameric conformation are not substrates for adenosine deaminase prompted the synthesis of nucleosides designed to restrict rotation around the glycosyl bond. A prior report from our laboratory[11] described the isolation of 1-methyl- (**3**) and 2-methylformycin (**4**) from the reaction of formycin under



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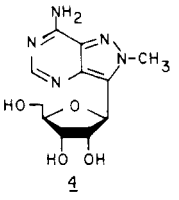

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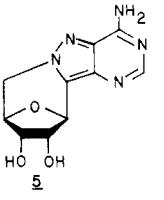
basic conditions with methyl iodide. Unlike the methylation of adenosine, N-methylation of formycin does not result in quaternization of the ring system or require the existence of the imino form. Observations from CPK molecular models indicated that **4** would most likely exist with a predominant syn relationship between sugar and base, whereas **3** should have unrestricted rotation about the glycosidic bond. Therefore, **3** should more clearly resemble formycin with respect to its rotameric preference. X-ray analysis[12] of 2-methylformycin (**4**) confirmed the high degree of syn character, at least in the solid state. Thus, it was expected that **4** would be somewhat resistant toward enzymic deamination and show enhanced chemotherapeutic activity when compared to **3** or **1**. However, **4** exhibited essentially the same activity (T/C = 127) when compared to **3** (T/C = 122) as an antileukemic agent.

Two recent studies[13,14] have used 2,5′-anhydroformycin (**5**) and 4,5′-anhydroformycin (**6**) as models for the anti and syn rotamers of formycin, respectively. Calf intestinal mucosa adenosine deaminase and Takadiastase adenosine deaminase accepted **5** (the anti model) as a substrate while **6** (the syn



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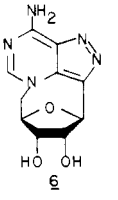

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model) was not deaminated. It was also noted that **5** was less susceptible to deamination than formycin.

In direct contrast to these findings, 2-methylformycin (**4**) (a syn nucleoside) is deaminated by calf intestinal and human erythrocytic adenosine deaminase while 1-methylformycin (**3**) is resistant to deamination[15,16] To examine this paradox, we initiated a study to determine the biological effects, if any, attendant upon the changes arising from specific alkylation of the base moiety of formycin in contrast to the syn,anti conformational relationship.

In order to accomplish this study, we needed 4-methylformycin (**7**) since CPK models indicated that this derivative could not easily assume a syn conformation. Thus, **7** would be comparable to **6** with respect to the effects of alkylation of the heterocycle but would exist predominantly in the opposite rotameric conformation. A similar situation exists between **4** and **5**.

In addition to the previously discussed basic methylation of formycin,[11] two other methylation studies have been published.[13,17] 1-Methylformycin was the only *N*-methyl isomer isolated, in excellent yield, from the treatment of formycin with dimethylformamide dimethyl acetal.[13] Reaction of formycin with diazomethane gave both 1-methyl- (**3**) and 2-methylformycin (**4**) in addition to several products methylated in the sugar moiety.[17] However, two early studies[18] of the 7-amino-

† The structures of the methylated formycins as shown in the text were drawn for graphic convenience and are not intended to imply a specific rotameric conformation.

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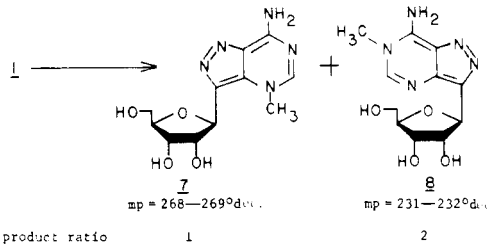
pyrazolo[4,3-*d*]pyrimidine system concluded that the nitrogen at position 4 was most basic, and on this basis we expected to obtain **7** under neutral methylation conditions.

We should now like to report the first synthesis of 4-methylformycin (**7**) and 6-methylformycin (**8**) as well as a comparison of the physicochemical properties for all five of the isomeric *N*-methylformycins. Formycin, in dimethylformamide solution, was treated with excess methyl iodide. Thin layer chromatographic analysis showed that two major UV-absorbing products had been formed. These compounds were separated by fractional crystallization. The major product (mp 231–232 °C dec) was obtained in 50% yield and the minor product (mp 268–269 °C dec) in 17% yield based on the assumption that they were monomethylformycin derivatives. The ratio of the products as determined by UV analysis[19] of the reaction mixture was found to be 2:1 (low melting:high melting).

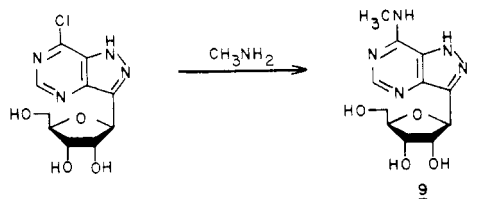
Scheme I



^{1}H NMR analysis confirmed that both products were monomethylated derivatives of formycin. The major isomer had a methyl absorption peak at δ 3.47, and the minor isomer at δ 4.04. These chemical shifts suggested that the products were neither 1-methyl- (**3**) (δ_{NCH_3} 4.28) nor 2-methylformycin (**4**) (δ_{NCH_3} 4.18), and chromatographic comparisons (see Table V) confirmed this fact. In the adenines, methylation of the exocyclic amine (i.e., 6-methylaminopurine) gives a methyl signal, in the ^{1}H NMR, which is upfield from the methyl signals observed for ring nitrogen methylated derivatives.[20] This upfield signal is additionally coupled to the remaining proton on the exocyclic amine. The methyl signal for the major product obtained in our study appeared as a singlet. However, the upfield chemical shift of this methyl signal suggested that this isomer might be the *N*-7-methyl derivative **9**. A previous report[21] on the synthesis of **9** did not include ^{1}H NMR data, which prompted the resynthesis of **9** for this study.

This resynthesis of *N*-7-methylformycin (**9**) was accomplished via a more facile and convenient route involving a nucleophilic displacement of the chloro group in 7-chloro-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine[21,22] with ethanolic methylamine. The nucleoside **9** showed a doublet for the methyl absorption peak at δ 3.07 which did not correspond to the chemical shift observed for the methyl group of the major isomer obtained in the methylation reaction. The spectral data and chromatographic comparisons (Table V) eliminated *N*-7-methylformycin (**9**) as the major isomer.

Scheme II



Selected ^{1}H NMR spectral data for the five isomeric methylated formycins are compiled in Table I. It is of some interest that the signal for the anomeric ($H_{1'}$) proton of the two isomers wherein rotation about the glycosidic bond (C3-C1′) is restricted (**4** and **7**) are observed approximately 0.2 ppm downfield from the anomeric signal observed for the other three methyl isomers.

Table I. Selected ^{1}H NMR Spectral Data for *N*-Methylformycin[a]

	NCH_3	H_5	$H_{1'}$
formycin (**1**)		8.21	5.03
1-methylformycin (**3**)	4.28	8.12	4.96
2-methylformycin (**4**)	4.18	8.09	5.20
4-methylformycin (**7**)	4.04	8.21	5.20
6-methylformycin (**8**)	3.47	7.88	4.90
N-7-methylformycin (**9**)	3.07	8.20	4.98

[a] Me_2SO-d_6 was used as a solvent and chemical shifts are in parts per million from an internal standard (DSS).

A comparison of the UV spectral characteristics of the five isomers (Table II) with the published data for the cyclonucleosides **5** and **6** allowed us to make a structural assignment for the minor isomer obtained in the methylation reaction as 4-methylformycin (**7**). This isomer is the only *N*-methyl isomer to show a definite long-wavelength maximum in the 310–315-nm region of the pH 11 spectrum and is comparable in this regard to 4,5′-anhydroformycin (**6**). Thus, by default, the major isomer obtained in the methylation reaction was assumed to be 6-methylformycin (**8**).

These structural assignments were also confirmed by ^{13}C NMR analysis. Selected ^{13}C NMR data for all five of the monomethyl isomers are compiled in Table III. The coupled ^{13}C NMR spectrum of the major and minor isomers showed a doublet for the C5, each half being further split (quartet) by three-bond coupling[23] to methyl protons. This confirmed that the *N*-methyl groups for both isomers were residing in the pyrimidine ring. The C7 of the minor isomer exhibited only a doublet from a three-bond coupling to the C5 proton which corroborated our assignment of this isomer as 4-methylformycin (**7**). The C7 of the major isomer, although partly obscured, showed a multiplet as a result of three-bond coupling to both the C5 proton and the methyl protons. This observation confirmed our initial structural assignment of 6-methylformycin (**8**) for this isomer.

The pK_as of **7** and **8** were determined spectrophotometrically.[24] 4-Methylformycin has a basic pK of 6.4 while 6-methylformycin has a basic pK of 6.8 and an acidic pK of 10.3. A comparison of these values with the published[17] pK values for 1-methyl- (**3**) (4.02) and 2-methylformycin (**4**) (4.87) indicates that methylation in the pyrimidine ring significantly increases the basicity of formycin (**1**) (pK = 4,4, 9.7),[4b] whereas methylation in the pyrazole ring does not greatly change the basicity of **1**. It is of some interest that 6-methylformycin exhibited an acidic pK which would suggest a significant contribution from or ready tautomerism to the imino form. We were unable to observe an acidic pK for the other ring-methylated isomers. This same pattern has been observed in certain methylated adenines[25] and methylated 4-aminopyrazolo[3,4-*d*]pyrimidines[26] in that only isomers wherein the methyl group resides on a nitrogen ortho to the exocyclic amino group (i.e., 1-methyladenine) displayed acidic pK_as. The demonstration of an acidic pK for **8** would tend to provide additional proof for our structural assignment. Initial attempts to obtain pKs for **8** were unsuccessful since only variable data was recorded. We then found that **8** was unstable in hot aqueous solution, which required us to prepare solutions of **8** for pK determinations by dissolving **8** in a large volume of water to avoid all but minimum warming of the solution followed by a rapid acquisition of data thereafter. The pK data from samples of **8** prepared in this manner were reliably consistent.

Mass spectral abundance for the six most predominant ions from fragmentation of the isomeric *N*-methylformycins are compiled in Table IV. Although some differences in the frag-

Table II. Ultraviolet Absorption Spectra

compd	pH 1 spectrum, λ_{max}, nm ($\epsilon \times 10^{-3}$)					pH 11 spectrum, λ_{max}, nm ($\epsilon \times 10^{-3}$)				
1[a]		295			232.5		302			234
(p*K* = 4.4, 9.7)		(10.1)			(8.28)		(7.9)			(17.9)
3[b]		302			236	314 sh	301	293 sh		232
(p*K* = 4.0)[c]		(6.32)			(7.03)	(3.93)	(6.46)	(6.19)		(6.51)
4[b]		305		270	231	317 sh	305	295 sh		237
(p*K* = 4.8)[c]		(11.2)		(5.9)	(6.05)	(10.9)	(8.45)	(12.9)	(11.2)	(5.61)
5[d]		304			231		305			234 sh
		(11.2)			(13.7)		(12.5)			(9.7)
6[d]		304			245	317			272	253
		(14.3)			(7.93)	(7.99)			(9.78)	(9.86)
7	325 sh	307	300		242	314			270	246
(p*K* = 6.4)	(4.08)	(10.8)	(11.0)		(5.91)	(6.47)			(9.7)	(10.5)
8			293		229		303 sh	288	278.5 sh	238.5
(p*K* = 6.8, 10.3)			(7.78)		(14.5)		(7.03)	(11.1)	(10.3)	(15.7)
9	324 sh	307	297.5		241.4 sh	317 sh	300.5			240
	(5.69)	(15.9)	(17.1)		(10.6)	(7.63)	(12.9)			(14.5)

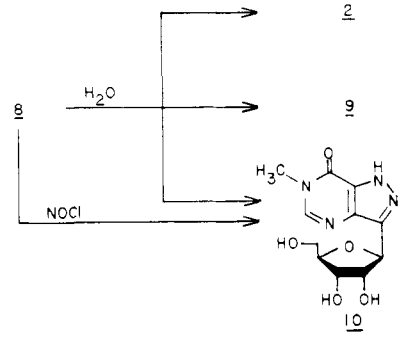
[a] Data from ref 4b. [b] Data from ref 11. [c] Data from ref 17. [d] Data from ref 14.

Table III. ^{13}C NMR Data[a]

	NCH_3	C_3	C_{3a}	C_5	C_7	C_{7a}	C_1'
1[b]		143.2	138.3	151.4	151.6	123.4	78.2
3[b]	39.0	142.1	140.2	150.1	151.3	122.1	78.2
		(−1.1)	(+1.9)	(−1.3)	(−0.3)	(−1.3)	
4	39.0	133.4	136.1	152.1	155.8	129.9	75.9
		(−9.8)	(−2.2)	(+0.7)	(+4.2)	(+6.5)	(−2.3)
7	39.2	136.9	126.7	146.0	155.7	130.3	78.6
		(−6.3)	(−11.6)	(−5.4)	(+4.1)	(+6.9)	(+0.4)
8	34.3	141.3	130.5	146.1	150.2	130.1	77.2
		(−1.9)	(−7.8)	(−5.3)	(−1.4)	(+6.7)	(−1.0)
9	27.0	143.6	137.4	151.3	151.3	123.3	77.8
		(+0.4)	(−0.9)	(−0.1)	(−0.3)	(−0.1)	(−0.4)

[a] Chemical shifts are in parts per million with respect to Me_4Si ($\Delta\delta$ from formycin). Spectra were recorded in Me_2SO-d_6 with *p*-dioxane added as an internal standard. Chemical shifts were assigned from the coupled spectra and by analogy to the spectra of methylated adenines. [b] Data from ref 23.

Scheme III



mentation patterns were observed, these differences were not sufficient to delineate structural features of a particular isomer.

Reverse phase LC analysis of aqueous solutions of **8** always showed the presence of three additional products. Since samples of **8** for LC analysis were prepared by dissolution in hot water, we initiated a study designed to establish the structure of the products formed from **8** in water. Boiling water (4–6 h) converted **8** into the three products as shown in Scheme III. The three compounds were separated and isolated by column chromatography. Two of the products were identified as *N*-7-methylformycin (**9**) and formycin B (**2**) by spectroscopic (UV) and chromatographic (TLC) comparison with authentic samples of **9** and **2**. The third product has a UV λ_{max} (pH 1) of 272 nm and λ_{max} (pH 11) of 280 nm. A mass spectra of this third product showed M^+ *m/e* 282, indicating that it was a monomethylated formycin B derivative. The structure 6-methylformycin B (**10**) was assigned to this product from

Table IV. Mass Spectral Data for the Isomeric *N*-Methylformycins

	3	**4**	**7**	**8**	**9**
M^+ (rel	281	281	281	281	281
abundance, %)	(14)	(25)	(6)	(17)	(20)
m/e of the six	193	192	192	192	192
most abundant	(25)	(100)	(73)	(80)	(87)
ions	192	190	178	179	179
(rel	(100)	(31)	(100)	(47)	(46)
abundance, %)	179	179	163	178	178
	(51)	(70)	(22)	(100)	(100)
	178	178	44	57	163
	(100)	(100)	(52)	(18)	(24)
	162	162	42	44	57
	(43)	(51)	(44)	(21)	(33)
	42	42	36	42	42
	(26)	(26)	(38)	(100)	(22)

mechanistic considerations. However, a comparison of the UV spectra of the assumed 6-methylformycin B with the spectra of 3,6-dimethylpyrazolo[4,3-*d*]pyrimidin-7-one[27] failed to confirm this assignment since 3,6-dimethylpyrazolo[4,3-*d*]-pyrimidin-7-one does not exhibit an acid–base shift in its UV spectra [λ_{max} (pH 1) 281.5, λ_{max} (pH 11) 281.5 nm]. This prompted us to prepare 6-methylformycin B (**10**) by an alternate route so that a direct comparison with the hydrolysis product could be accomplished.

6-Methylformycin B (**10**) was obtained from a deamination of **8** with nitrosyl chloride by the method described[28] for the preparation of isoinosine. Although the UV maxima were not precisely coincidental, the nucleoside prepared by this method

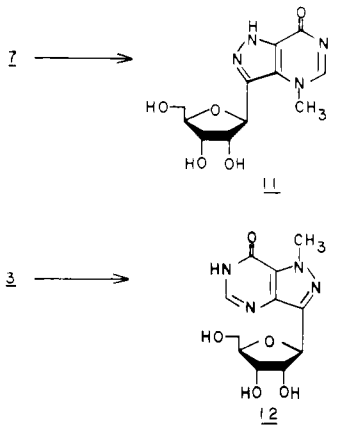
Table V. Comparative TLC[a] Data for the *N*-Methyl Derivatives of **1** and **2**

	solvent A[b]	solvent B[b]
3	$R_1 = 0.91$	$R_1 = 0.00$
4	$R_1 = 0.89$	$R_1 = 1.36$
7	$R_1 = 0.73$	$R_1 = 0.17$
8	$R_1 = 0.80$	$R_1 = 0.33$
9	$R_1 = 0.81$	$R_1 = 1.47$
10	$R_2 = 0.82$	$R_2 = 1.20$
11	$R_2 = 0.85$	$R_2 = 0.42$
12	$R_2 = 0.94$	$R_2 = 1.38$

[a] Chromatograms were obtained from glass plates coated (0.25 mm) with SilicAR-7GF. [b] Solvent A, H_2O; solvent B, chloroform–methanol (3/1 v/v).

was chromatographically identical with the third product obtained from the water hydrolysis of **8** and confirmed our initial assignment of structure **10** to that product.

4-Methylformycin (**7**) and 1-methylformycin (**3**) were similarly deaminated to give 4-methylformycin B (**11**) and 1-methylformycin B (**12**), respectively. 2-Methylformycin B



has previously been prepared by chemical[11] and enzymatic[17] deamination of **4**. The syntheses of **10, 11,** and **12** thus complete the set of monomeric *N*-methyl derivatives of formycin B.

As was observed with the methylformycins, the ^1H NMR methyl signal for **10** (δ 3.52) is upfield from the methyl signals for **11** (δ 3.93), **12** (δ 4.14), and 2-methylformycin B (δ 4.11).[29] Similarly, the H_1' signals of the isomers wherein rotation about the glycosidic (C_3-C_1') bond is restricted, **11** (δ 5.03) and 2-methylformycin (δ 5.12),[29] are downfield from those of the isomers with unrestricted rotation, **10** (δ 4.93) and **12** (δ 4.87).

Comparative TLC mobilities of the methylated formycin and formycin B isomers are presented in Table V.

One of the primary reasons for this investigation was to prepare a formycin derivative which would not undergo an enzymic deamination as readily as formycin. The five isomeric mono-*N*-methylformycins (**3, 4, 7, 8,** and **9**) have now been studied[30] in regard to their reactivity with human erythrocytic adenosine deaminase (ADA), their ability to form intraerythrocytic nucleotides, and their cytotoxicity to L-1210 cells. The nucleosides **3, 4,** and **9** showed significant cytotoxicity to L-1210 cells in culture and are the only isomers incorporated into erythrocytic nucleotide pools. 6-Methylformycin (**8**) displayed very weak cytotoxicity to L-1210, but the fact that this cytotoxicity may be due to a rearrangement of **8** into **9** under the test conditions has not been ruled out.

Following our communications[16,31] on the preliminary results of these studies, another report[32] on the enzymatic deamination of certain 1- and 2-alkylformycins has appeared which showed[32] that both 1-methyl- (**3**) and 2-methylformycin (**4**) were deaminated by Takadiastase ADA and calf intestinal mucosa ADA. The observed rate of deamination of **3** was found[32] to be approximately equal to the observed rate of deamination of formycin (**1**). By contrast, the rate of deamination of **4**, by both enzymes, was shown[32] to be very much slower than the rate of deamination of **1** and **3**. In fact, no detectable deamination of 2-methylformycin was observed at enzyme concentrations which resulted in complete deamination of **1** and **3**.[32] This most recent study[32] also reported that neither 1-isopropyl- nor 2-isopropylformycin was a substrate for either deaminase. The results concerning the deamination of the isopropyl derivatives are not relevant to the present discussion as no information regarding their ability to act as substrates for a kinase or their activity against L-1210 was reported.

To discuss the effects on biological activity resulting from alkylation of formycin, two factors in addition to the rotational conformation about the glycosidic (C3-C1′) bond must also be considered: (1) electronic changes resulting from alkylation of the base and (2) steric inhibition to enzyme binding caused by alkylation on or near a binding site in the substrate. It is of importance that structural variations which not only inhibit deamination but also result in loss of biological activity are of less significance from a therapeutic standpoint.

With the exception of methylation at the 6 position, methylation of the ring nitrogens of **1** eliminates tautomerism and results in molecules with a more fixed bond character. Methylation in the pyrimidine ring, as in **7** and **8**, significantly increases the basicity of the system. Neither **7** nor **8** is a substrate for ADA and significantly neither is incorporated into nucleotide pools. Methylation in the pyrazole ring, as in **3** and **4**, causes a comparatively much smaller increase in the basicity of the system. Both **3** and **4** are phosphorylated but only **4** is a substrate for the deaminase enzyme. It is not possible to attribute the deamination study results conclusively to electronic effects attendant upon alkylation since possible steric inhibition to enzyme binding at N_1 and N_6 must be considered for isomers **3** and **8**, respectively. However, it should be noted that **8** is not a substrate for ADA or a kinase, whereas methylation at the 1 position only inhibits deamination.

Isomers **2**, **8**, and **9** have unrestricted rotation about the C3-C1′ bond. A methyl group on the exocyclic amine, as in nucleoside **9**, does not present the same degree of steric inhibition for enzyme binding as discussed previously for **3** and **8**. In fact, nucleoside **9** is the only methyl isomer with unrestricted rotation about the glycosidic bond which is deaminated, albeit at a much reduced rate when compared to formycin. Considering the two isomers with restricted rotation about the C3-C1′ bond, the more basic, pyrimidine methylated isomer **7** is neither deaminated nor phosphorylated, and the less basic, pyrazole methylated **4** is both a substrate for ADA and a kinase.

This finding that the syn nucleoside **4** is deaminated while **3** is not deaminated parallels another study[15] using calf intestinal ADA, one of the enzymes used in studies[13,14] involving the cyclonucleosides **5** and **6**. For consistency with the syn–anti postulates from the studies of **5** and **6**, the deamination of **4** has been explained[17] by assuming that **4** can adopt an anti conformation on interaction with ADA. Examination of CPK models show that an anti conformation for **4** is possible, although sterically unfavorable, and that the anti conformation for **7** is highly favored. Thus, the fact that **7** is not deaminated raises serious doubts about the syn–anti postulates for deamination based solely on the studies of the anhydroformycin models.

The findings from this deamination study of these methylated formycins do not establish that formycins with a syn relationship are deaminated and anti nucleosides are resistent to deamination. Further, this study does not conclusively invalidate the assumptions advanced from the deamination studies of **5** and **6** since deamination of these anhydronucle-

osides by human erythrocytic ADA has not been investigated. However, it appears from the findings of this study[30] that the electronic effects attendant upon alkylation of the 4 position of the base to produce the syn model **5** are possibly more significant than the conformation about the glycosidic bond in determining substrate acceptability by ADA.

A further point of interest was the contrast between the results from the present study of *N*-7-methylformycin (**9**) and those of an earlier study. Kunimoto et al.[33] reported that **9** was inactive against Yoshida sarcoma cells in vitro, which led to the speculation[4a] that perhaps di- and triphosphates of **9** were not biosynthesized. Our studies[30] have shown that **9** is indeed converted into a triphosphate in human erythrocytes, although at a slower rate than that observed for the formation of formycin 5′-triphosphate. Additionally, **9** had a relative ID_{50} against L-1210 cell cultures of only twice that observed for formycin.[30]

Experimental Section

UV spectra were recorded on a Beckman Acta C-III spectrophotometer. ^1H NMR spectra were obtained for both Me_2SO-d_6 and Me_2SO-d_6/D_2O solutions of the compounds and were recorded on a Varian EM-390 instrument. ^{13}C NMR spectra were obtained by the techniques described in ref 23. Electron impact, low-resolution mass spectra were obtained by direct inlet on underivatized compounds and were recorded on a Varian MAT 1125 instrument.

Elemental analysis were obtained from Heterocyclic Chemical Corp., Harrisonville, Mo., or M-H-W Laboratories, Phoenix, Ariz. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected.

TLC was performed on glass plates coated (0.25 mm) with SilicAR-7GF. SilicAR-CC-7 (Mallinckrodt) was used as the stationary phase in normal column chromatography. Dry column chromatography was performed on J. T. Baker no. 5-3405 silica gel, to which 0.5% of a phosphor (Du Pont no. 609) had been added, packed in Nylon columns of required diameter and sealed at one end. The sealed end was perforated and the solvent and products were allowed to elute from the column. The progress of the UV-absorbing components were monitored with a short-wave (254 nm) UV Mineralight. A 4 × 300 mm μ-Bondapak C_{18} (Waters) column was used for LC analysis with water as the eluting solvent.

The pK_a data were obtained by the spectroscopic method described by Albert and Serjeant.[24]

Unless otherwise specified, evaporations were conducted on a rotary evaporator using a hot water both as a heat source and under aspirator vacuum for low-boiling (bp ≤ ethanol) solvents or high vacuum for higher boiling solvents.

4-Methylformycin (7) and 6-Methylformycin (8). Formycin (**1**, 2 g) was dissolved in dry dimethylformamide (60 mL), methyl iodide (2 mL) was added, and the mixture was then stirred at room temperature for 25.5 h. The reaction mixture was evaporated in vacuo on a steam bath. The residual oil was dissolved in ethanol (50 mL), and the solution again evaporated in vacuo. The residue was triturated with hot diethyl ether (3 × 50 mL), the diethyl ether was discarded, and the residue was dissolved in water (30 mL). The pH of the resulting solution was adjusted to approximately 9.5 by the addition of 1 N NH_4OH. A white solid separated after stirring at room temperature for 0.5 h, and this was followed by refrigeration for 1.5 h. The solid was collected by filtration, washed with cold water (10 mL), and air dried to yield 1.2 g (50%) of **8**, mp 224–227 °C dec. Pure **8** was obtained by two recrystallizations from water, 650 mg, mp 231–232 °C dec. Anal. ($C_{11}H_{15}N_5O_4 \cdot 2H_2O$) C, H, N. Hydration of **8** was verified by ^1H NMR.

The aqueous solution (filtrate and washings), after removal of **8**, was evaporated to dryness in vacuo on a steam bath. The residue was redissolved in water (25 mL), and Dowex 1X8 (^-OH) (15 g) was added. The mixture was stirred until a negative halogen test ($AgNO_3$) was obtained. The resin was removed by filtration and washed with water (100 mL). The water solutions were combined and evaporated to dryness in vacuo on a steam bath. The solid residue was stirred with ethanol (10 mL), and the mixture allowed to stand for 14 h to give **7** (250 mg, 12%). An additional quantity (120 mg) of **7** was obtained by extraction of the Dowex 1X8 (^-OH) resin with a water–concentrated NH_4OH mixture (4:1 v:v) (4 × 50 mL) followed by evaporation of the extracts and stirring the resulting residue with ethanol (50 mL). The total yield of **7** was 370 mg (17.5%). Pure **7** was obtained by recrystallization from aqueous ethanol and drying in vacuo at 110 °C, 200 mg, mp 268–269 °C dec. Anal. ($C_{11}H_{15}N_5O_4$) C, H, N.

Spectroscopic Analysis of the Product Ratio from the Methylation of Formycin. Formycin (1 g) was methylated as described above. After removal of the solvent and the addition of 1 N NH_4OH, the mixture was evaporated to dryness in vacuo. The resulting residue was coevaporated with ethanol (3 × 25 mL), triturated with chloroform (2 × 50 mL), and then dissolved in aqueous methanol. An aliquot was applied to a SilicAR-7GF TLC plate, and the chromotogram developed with methanol. The band containing a mixture of **7** and **8** was eluted from the TLC plate with a methanol/water mixture (3:2 v:v). A UV spectrum of the mixture was obtained in pH 11 buffer and analyzed by solving the simultaneous equations[19]

$$A_{m\lambda_1} = (a_{7\lambda_1}C_7 + a_{8\lambda_1}C_8)b$$

$$A_{m\lambda_2} = (a_{7\lambda_2}C_7 + a_{8\lambda_2}C_8)b$$

to obtain

$$C_7 = \frac{a_{8\lambda_2}A_{m\lambda_2} - a_{8\lambda_1}A_{m\lambda_2}}{b(a_{7\lambda_1}a_{8\lambda_2} - a_{8\lambda_1}a_{7\lambda_2})}$$

where A_m is the observed absorption of the mixture of **7** and **8** at the chosen wavelengths λ_1 and λ_2, a_7 and a_8 are the absorptivities of **7** and **8** at the chosen wavelengths, and b is the cell path length in cm; 314 nm where a_7 is at a maximum and $a_7 > a_8$ and 288 nm where a_8 is at a maximum and $a_8 > a_7$ were chosen as λ_1 and λ_2, respectively.[19] The concentrations (C) of **8** and **7** were calculated and C_8/C_7 was found to be 2.24.

Water Hydrolysis of 8. An aqueous solution of **8** (100 mg/10 mL) was heated at reflux for 24 h. The course of the reaction was monitored by TLC using a chloroform/methanol mixture (9:2 v:v) as the developing solvent. At the end of 6-h reflux only a trace of unreacted **8** remained, and three new products had been formed as determined by TLC. No further change was noted at the end of 24-h reflux. The reaction solution was evaporated to dryness in vacuo, and the residue was dissolved in ethanol (20 mL). Silica gel (1 g) was added to the solution, and the mixture evaporated to dryness in vacuo. The dry powder was placed on a column (2.5 × 25 cm) of silica gel which had been equilibrated with 5% methanol in chloroform. The column was developed with 5% methanol in chloroform (150 mL), then 10% methanol in chloroform, collecting 10–15-mL fractions. Fractions containing a single product as determined by TLC were evaporated under reduced pressure. The first product eluted from the column (UV λ_{max} (pH 1) 272, λ_{max} (pH 11) 280 nm; MS M^+ *m/e* 282) was identified as 6-methylformycin B (**10**) by TLC comparison in three solvent systems to **10** prepared by the nitrosyl chloride reaction with **8.** The second product eluted from the column was identified as *N*-7-methylformycin (**9**) by its UV spectra and TLC comparison to authentic **9**. The third product was identified as formycin B (**2**) by its UV spectra, MS (M^+ *m/e* 268), and TLC comparison to authentic formycin B.

***N*-7-Methylformycin (9).** 3-(β-D-Ribofuranosyl)-7-chloropyrazolo[4,3-*d*]pyrimidine [22] (500 mg) was mixed with methylamine hydrochloride (130 mg), anhydrous sodium acetate (500 mg), and ethanol (50 mL). The mixture was stirred and heated at reflux for 2 h. The reaction mixture was evaporated to dryness in vacuo; the residue was coevaporated with water (2 × 10 mL) and then 2-propanol (3 × 20 mL). The residue was extracted with hot 2-propanol (2 × 50 mL) and a small amount of insoluble material was removed by filtration. The combined extracts were evaporated to dryness in vacuo and the residue was then dissolved in methanol (20 mL). Silica gel (J. T. Baker no. 5-3405) was added to the methanol solution, and the mixture was evaporated to dryness in vacuo. The dry powder was placed on a silica gel dry column (1 × 12 in.), the column eluted with an ethyl acetate–water–1-propanol mixture (4:2:1 v/v/v, upper phase) and the UV-absorbing material eluting from the column collected. The solution was then evaporated to dryness in vacuo, and the residue recrystallized twice from an ethanol–ethyl acetate mixture to give 200 mg (38%) of **9**, mp foams above 145 °C. The solid was dried in vacuo at 110 °C. Anal. ($C_{11}H_{15}N_5O_4 \cdot H_2O$) C, H, N. The hydration of **9** was verified by ^1H NMR.

6-Methylformycin B (10). 6-Methylformycin dihydrate (**8**, 150 mg) was mixed with pyridine (5 mL) and chloroform (5 mL). The mixture was stirred and heated in an oil bath at 60 °C (bath temperature). A solution of nitrosyl chloride in chloroform (5 mL, 1.4 mmol/mL) was

added over a 10-min period. The resulting mixture was stirred and heated at 70 ± 5 °C (bath temperature) for 2 h. The reaction mixture was evaporated in vacuo, and the resulting oily residue was coevaporated with ethanol (3 × 20 mL). The residue was dissolved in water (5 mL), and the pH of the solution was adjusted (pH >10) by the addition of a few drops of concentrated NH_4OH. The basic solution was applied to a column of Dowex 1X8 ($HCOO^-$) (50 mL), and the column washed with water (400 mL). The column was then eluted with 0.1 N HCOOH (400 mL) collecting 20-mL fractions. The fractions containing UV-absorbing material with λ_{max} 272 nm were pooled and evaporated to dryness in vacuo. The residue was coevaporated with ethanol (4 × 20 mL) and then recrystallized from ethanol to obtain 60 mg (45%) of **10**, mp 201–203 °C. The compound was dried at 110 °C in vacuo. Anal. ($C_{11}H_{14}O_5N_4$) C, H, N. UV: λ_{max} (nm) ($\epsilon \times 10^{-3}$) pH 1, 275 (9.0); pH 11, 281.5 (6.2); 230 (23.6). ^1H NMR: δ 3.52 (s, NCH_3), 4.93 (d, H_1'), 8.16 (s, H_5).

4-Methylformycin B (11). 4-Methylformycin (**7**, 150 mg) was mixed with pyridine (5 mL) and chloroform (5 mL). The mixture was stirred and heated in an oil bath at 60 °C. A solution of nitrosyl chloride in chloroform (5 mL, 1.4 mmol/mL) was added over an interval of 5 min. The mixture was stirred and heated at 65–70 °C (bath temperature) for 2 h. The mixture was then evaporated in vacuo, and the oily residue was coevaporated with ethanol (3 × 25 mL). The residue was dissolved in water (5 mL), and the pH of the solution adjusted (pH >10) by the addition of a few drops of concentrated NH_4OH. The solution was then applied to a column of Dowex 1X8 ($HCOO^-$) (50 mL). The column was washed first with water (400 mL) and then eluted with 0.1 N HCOOH collecting 20-mL fractions. The fractions containing UV-absorbing material with λ_{max} 285 nm were pooled and evaporated to dryness in vacuo. The residue was coevaporated with ethanol (2 × 25 mL) and then recrystallized from ethanol to obtain 56 mg (37%) of **11**, mp 239–240 °C. The compound was dried in vacuo at 110 °C for analysis. Anal. ($C_{11}H_{14}O_5N_4$) C, H, N. UV: λ_{max} (nm) ($\epsilon \times 10^{-3}$) pH 1, 277.5 (10.3); pH 11, 302 (7.9), 263.5 sh (7.2), 237.5 (11.4). ^1H NMR: δ 3.93 (s, NCH_3), 5.03 (d, H_1'), 8.13 (s, H_5).

1-Methylformycin B (12). 1-Methylformycin (**3**, 300 mg) was mixed with chloroform (10 mL) and pyridine (10 mL). The mixture was stirred and heated in an oil bath at 60 °C (bath temperature). A solution of nitrosyl chloride in chloroform (10 mL, 1.4 mmol/mL) was added over a period of 10 min. The resulting mixture was stirred and heated at 65 °C (bath temperature) for 2 h. The reaction mixture was evaporated in vacuo and the residue coevaporated with ethanol (3 × 20 mL). The residue was dissolved in water (5 mL), and the pH of the solution adjusted (pH >10) by the addition of a few drops of concentrated NH_4OH. The solution turned a deep wine red in color once basic. The solution was applied to a column of Dowex 1X8 ($HCOO^-$) (50 mL). The column was washed with water (400 mL) and then eluted with 0.1 N HCOOH collecting 20-mL fractions. The UV-absorbing fractions had in addition to absorption at λ_{max} 282 nm a complex absorption pattern at long wavelength with two prominent maxima at 379 and 397 nm. The UV absorption at the longer wavelengths was greater in earlier fractions and later fractions where the recorded UV absorbance at 282 nm was equal to or greater than the recorded absorbance at 397 nm were pooled and evaporated. The residue was coevaporated with ethanol (3 × 50 mL). TLC in chloroform–methanol (3/1 v/v) showed one major component with trace impurities indicating that the component responsible for the long-wavelength absorption has a much higher extinction coefficient than the major component of the mixture. The solid residue was recrystallized from an ethanol–ethyl acetate mixture to yield 40 mg of pure **12**. The solid was dried at 110°C in vacuo, mp 189–192 °C dec. Anal. ($C_{11}H_{14}O_5N_4$) C, H, N. UV: λ_{max} (nm) ($\epsilon \times 10^{-3}$) pH 1, 280 broad (4.8), 226 (9.7); pH 11, 308 sh (3.0), 290 broad (5.7), 230 (2.1). ^1H NMR: δ 4.14 (s, NCH_3), 4.87 (d, H_1'), 7.83 (s, H_5).

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References and Notes

(1) Hori, M.; Ito, E.; Takita, T.; Koyama, G.; Takeuchi, T.; Umezawa, H. *J. Antibiot., Ser. A* **1964,** *17,* 96.
(2) Robins, R. K.; Townsend, L. B.; Cassidy, F. C.; Gerster, J. F.; Lewis, A. F.; Miller, R. L. *J. Heterocycl. Chem.* **1966,** *3,* 110.
(3) Koyama, G.; Maeda, K.; Umezawa, H.; Iitaka, Y. *Tetrahedron Lett.* **1966,** 597.
(4) (a) Suhadolnik, R. J. "Nucleoside Antibiotics"; Wiley-Interscience: New York, 1970. (b) Townsend, L. B. In "Handbook of Biochemistry and Molecular Biology", "Nucleic Acids", 3rd ed.; Fasman, D. G., Ed.; CRC Press: Cleveland, 1975; Vol. I, pp 271–401.
(5) Sawa, T.; Fukagawa, Y.; Shimauchi, Y.; Ito, K.; Hamada, M.; Takeuchi, T.; Umezawa, H. *J. Antibiot., Ser. A* **1965,** *18,* 259.
(6) Ward, D. C.; Cerami, A.; Reich, E.; Acs, G.; Altwerger, L. *J. Biol. Chem.* **1969,** *244,* 3243.
(7) Ikehara, M.; Murao, K.; Harada, F.; Nishimura, S. *Biochim. Biophys. Acta* **1968,** *174,* 696.
(8) Brinkley, S. A.; Lewis, A. F.; Critz, W. J.; Witt, L. L.; Townsend, L. B.; Blakley, R. L. *Biochemistry* **1978,** *17,* 2350.
(9) Sawa, T.; Fukagawa, Y.; Homma, I.; Takeuchi, T.; Umezawa, H. *J. Antibiot., Ser. A* **1967,** *20,* 317.
(10) Ogilvie, K. K.; Slotin, L.; Rheault, P. *Biochem. Biophys. Res. Commun.* **1971,** *45,* 297.
(11) Townsend, L. B.; Long, R. A.; McGraw, J. P.; Miles, D. W.; Robins, R. K.; Eyring, H. *J. Org. Chem.* **1974,** *39,* 2023.
(12) Abola, J. E.; Sims, M. J.; Abraham, D. J.; Lewis, A. F.; Townsend, L. B. *J. Med. Chem.* **1974,** *17,* 62.
(13) Zemlicka, J. *J. Am. Chem. Soc.* **1975,** *97,* 5896.
(14) Makage, O.; Nakamura, M.; Umezawa, S. *J. Antibiot.* **1975,** *28,* 492.
(15) Giziewicz, J.; DeClercq, E.; Luczak, M.; Shugar, D. *Biochem. Pharmacol.* **1975,** *24,* 1813.
(16) Crabtree, G. W.; Agarwal, R. P.; Parks, R. E. Jr.; Lewis, A. F.; Townsend, L. B. *Proc. Am. Assoc. Cancer Res.* **1977,** *18,* 181.
(17) Giziewicz, J.; Shugar, D. *Acta Biochim. Pol.* **1977,** *24,* 231.
(18) (a) Lynch, B. M.; Robins, R. K.; Cheng, C. C. *J. Chem. Soc.* **1958,** 2973. (b) Pullman, A.; Pullman, B.; Nakajima, T. *Bull. Soc. Chim. Fr.* **1959,** 590.
(19) Meites, L.; Thomas, H. C. "Advanced Analytical Chemistry"; McGraw-Hill: New York, 1958; Chapter 8.
(20) Townsend, L. B. "Synthetic Procedures in Nucleic Acid Chemistry", Zorbach, W. W., Tipson, R. S., Eds.; Wiley-Interscience: New York, 1973; Vol. 2, Chapter 7.
(21) Watanabe, S.; Matsuhashi, G.; Fukatsu, S.; Koyama, G.; Maeda, K.; Umezawa, H. *J. Antibiot., Ser. A* **1966,** *19,* 93.
(22) Long, R. A.; Lewis, A. F.; Robins, R. K.; Townsend, L. B. *J. Chem. Soc. C* **1971,** 2443.
(23) Chenon, M.-T.; Panzica, R. P.; Smith, J. C.; Pugmire, R. J.; Grant, D. M.; Townsend, L. B. *J. Am. Chem. Soc.* **1976,** *98,* 4736, and reference cited therein.
(24) Albert, A.; Serjeant, E. P. "The Determination of Ionization Constants"; Chapman and Hall: London, 1971; Chapter 4.
(25) Dreyfus, M.; Dodin, G.; Bensaude, O.; Dubois, J.-E. *J. Am. Chem. Soc.* **1977,** *99,* 7027.
(26) Dodin, G.; Dreyfus, M.; Bensaude, O.; Dubois, J.-E. *J. Am. Chem. Soc.* **1977,** *99,* 7257.
(27) Lewis, A. F.; Townsend, L. B. "Nucleic Acid Chemistry; Improved and New Synthetic Procedures, Methods, and Techniques", Townsend, L. B., Tipson, R. S., Eds.; Wiley-Interscience: New York, 1978; p 121.
(28) Wolfenden, R.; Sharpless, T. K.; Ragade, I. S.; Leonard, N. J. *J. Am. Chem. Soc.* **1966,** *88,* 185.
(29) Long, R. A.; Townsend, L. B., unpublished results.
(30) Crabtree, G. W.; Agarwal, R. P.; Parks, R. E. Jr.; Lewis, A. F.; Wotring, L. L.; Townsend, L. B. *Biochem. Pharmacol.* **1979,** *28,* 1491.
(31) Lewis, A. F.; Townsend, L. B. "Abstracts of Papers", 175th National Meeting of the American Chemical Society, Anaheim, Calif., March 1978; American Chemical Society: Washington, D.C., 1978; MEDI 42.
(32) Makabe, O.; Miyadera, A.; Kinoshita, M.; Umezawa, S.; Takeuchi, T. *J. Antibiot.* **1978,** *31,* 456.
(33) Kunimoto, T.; Wakashiro, T.; Okamura, I.; Asajima, T.; Hori, M. *J. Antibiot., Ser. A* **1968,** *21,* 468.