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# Structure of the Nucleoside Antibiotics Formycin, Formycin B and Laurusin (1)

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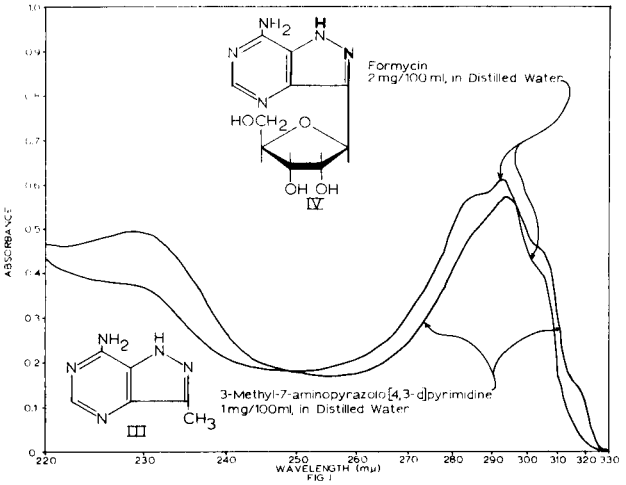
*Sir*:

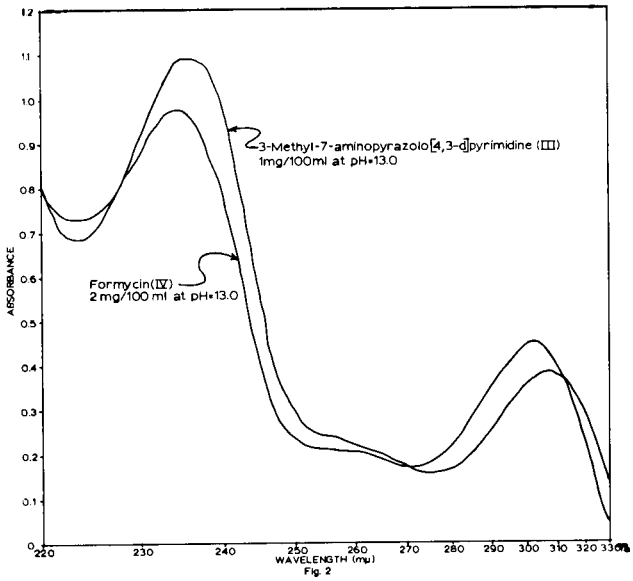
We wish to report the structure of the nucleoside antibiotic formycin as 7-amino-3-(β-D-ribofuranosyl)-pyrazolo[4,3-d]pyrimidine (IV) and the structure of formycin B and laurusin as 3-(β-D-ribofuranosyl)-pyrazolo[4,3-d]-7-pyrimidone (V). These antibiotics represent a new class of *C*-nucleoside derivatives isolated from natural sources.

Formycin (2) is an antibiotic first isolated from *Norcardia interforma*. Formycin has been found to exhibit significant antitumor activity against Ehrlich carcinoma in mice, Yoshida rat sarcoma in tissue and to inhibit HeLa cells (2). From the same microorganism has recently been isolated (3) a related antibiotic designated as formycin B.

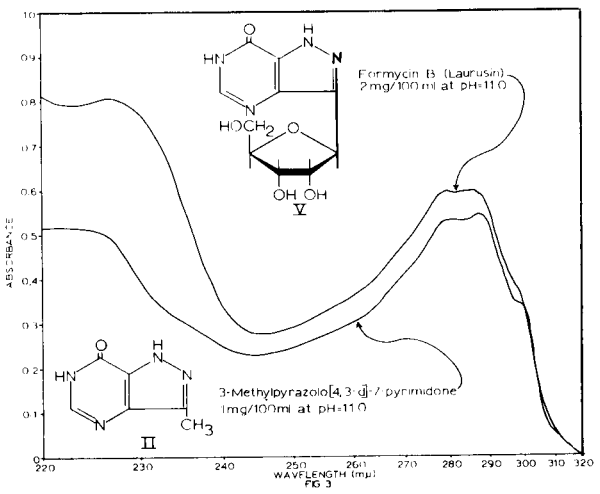
Formycin ($C_{10}H_{13}N_5O_4$) possesses an empirical formula isomeric with that of adenosine and in fact formycin is converted to formycin B by treatment with nitrous acid or adenosine deaminase (3, 4, 5). These data suggest a possible nucleoside type structure for formycin and formycin B. Recently the antibiotic laurusin, has been isolated from *Streptomyces lavendulae* (6) and has been shown to be identical to formycin B (2, 5, 7). The ultra-violet absorption spectra for formycin are unique among nucleoside antibiotics in that the absorption maximum in 0.1 *N* sodium hydroxide solution is recorded as 305 mμ (2). This absorption occurs at notably longer wave lengths than most naturally occurring nucleoside derivatives. Visual inspection of the ultra-violet absorption spectrum of formycin (8) and 3-methyl-7-aminopyrazolo[4,3-d]pyrimidine (9) (III) in distilled water revealed a striking number of similarities (see Fig. 1). Comparison of the u.v. spectra of both III and IV at *p*H 13 again revealed the spectra to be decidedly similar (see Fig. 2). The ultra-violet absorption spectrum of formycin B (laurusin) at *p*H of 11 strongly resembles that for 3-methylpyrazolo[4,3-d]-7-pyrimidone (9) (II), (see Fig. 3). It was noted that in strong sodium hydroxide formycin B again showed maxima similar to those exhibited by II under similar conditions (Fig. 4).

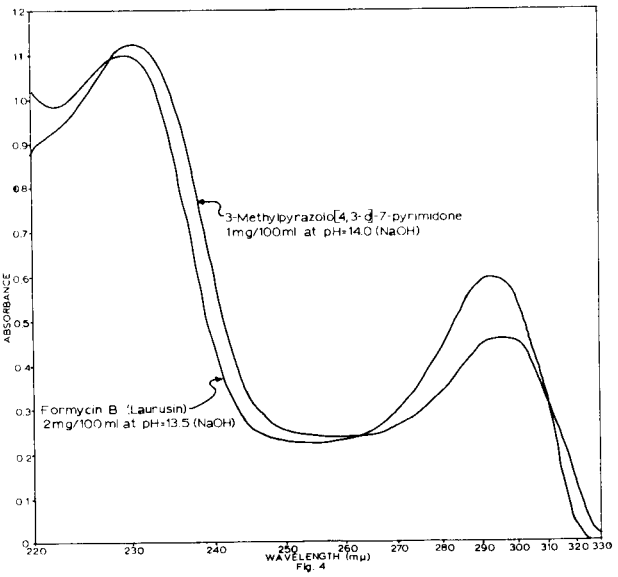
A study of the p.m.r. spectra of formycin in $D_6$ dimethylsulfoxide (DSS as an internal standard) revealed a very sharp singlet at 8.3 δ due to one aromatic proton at $C_5$ and a broad peak (2H) at 7.5 δ due to $NH_2$. A very broad peak of low absorption (1H) was observed at 12.8 δ. This latter peak is typical of an imidazole or pyrazole "NH" absorption. The remainder of the spectrum consists of a series of peaks from 3.5 to 5.3 δ (9H). The addition of a few drops of $D_4$ acetic acid gave a p.m.r. spectrum (Fig. 5) which resulted in exchange with deuterium

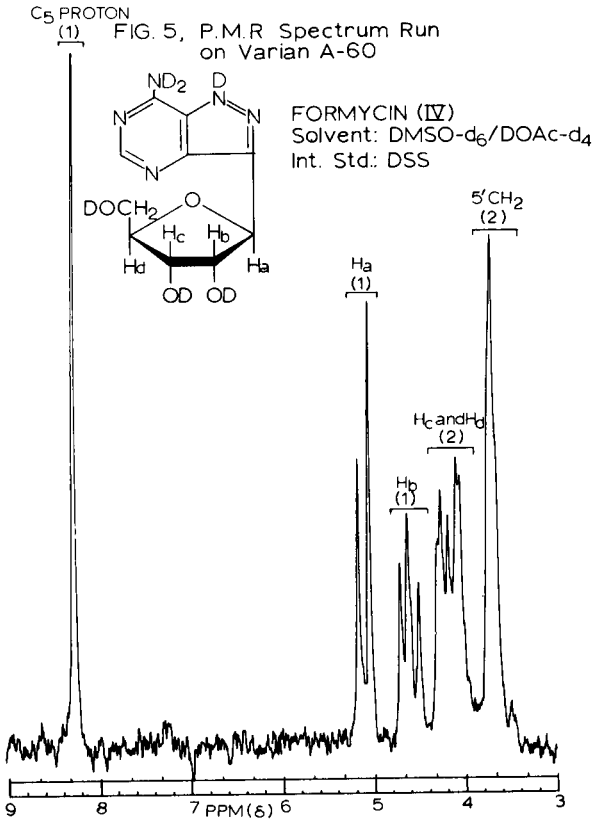


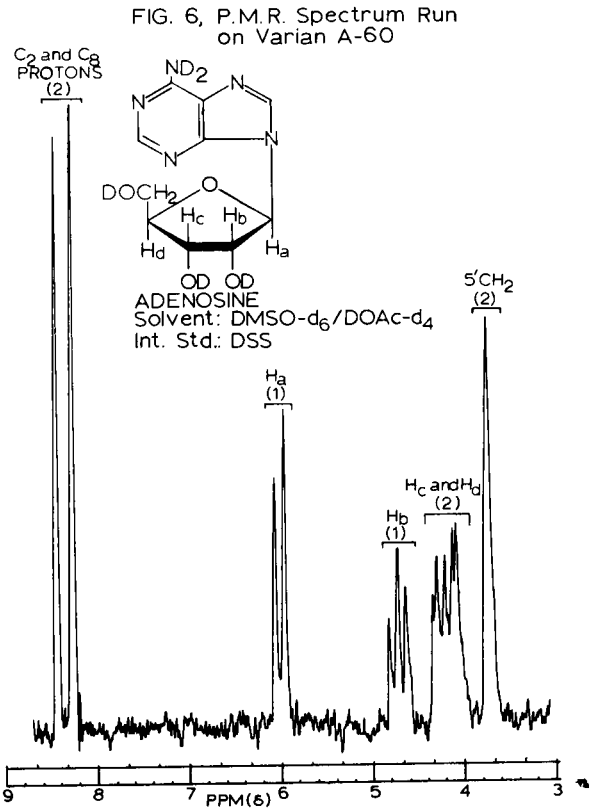



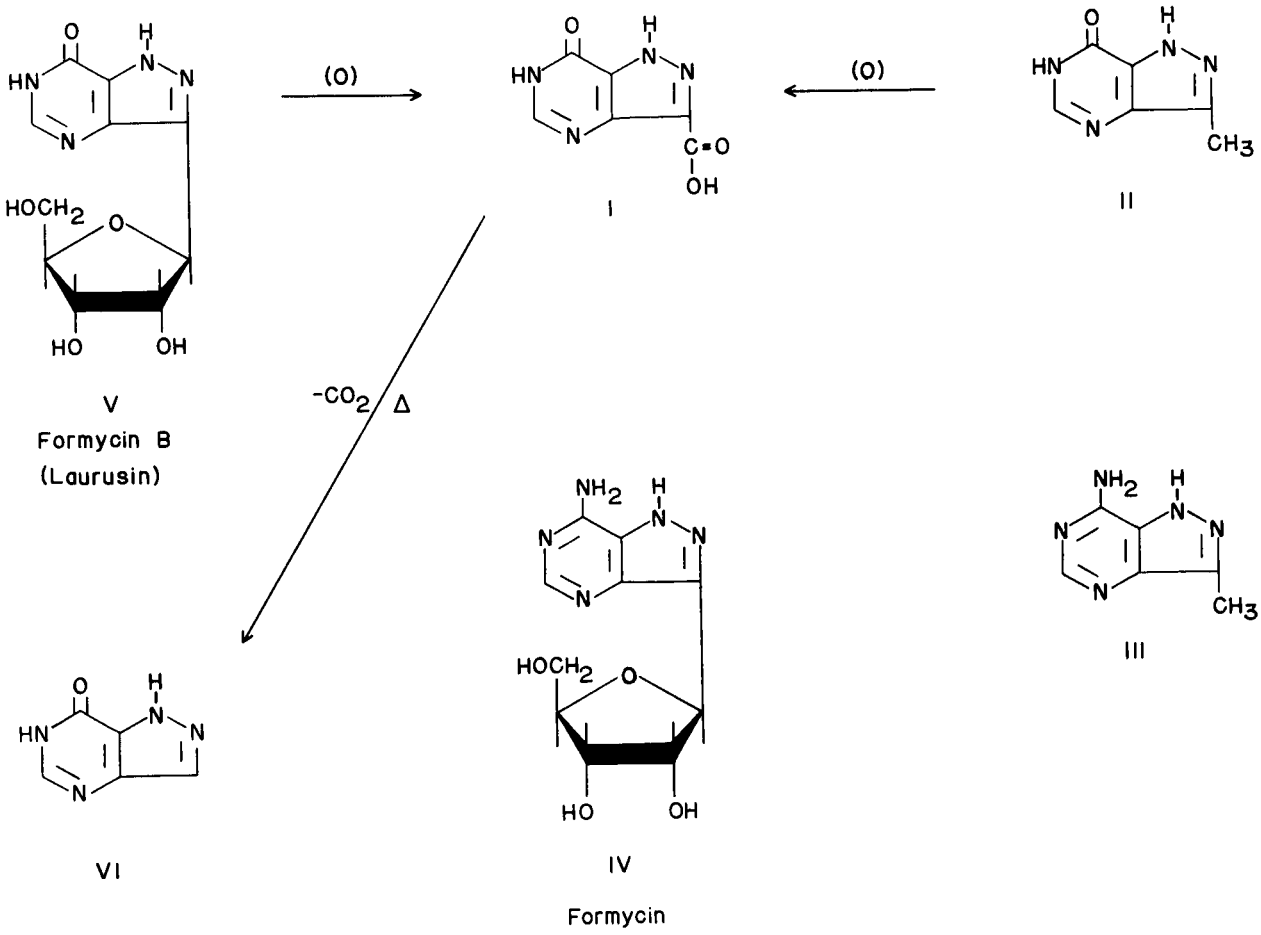
of the protons on nitrogen at 12.8 and 7.5 δ and an exchange of 3 protons in the 3.5 to 5.3 δ region. This data is consistent with the exchange of 3 hydroxyl protons. The p.m.r. spectrum of adenosine under similar conditions is given in Fig. 6. There is a very striking similarity in the carbohydrate portion of the two spectra (Fig. 5 and Fig. 6) except for the position of the anomeric proton of adenosine which is at 6.15 δ as compared to the anomeric proton of formycin at 5.14 δ. The presence of the anomeric proton at 5.14 δ is consistent with the assignment of the sugar moiety as attached to carbon instead of nitrogen. Thus the anomeric proton of pseudouridine (10) appears under similar conditions of solvent, etc. at 5.08 δ. The presence of D-ribofuranose as part of the formycin molecule is indicated by the great similarity of the p.m.r. spectra with those of adenosine. This identity is established by the ready assignment of the various



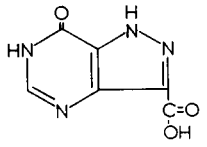



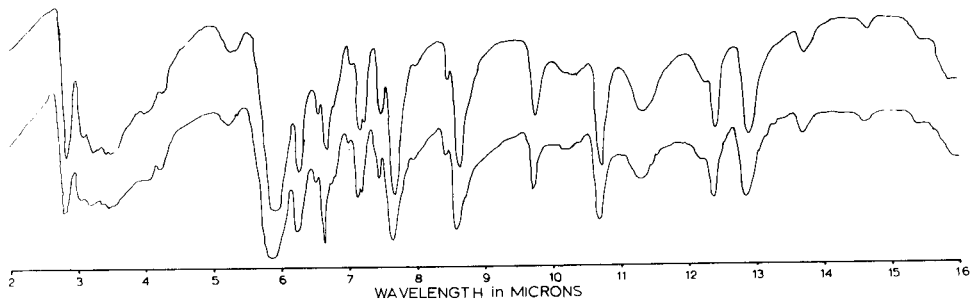






REACTION SCHEME

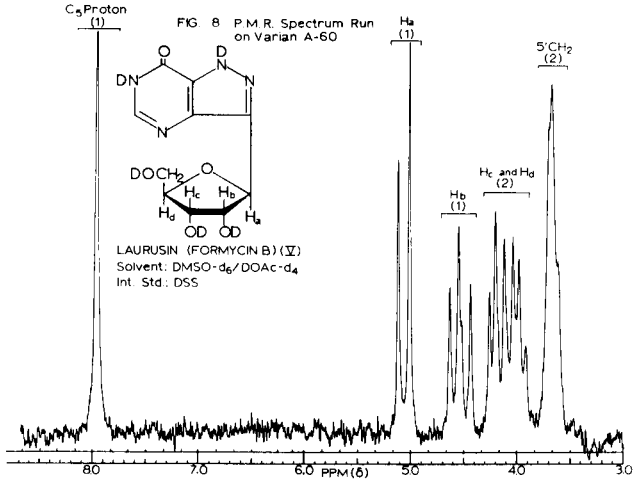
Fig. 7, IR Spectrum of 3-Carboxypyrazolo[4,3-d]-7-pyrimidone (I)

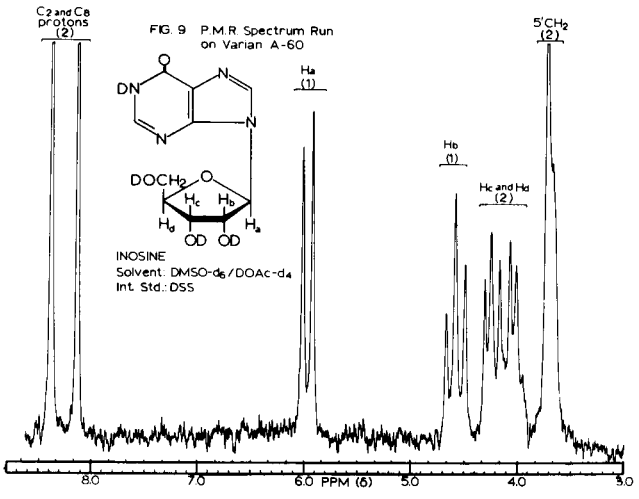





Obtained by oxidation of 3-Methylpyrazolo-[4,3-d]-7-pyrimidone (II)

Obtained by oxidation of Laurusin (Formycin B) (V)




C-protons relative to those in the D-ribofuranose moiety of adenosine. The anomeric proton at $C_1'$ of formycin is split into a sharp doublet, (J = 7 cps). This is consistent with attachment of the sugar to an aromatic position which is not adjacent to another aromatic proton. This, too is consistent with structure IV proposed for formycin, since the presence of such an aromatic proton adjacent to the position of attachment of the sugar (in a *C*-nucleoside) results in additional splitting of the anomeric proton (10). The attachment of the sugar at position 3 receives strong support from the ultra-violet absorption data of Figures 1-4. It is of interest that Robins *et al.* have noted (9) that a 3-substituted (alkyl)pyrazolo[4,3-d]pyrimidine exhibits a small but definite bathochromic shift of the ultra-violet absorption maxima of about 2-6 mμ over that of the parent pyrazolo[4,3-d]pyrimidine (11).

Rigorous proof for the assignment of a *C*-nucleoside with attachment at position 3 was provided by oxidation of laurusin (12) (formycin B) with aqueous potassium permanganate or chromium trioxide in sulfuric acid to obtain 3-carboxypyrazolo[4,3-d]-7-pyrimidone (I). The unambiguous synthesis of I was best accomplished by direct oxidation of 3-methylpyrazolo[4,3-d]-7-pyrimidone (9) (II) with chromium trioxide in sulfuric acid at room temperature.

3-Methylpyrazolo[4,3-d]-7-pyrimidone (9) (II, 0.95 g.) was dissolved in 20 ml. concentrated sulfuric acid. Chromium trioxide (1.0 g.) was added over a period of 4 hours while the solution was stirred. The temperature was maintained at 32° for an additional 15 hours. The solution was then poured over 200 g. of crushed ice and the white product filtered and washed with water to give 0.66 g. (52%) of crude product. This product (I) was purified by precipitation from hot dilute sodium hydroxide with dilute hydrochloric acid to give 0.45 g., m.p. 335° dec.

*Anal.* Calcd. for $C_6H_4N_4O_3 \cdot H_2O$: C, 36.4; H, 3.03; N, 28.3. Found: C, 36.7; H, 3.03; N, 28.7. The ultraviolet absorption spectrum λ max (*p*H 1), 279 mμ ε, 7,920; λ max (*p*H 11), 291 mμ, ε, 8,020 and λ max (1*N* NaOH), 300 mμ, ε, 9,840 with a shoulder at 293 mμ ε, 9,160 and a shoulder at 312 mμ ε, 6,290. The presence of a carboxylic acid was indicated by a $PK_a$ determination of 3.6 (potentiometric).

Laurusin (6,12), (85 mg.) was dissolved in 1.85 ml. of concentrated sulfuric acid and oxidized with 200 mg. of chromium trioxide in a similar manner to give 25 mg. (40%) of chromatographically pure 3-carboxypyrazolo[4,3-d]-7-pyrimidone (I), m.p. 329-330° dec. This product was shown to be the same as I prepared from II by a comparison of u.v. spectral data, mixed melting point (332° dec.), TLC chromatography in three separate systems and by comparison of identical infrared spectra (see Fig. 7).

Dilute aqueous potassium permanganate was also utilized to oxidize V to 3-carboxypyrazolo[4,3-d]-7-pyrimidone (I) which was identified by ultra-violet absorption spectra and TLC chromatography (Table I). 3-Carboxypyrazolo[4,3-d]-7-pyrimidone (I, 50 mg.) was decarboxylated by gentle fusion for 3-4 minutes with a free flame to give a residue which was identified as pyrazolo[4,3-d]-7-pyrimidone (VI) by rigorous comparison of u.v. spectra, TLC in 4 solvent systems and infrared spectra of an authentic sample (11).

TABLE I

Comparison of Oxidation Products by TLC

| Compound | $R_f$ values A | B | C |
|---|---|---|---|
| I ($CrO_3$ oxidation of II) | 0.65 | 0.80 | 0.29 |
| I ($CrO_3$ oxidation of V) | 0.65 | 0.80 | 0.29 |
| I ($KMnO_4$ oxidation of V) | 0.65 | 0.81 | 0.29 |

Solvent systems: A, 5% aqueous $NH_4CO_3$; B, 20% aqueous $(NH_4)_2CO_3$ and C, Isopropanol, 28% aqueous ammonia, water vol:vol, 40:30:30. Compounds were detected by ultraviolet light. 254 mμ Absorbent used was alumina HF254. All products were first converted to the potassium salt before chromatography.

Since formycin is readily deaminated to formycin B which is identical to laurusin (3, 4, 5, 6, 7) the attachment of the sugar at position 3 in formycin is also definitely established. The assignment of D-ribofuranose to position 3 accounts for the unusual chemical stability of V and IV and the failure to detect D-ribose by the usual hydrolysis procedures (2, 3). Additional support for the structure of formycin B as V is found from a study of the acidic $PK_a$'s. Umezawa (3) records a $PK_a$ of 8.8 for V, which is consistent with removal of the proton from $N_6$. Inspection of formula V would suggest removal of the proton from $N_1$ at a higher *p*H. This expectation has been realized and an additional $PK_a$ of 10.4 has now been determined for formycin B. The $PK_a$ of formycin in the region 9.5 (2) is now readily explained as removal of a proton from the pyrazole nitrogen.

The presence of two distinct "NH" protons in laurusin is readily detected by the p.m.r. spectra in $D_6$ dimethyl sulfoxide. The pyrazole "NH" occurs as a low broad absorption at 14.0 δ and the amide "NH" as a wide peak at 12.3 δ. The remaining p.m.r. spectrum in $D_6$ dimethyl sulfoxide is very similar to that of formycin except for the conspicuous absence of the $NH_2$ absorption at 7.5 δ.

The addition of a small amount of $D_4$ acetic acid to the p.m.r. spectrum of laurusin in $D_6$ dimethyl sulfoxide provided a ready deuterium exchange of the two "NH" protons at 14.0 and 12.3 δ and an exchange of 3 protons in the 3.3-5.3 δ region. (It is worthwhile to note that this type of information could not be obtained by exchange with deuterium oxide since the HDO peak occurs right in the region of most interest.) The remaining peaks (Fig. 8) were then readily assigned by direct comparison with a p.m.r. spectrum of inosine (Fig. 9) in the same solvent system. In fact with the exception of the anomeric proton of V at 5.07 δ this part of the two p.m.r. spectra was virtually identical (Fig. 8 and Fig. 9).

With regard to the anomeric configuration of formycin and formycin B it can be stated that the great similarity of p.m.r. spectra with the corresponding natural purine nucleosides adenosine and inosine is consistent with β-assignment. The fact that formycin is attacked by adenosine deaminase is additional support for β-assignment since Lepage and Junga (13) recently found that adenosine deaminase from mouse tissues does not attack the α-anomer of a number of adenosine analogs but readily deaminates the corresponding β-anomer. The structural assignment for formycin (IV), and formycin B (laurusin) (V) presented here, has very recently been independently confirmed by X-ray analysis (14) of formycin hydrobromide. Stuart molecular models of formycin and adenosine exhibit a most striking steric similarity which would predict that this class of antibiotics should resemble the purine nucleosides very closely in the enzymatic reactions of various biochemical systems. It is of considerable interest that although 7-aminopyrazolo-[4, 3-d]pyrimidine and pyrazolo[4, 3-d]-7-pyrimidone were prepared as purine analogs and reported in 1956 (11) no biological activity has been noted to date for these heterocyclic bases. These antibiotics represent the first instance of the pyrazolo[4, 3-d]-pyrimidine ring occurring in nature. The chemical synthesis of 7-amino-3-(β-D-ribosuranosyl)pyrazolo-[4, 3-d]pyrimidine (IV) offers an interesting challenge and is currently under active investigation in our Laboratory.

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Received February 11, 1966

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