THE SYNTHESIS AND STUDY OF CERTAIN DERIVATIVES OF THE C-NUCLEOSIDES FORMYCIN AND FORMYCIN B

by

Arthur Frank Lewis

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ABSTRACT

Methylation of formycin under non-basic reaction conditions has afforded 4-methylformycin and 6-methylformycin. The structures assigned to these two mono-N-methyl isomers of formycin were based on ultraviolet and nuclear magnetic resonance spectral studies. N^7-methylformycin has been resynthesized by an alternate and more facile route. Some of the physico-chemical properties of the five N-methyl isomers of formycin have been tabulated and compared. Certain spectral characteristics were found to distinguish 6-methylformycin from the other ring-methylated isomers. Additionally, 6-methylformycin was found to be unstable in aqueous solution, yielding three products, formycin B, N^7-methylformycin and 6-methylformycin B. The first two products of this reaction were identified by direct comparisons to authentic materials. A confirmation that 6-methylformycin B was the third product involved the unambiguous synthesis of 3,6-dimethylpyrazolo[4,3-d]pyrimidin-7-one and followed by a comparative study of its ultraviolet spectra. However, the structure of the third product was not confirmed by this method and led to the synthesis of 6-methylformycin B by a reaction of nitrosyl chloride with 6-methylformycin. A comparison of the 6-methylformycin B prepared in this manner with the third product obtained from the reaction of boiling water with 6-methylformycin confirmed that the third product was indeed 6-methylformycin B. The two remaining,

unknown, mono-N-methyl formycin B derivatives have also been synthesized by the reaction of nitrosyl chloride with 4-methylformycin and 1-methylformycin.

Studies have shown that formycin derivatives methylated in the pyrimidine ring do not inhibit the growth of L-1210 cell cultures. Associated studies have also found that 4-methylformycin and 6-methylformycin were not substrates for either adenosine deaminase or adenosine kinase from human erythrocytes. Certain structure activity relationships for the methylated formycins have been drawn from these results.

Formycin-5'-triphosphate has been synthesized using standard methods of nucleotide preparation. A collaborative study has investigated the interaction of formycin-5'-triphosphate with the nucleotide reductase from Lactobacillus leichmannii. The preliminary results of this study indicate that the enzymatic reduction of formycin is a viable alternate to established routes for the synthesis of 2'-deoxyformycin.

The versatile synthetic intermediate 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide has been resynthesized by an alternate, more efficacious route which involves the acidic ring fission of the pyrimidine moiety of formycin-N^6-oxide. This amino analog of the C-nucleoside antibiotic pyrazofurin was not inhibitory to the growth of L-1210 cell cultures.

Annulation of 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide has afforded 5-amino-3(β-D-ribofuranosyl)pyrazolo[4,3-d]-

pyrimidin-7-one (5-aminoformycin B), a C-nucleoside analog of guanosine. The synthesis of 5-aminoformycin B was preceded by model studies which produced the guanine analog, 5-aminopyrazolo[4,3-d]-pyrimidin-7-one.

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I would like to especially recognize the efforts of Professor R. L. Blakley who was the directing force responsible for the enzymatic reduction studies. The biological studies discussed in this manuscript were the result of rewarding associations with Drs. R. E. Parks, Jr., G. W. Crabtree, R. P. Agarwal and L. L. Wotring.

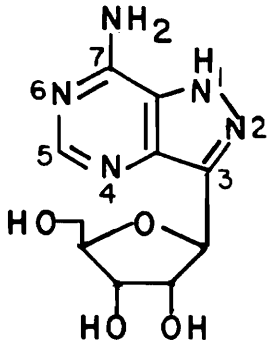
Most importantly, I wish to thank Professors Leroy B. Townsend and Roland K. Robins for twenty years of professional council and friendship.

CHAPTER 1

INTRODUCTION

Formycin (1) was initially isolated[1] from the rice mold, Norcardia interforma and has been identified as a C-nucleoside,[2,3] isomeric with the natural nucleic acid constituent, adenosine. The numbering, as shown, of the pyrazolo[4,3-d]pyrimidine heterocyclic moiety of this nucleoside differs from that of the more common isomeric purines. Formycin (1), minimycin (oxazinomycin, 2),[4] pseudouridine (3),[4] showdomycin (4)[4] and pyrazofurin (5)[4] are related in that they all belong to the relatively new C-nucleoside class of compounds. These nucleosides contain a carbon to carbon bond between the carbohydrate and aglycon moieties rather than the more common carbon to nitrogen glycosidic bond.

Formycin (1) has demonstrated antitumor, antibacterial, antifungal and antiviral activity.[5] Formycin is a substrate for many adenosine specific enzymes; e.g., adenosine kinase.[6] The C-nucleoside 1 and other closely related C-nucleoside derivatives do not readily undergo acidic hydrolysis of the glycosidic bond nor are they substrates for the nucleic acid enzymes which cleave the glycosidic bond.[5] Unfortunately, 1 is a good substrate for the adenosine catabolic enzyme adenosine deaminase (ADA).[7] The therapeutic effectiveness of 1 is thus diminished by the facile deamination of



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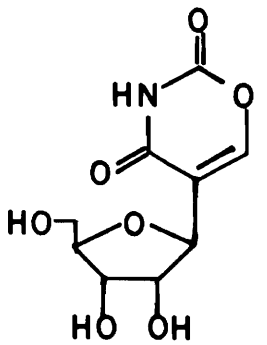

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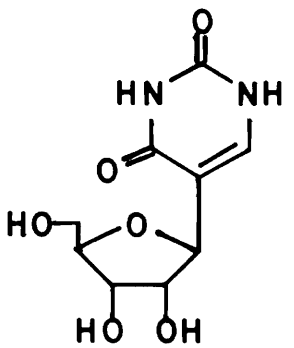

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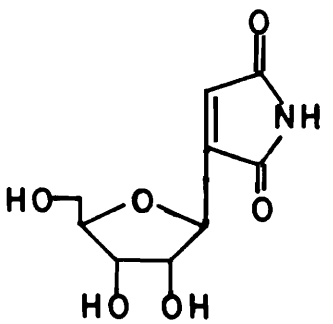

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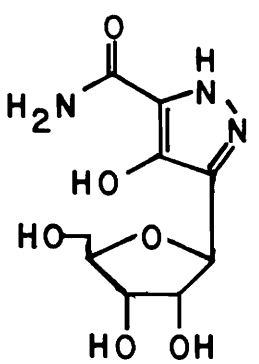

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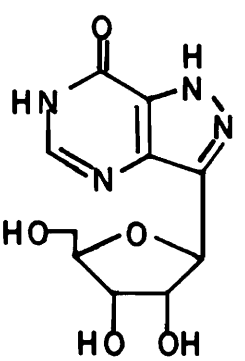

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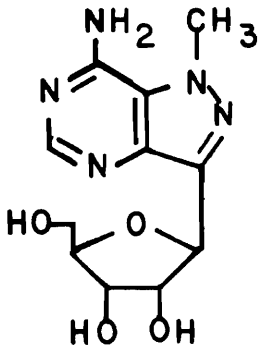
1 into the nearly inactive formycin B (6).

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[8] which suggested that adenosine derivatives in the syn rotameric conformation are not substrates for adenosine deaminase prompted the synthesis of formycin nucleosides designed to restrict rotation around the glycosyl bond. To this end, Townsend, et al.,[9] described the isolation of 1-methyl- (7) and 2-methylformycin (8) from the reaction of formycin under basic conditions with methyl iodide. Observations from CPK molecular models indicated that 8 would most likely exist with a predominant syn relationship between sugar and heterocycle whereas 7 should have unrestricted rotation about the glycosidic bond. Therefore, 7 should more nearly resemble formycin with respect to its rotameric preference. X-ray analysis[10] of 2-methylformycin (8) confirmed the high degree of syn character, at least, in the solid state. Thus, it was expected that 8 would be somewhat resistant towards enzymic deamination and show enhanced chemotherapeutic activity when compared to 7 or 1. This expectation was not realized since 8 exhibited essentially the same activity (T/C = 127) when compared to 7 (T/C = 122) as an antileukemic agent.[11]

Two recent studies[12,13] have suggested that, like adenosine, formycin derivatives existing in the anti rotamer are substrates for adenosine deaminase, and derivatives which possess a syn relationship between the heterocyclic and sugar moieties are not deaminated. These studies used 2,5'-anhydroformycin (9) and 4,5'-anhydroformycin



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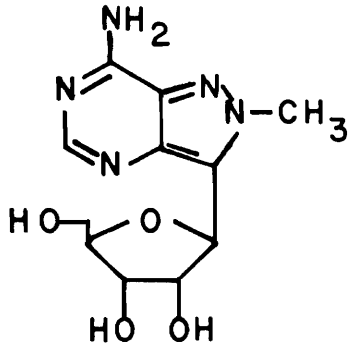

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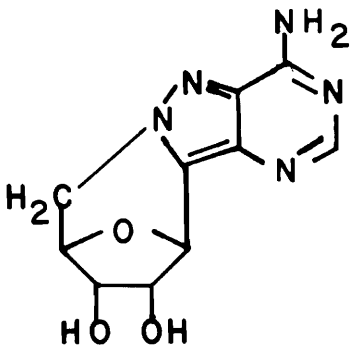

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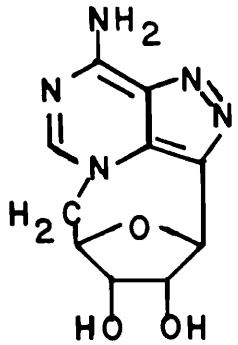

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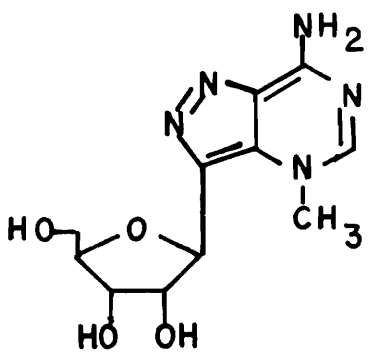


11

(10) as models for the anti and syn rotamers, respectively. Calf intestinal mucosa adenosine deaminase[12,13] and Takadiastase adenosine deaminase[13] accepted 9 (the anti model) as a substrate while 10 (the syn model) was not deaminated. It was also noted[13] that 9 was less susceptible to deamination than formycin with calf intestinal mucosa adenosine deaminase but not with Takadiastase adenosine deaminase.

In direct contrast to these findings, are two recent reports[14,15] that 2-methylformycin (8) (a syn nucleoside) is deaminated by calf intestinal and human erythrocytic adenosine deaminase and that 1-methylformycin (7) is resistant to deamination. To examine this paradox, a study was initiated to determine the biological effects, if any, attendant upon the changes arising from specific alkylation of the heterocyclic moiety of formycin in contrast to the syn, anti conformational relationship.

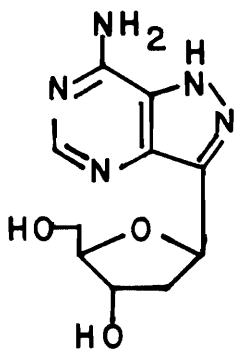
For this study, the synthesis of 4-methylformycin (11) was investigated since CPK models indicated that this derivative could not easily assume a syn conformation. Thus, 11 would be comparable to 10 with respect to the effects of alkylation of the heterocycle but would exist predominantly in the opposite rotameric conformation. A similar situation exists between 8 and 9. Additionally, the synthesis of 11 would allow a comparison of the physico-chemical and biological differences between formycin derivatives alkylated in the pyrimidine ring and those alkylated in the pyrazole ring.

It has been reported[16] that formycin is incorporated into the DNA of L-cells, indicating that a formycin nucleotide is a substrate

for a nucleotide reductase and that a 2'-deoxyformycin derivative is formed *in vivo*. Although there have been extensive studies of the biological activities of 1 and various derivatives of 1, little is known about the activity of 2'-deoxyformycin (12) and derivatives; perhaps because of the paucity of 12. 2'-Deoxyformycin (12) has been synthesized[17,18] by chemical conversions involving the parent nucleoside, formycin. However, the methods of synthesis producing 12 also produce 3'-deoxyformycin (13), with the latter isomer being the predominant product.

To investigate the effectiveness of obtaining 2'-deoxyformycin (12) *via* an *in vitro* enzymatic reduction of 1, a collaborative program was instituted with Professor R. L. Blakley at The University of Iowa. The purpose of this collaborative study was to obtain preliminary data regarding the possible production of 12 from the reduction of formycin-5'-triphosphate with the readily available nucleoside triphosphate reductase of *Lactabacillus leichmannii*. The interaction of formycin nucleotides with the reductase enzyme and also attempts to determine if 2'-deoxyformycin nucleotides could effectively mimic 2'-deoxyadenosine-5'-triphosphate as a physiological regulator of the reductase system were additional goals of this collaborative study.

Only a few derivatives of 1 and/or 6 with a substituent in the 5-position are known.[21a] With the exception of the inactive catabolite of 6, oxoformycin (14), only limited data is available on the biological activities of 5-substituted derivatives of 1 or 6. The synthesis of such derivatives requires a suitable, easily attainable



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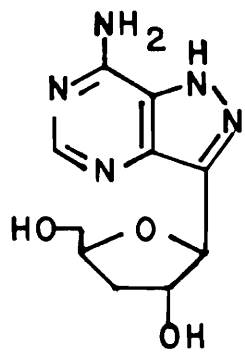

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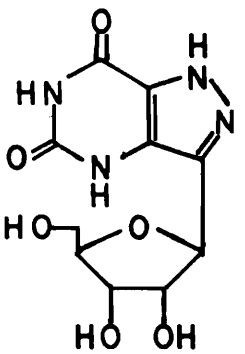

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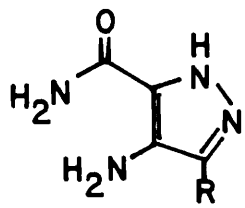
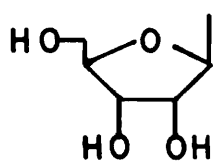
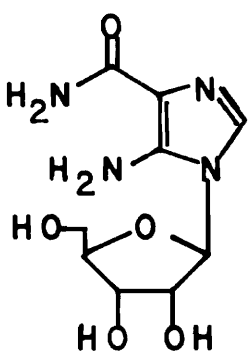


15 R = H
16 R = CH_3

17 R = 



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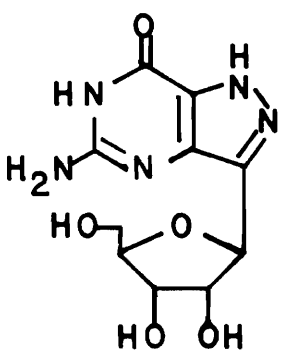

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intermediate. The pioneering work of Robins and coworkers[19,20] has described the syntheses of a variety of 5,7-disubstituted pyrazolo-[4,3-d]pyrimidines. For these syntheses, 4-aminopyrazole-3-carboxamide (15)[19] and 4-amino-3-methylpyrazole-5-carboxamide (16)[20] were found to be most valuable intermediates. Long[21] has demonstrated that 6 can be converted into 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide (17), which has been given the sobriquet of ΨAPCA-riboside. The o-aminoamide 17 could be a versatile intermediate for the syntheses of 5-substituted derivatives of 1 and 6. However, 17 was not obtained in substantial yield by the published method[21a] and an investigation into possible alternate routes for the synthesis of 17 was instituted for this study.

In addition to being a valuable intermediate for the syntheses of formycin and formycin B derivatives, 17 *per se* was of biological interest. ΨAPCA-riboside (17) is an amino analog of pyrazofurin (5), an antiviral agent,[22] and is an isomer of 5-amino-1-(β-D-ribofuranosyl)imidazole-4-carboxamide (AICA-riboside, 18). The 5'-phosphate derivative of 18 is a key intermediate in the purine nucleotide *de novo* pathway. Thus, obtaining sufficient quantities of 17 for biological study gave additional impetus to the investigation of alternate, more efficacious methods for the synthesis of 17.

It should be possible to obtain various 5-substituted derivatives of 6 from annulation of the o-aminoamide 17. 5-Amino-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidin-7-one (19), a guanosine analog, was the derivative of major interest and methods for the synthesis of 19 were investigated. Derivatives of guanosine have been widely

studied as antitumor agents and as probes in biological reactions.[23] For a nucleoside to exhibit biological activity it is generally necessary that the nucleoside first be converted to a nucleotide. To obtain the nucleotide of a guanosine derivative or analog, the glycosidic bond must first be enzymatically cleaved by purine nucleoside phosphorylase (PNPase) and the base or aglycon must then be a substrate for hypoxanthine-guanine phosphoribosyl transferase (HGPRTase).[23] The latter enzyme catalyzes a condensation between the nitrogen heterocycle and phosphoribosylpyrophosphate (PRPP), resulting in the formation of a nucleotide. Formycin derivatives, by virtue of their stable carbon-carbon glycosidic bond, are not substrates for phosphorylases.[5] Thus, an analog such as 19 probably would not be converted to the biologically active nucleotide. However, if 19 mimics guanosine in the manner that formycin mimics adenosine in many enzymic reactions, 19 could be an inhibitor of PNPase and thus be a powerful probe in the study of nucleic acid metabolism and cell kinetics. The direct phosphorylation of certain guanosine derivatives has been reported.[24] These reports have not, however, been confirmed. A guanosine analog which is not a substrate for PNPase could act as a probe for the existence of a guanosine kinase.

Many studies[5] have shown that formycin (1) mimics adenosine in many biological processes, yet certain chemical and biochemical differences have also been noted.[5] The "anomalous" behavior of certain formycin derivatives have, at times, been attributed to the "paradoxical" nature of formycin. One of the goals of the studies outlined

herein was to provide some new insight into certain important biological reactions involving "paradoxical" formycin derivatives. The potential objective of all of the outlined studies was the development of new chemotherapeutic agents.

CHAPTER 2

HISTORICAL REVIEW

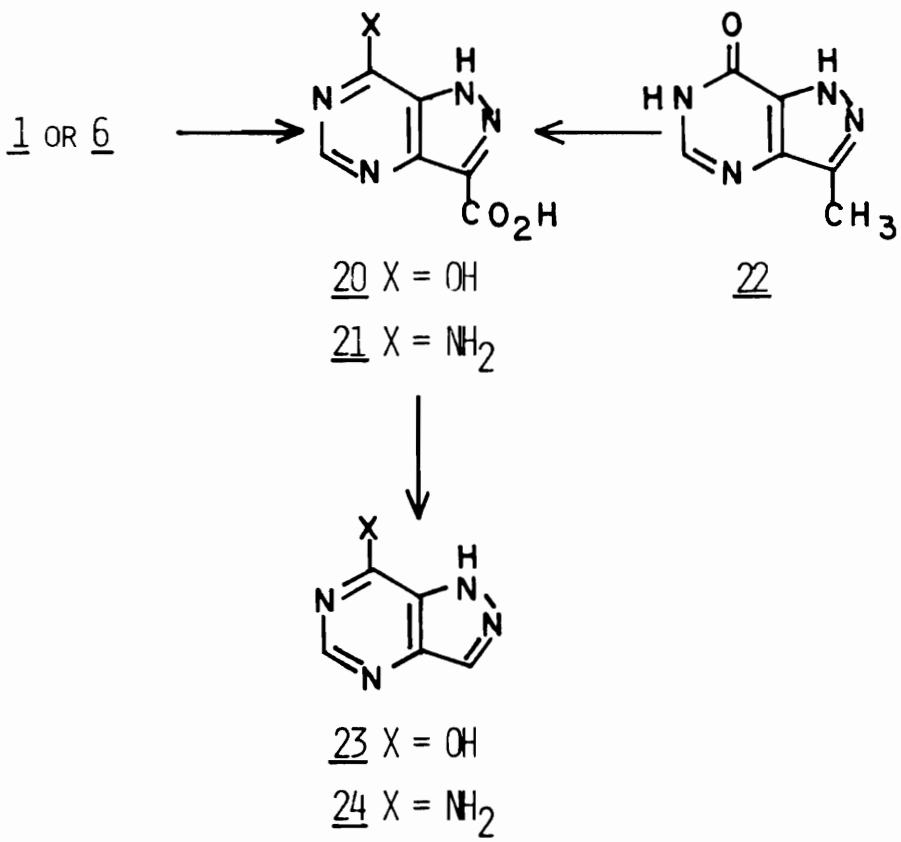
Formycin, Formycin B and Oxoformycin

The isolation,[1] in 1964, of the antibiotic, formycin (1), from the culture filtrates of Norcardia interforma was followed by the isolation[25] of a second new substance, formycin B (6), from the same medium. Formycin B (6) was found to be identical to the compound, Laurusin, which had been isolated[26] from the fermentation broth of a Streptomyces lavendulae varient. The relationship of formycin B (6) to formycin (1) was established from the observations that 1 could be deaminated to give 6.[27] A third product, oxoformycin (14), was later isolated from N. interforma[28] and also from the urine of mice and rabbits which had been injected with a buffered solution of formycin B (6).[29]

The structures of formycin (1) and formycin B (6) were established by chemical degradation[2,30] to known products and by X-ray crystallographic studies of formycin hydrobromide monohydrate.[3] Formycin (1) and formycin B (6) were found[25] to have molecular formulas isomeric with adenosine and inosine, respectively. The ultraviolet absorption spectra of 1 and 6 were found[2,3] to be comparable to known[19,20] pyrazolo[4,3-d]pyrimidines. Treatment of 6 with aqueous permanganate or chromium trioxide in sulfuric acid

furnished[2] an identical product, 3-carboxypyrazolo[4,3-d]pyrimidin-7-one (20), to that obtained by a similar oxidation of 3-methylpyrazolo[4,3-d]pyrimidin-7-one (22).[20] Compound 20 was decarboxylated[2] by gentle fusion to give pyrazolo[4,3-d]pyrimidin-7-one (23).[19] Similarly, compounds 1 and 6 have been treated with periodate and then sodium hydroxide to give 21 and 20.[30] Decarboxylation of 20 and 21 resulted in the formation of the known pyrazolo[4,3-d]-pyrimidines 23 and 24.[30] Comparison of the pmr spectra of 1 and 6 with the pmr spectra of adenosine and inosine established[2] that the carbohydrate portion of these nucleosides was a D-ribofuranose. The fact that 1 was a substrate for adenosine deaminase also suggested

Reaction Scheme 1



20 X = OH

21 X = NH_2

22

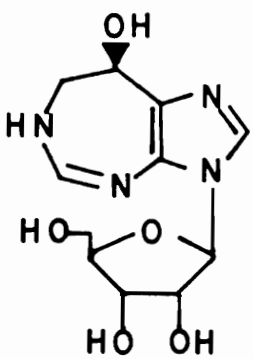
23 X = OH

24 X = NH_2

a β configuration. The assignment of anomeric configuration was corroborated by the X-ray crystallographic study[3] of formycin. Thus, the structures of formycin and formycin B were assigned as 7-amino-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidine (1) and 3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidin-7-one (6), respectively. Oxoformycin was assigned the structure, 3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidin-5,7-dione (14) from a study of its spectra and its relationship, as an oxidation product, to formycin B.[28,29]

Of considerable interest was the isolation of yet another product, coformycin (25), from the culture filtrates of N. interforma. Coformycin (25) was found to be an inhibitor of adenosine deaminase and was synergistic with formycin in inhibiting Ehrlich carcinoma and Escherichia coli.[5] Coformycin (25) was found not to be synergistic with 1 against Xanthomonas oryzae, which is inhibited by formycin B (6).[5] The structure, 3-(β-D-ribofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-d][1,3]diazepin-8-ol (25), has been determined[31] for coformycin.

Formycin and formycin B belong to a relatively new class of nucleosides, the C-nucleosdies. This class of compounds encompasses all glycosylated aromatic heterocycles incorporating a carbon-carbon



25

glycosidic bond rather than the more common carbon-nitrogen bond between the carbohydrate and aglycon moieties. Other members of this class of compounds are minimycin (2), pseudouridine (3), showdomycin (4) and pyrazofurin (pyrazomycin, 5).[4]

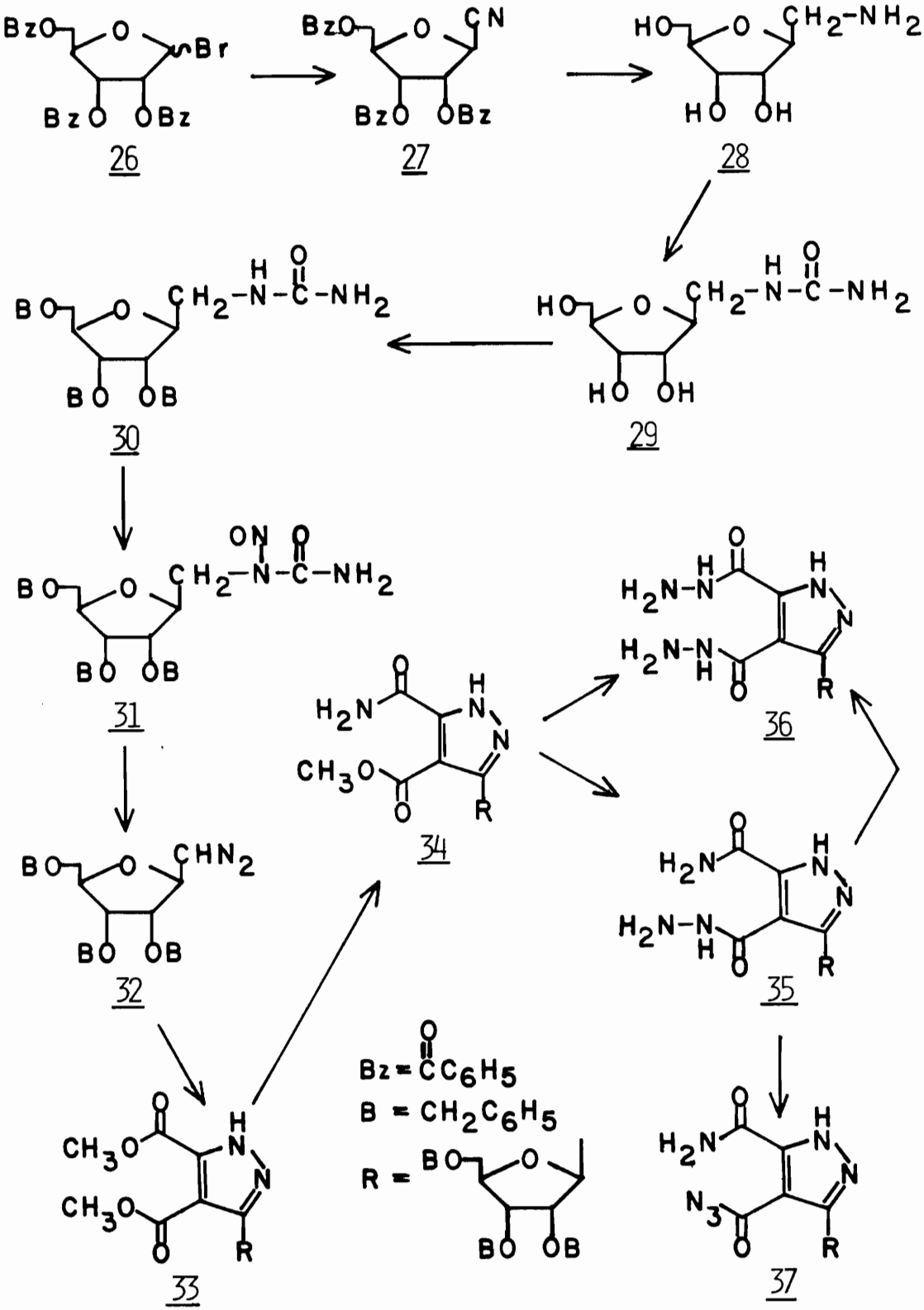
Certain spectral characteristics of 1 and 6 are attributable to their C-nucleoside structure. The anomeric ($H_{1'}$) signal in the pmr spectrum of 1 (δ5.14, DMSO-d_6) is found upfield from the corresponding signal for the N-nucleoside isomer, adenosine (δ6.15).[2] This upfield shift of the anomeric proton is a general characteristic of the C-nucleosides. A predominate base plus thirty (b + 30) fragment in the mass spectra of C-nucleosides is another general characteristic.[32] The b + 30 ion derives from a cleavage of the $C_{1'}$-$C_{2'}$ bond and the predominance of this ion is reflective of the stable glycosyl bond in C-nucleosides. In fact, the appearance of a predominate b + 30 ion in the mass spectrum is a diagnostic feature of C-nucleosides.[32]

In contrast to adenosine, 1 has both an acidic (9.7) and a basic (4.4) pKa.[4] The isomeric arrangement of the atoms in the chromophore, as reflected in 1 when compared to adenine, results in a notable bathochromic shift in the ultraviolet spectrum. A similar change is not noted when comparing the ultraviolet spectra of adenine with 4-aminopyrazolo[3,4-d]pyrimidine.[33] Under a variety of conditions, 1 is fluorescent, which makes 1 and nucleotides of 1 desirable biochemical probes in studies on nucleic acid structure.[5] Several studies delineating the conformational properties of formycin and certain derivatives have been published.[5,10,34-37]

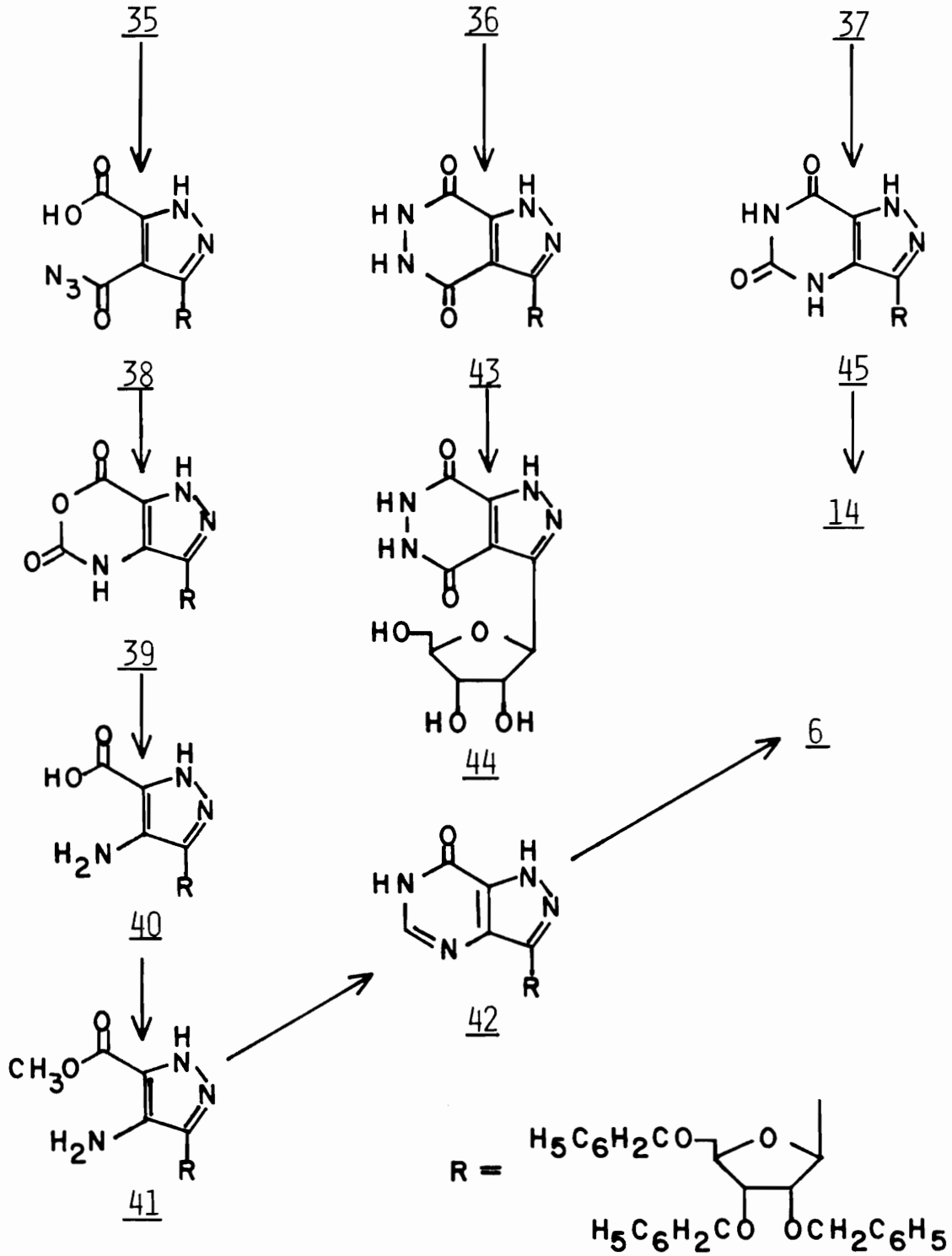
A recent conformational study of 1 by pmr[34] spectroscopy has concluded that 1 exists, in solution, in the syn rotameric conformation and that this conformation is stabilized by an internal hydrogen bond between $O_{5'}$ and N_4.

The total synthesis of a formycin derivative (oxoformycin, 14) was first communicated[38] in 1970. A full account of the synthesis of 14 appeared in 1972[39] and was preceded in 1971 with a description of the synthesis[40] of formycin B (6). A common intermediate, 35 was used for the syntheses of these two derivatives, 6 and 14.[38-40] The synthesis of 33 from 2,3,5-tri-O-benzoyl-β-D-ribofuranosyl cyanide (27) had been previously described by Bobek and Farkas.[41] The cyanide, 27, was obtained[42] from the versatile carbohydrate, 2,3,5-tri-O-benzoyl-D-ribofuranosyl bromide (26).[43] The diazo-sugar, 32, underwent 1,3-dipolar addition to dimethyl acetylenedicarboxylate to give 33.[39,40] Treatment of 33 with methanolic ammonia gave the monoamide 34,[38-40] which upon treatment with hydrazine hydrate gave a mixture of the mono- and dihydrazides, 35 and 36.[39] When 34 was treated with ethanolic hydrazine only 35 was obtained.[38] However, 35 was also converted to 36 by repeated treatment with hydrazine.[38] Treatment of 35 with nitrous acid produced the carboxazide, 37.[39] The reaction of 35 with dinitrogen tetroxide in acetic acid yielded the carboxazide, 38, which, upon heating, underwent a Curtius rearrangement and subsequent ring closure to give the 1,3-oxazine, 39.[40] The α-aminoacid, 40, was obtained by treatment of 39 with aqueous tetrahydrofuran and was esterified (41) and then heated

Reaction Scheme 2



Reaction Scheme 2 (continued)

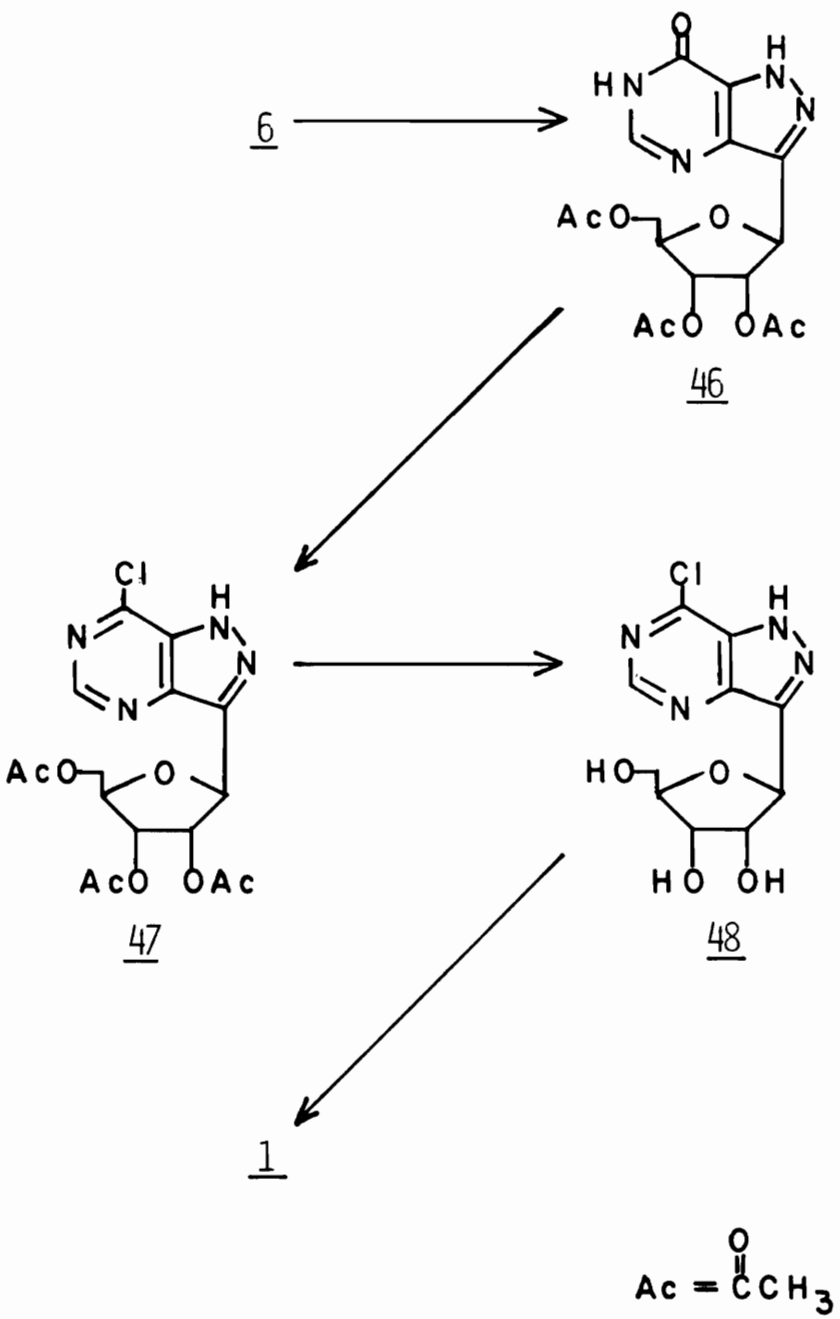


with formamide to form the pyrazolo[4,3-d]pyrimidine, 42.[40] Debenzylation of 42 gave the desired formycin B (6).[40] When the dihydrazide, 36, was treated with acid and the resulting product (43) debenzylated, a pyrazolopyridazine, 44, was obtained.[38,39] Upon heating, the azide 37 was converted to 45, which was debenzylated to give oxoformycin (14).[38,39]

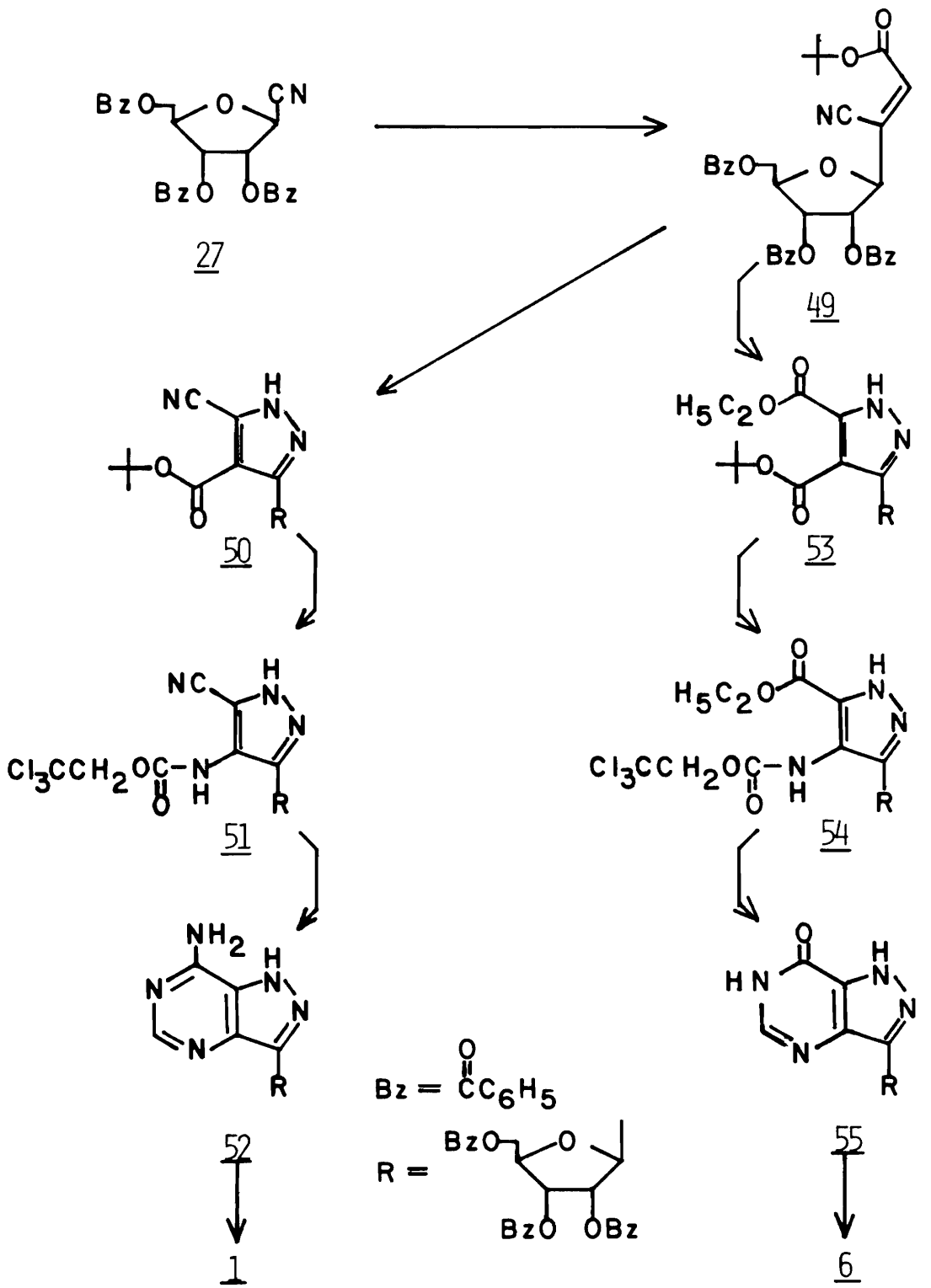
The conversion of 6 to 1 has been demonstrated by Long, et al.[44] Peracetylation of 6 gave the tri-O-acetyl derivative 46.[44,45] The reaction of 46 with phosphoryl chloride produced the 7-chloro derivative 47 which was deacetylated by careful treatment with methoxide.[44] Formycin (1) was obtained by heating 48 with liquid ammonia in a sealed reaction vessel.[44] Thus, the first reported synthesis of formycin entailed a twenty-step procedure.

A more direct method for the synthesis of 1 and 6 has recently been disclosed by Kalvoda.[46] Beginning with the nitrile 27, acid hydrolysis gave 2,3,5-tri-O-benzoyl-2,5-anhydro-D-allonic acid,[47] which was subsequently converted to the acid chloride. Without isolation, the acid chloride was reacted with t-butoxycarbonylmethylenetriphenylphosphorane in the presence of hydrogen cyanide to produce 49. The reaction of 49 with diazoacetonitrile or with ethyl diazoacetate gave the pyrazole nucleosides 50 and 53, respectively. Acid cleavage of the t-butylesters, followed by Curtius degradation, in the presence of trichloroethanol, gave the urethanes 51 and 54, respectively. Following reductive cleavage of the urethanes and ring closure with formamidine, 52 and 55 were

Reaction Scheme 3



Reaction Scheme 4



obtained from 51 and 54. Debenzoylation of 52 yielded formycin (1) and similarly, formycin B (6) was obtained from 55.[46]

Formycin belongs to another general class of compounds which are related by their biological activity and their structure; the nucleoside antibiotics. Other C-nucleosides which belong in this classification are pyrazofurin (5) and showdomycin (4).[5]

Formycin (1) has displayed antitumor, antiviral, antibacterial and antifungal activity.[4,5] Formycin B (6) is much less effective; does not inhibit animal tumors but does inhibit influenza virus and Xanthomonas oryzae.[5] Coformycin (25) was synergistic with formycin in its effect on Yoshida rat sarcoma cells,[5] and potentiated the in vitro and in vivo cytotoxicity and antitumor activity of many adenosine analogs.[48] Formycin (1) was found to be effective against the Simian malaria, Plasmodium knowlesi, by inhibiting in vitro incorporation of ^{14}C-methionine into protein and ^{14}C-adenosine or ^{14}C-orotic acid into RNA and DNA.[49] Inhibition of tobacco mosaic virus (TMV) multiplication by 6 has been reported.[50] The TMV inhibition was reversed by adenine, adenosine and adenylic acid but not by hypoxanthine or uracil.[50] Nucleotides of 6 were not incorporated into either the host RNA or the TMV-RNA[50] and thus, host RNA was synthesized in the presence of 6 while TMV multiplication was strongly inhibited.[50] Formycin B (6) has also been found to have a morphogenic effect on the cellular slime mold, Dictyostelium discordeum.[51] Formycin (1) has displayed some effectiveness as an immunosuppressive agent,[52] whereas the effectiveness of 6 was much

less except when allopurinol (a xanthine oxidase inhibitor) was present.[52] Even in the presence of coformycin (25), 1 only weakly inhibited ADP-induced platelet aggregation.[53] The effectiveness of 1 as an antischistosomal agent has also been evaluated.[54,55]

Biochemical studies have attempted to delineate the mechanism(s) of action of formycin derivatives. Because of their close structural relationship to adenosine and inosine, formycin derivatives have also been used to probe specific structural requirements for many biochemical processes. That 1 was a good substrate for adenosine deaminase was early recognized.[5] The fact that 6, the deamination product, was far less effective as an antitumor agent, limited the effectiveness of 1. The synergistic effect observed when 25 was used in combination with 1[5,48] is a direct reflection on the ability of 25 to inhibit adenosine deaminase. Such a synergistic effect was not seen in a combination of 25 and tubercidin (7-deazaadenosine)[48] since the latter is not a substrate for adenosine deaminase.[5]

A strain of N. interforma having a low productivity of 1, readily converted 6 into 14 (reaction A), whereas in the normal N. interforma strain, 6 was converted into 1 (reaction B).[28] Studies with these two strains showed[28] that reaction A was inhibited by allopurinol (a xanthine oxidase inhibitor) and a partial competitive inhibition was produced by hypoxanthine or xanthine. Reaction B was inhibited by hadacidin (an adenylosuccinate synthetase inhibitor) but not by azaserine (a glutamate antagonist).[28] Only N. interforma and Xanthomonas oryzae converted 6 into 1

(reaction B),[28] whereas N. interforma, Pseudomonas fluorscens and Streptomyces kasugaensis converted 6 into 14 (reaction A).[28] Oxoformycin (14) has displayed no significant biological activity and has been considered to be a catabolic end product of 1 and 6. However, the finding that 6 was converted into 14 and that this conversion was blocked by a xanthine oxidase inhibitor caused some initial confusion.[5] This confusion was due to the fact that formycin B (6) is not a substrate for nucleoside phosphorylase[5] and it had been shown[56] that nucleosides are not substrates for xanthine oxidase. This apparently anomalous behavior was resolved by the findings[57] that the oxidation of 6 was mediated by an aldehyde oxidase and that, in fact, 1 and 6 were not substrates for, but competitive inhibitors of, xanthine oxidase.

There is strong evidence that formycin (1) exerts its action on neoplastic cells at the nucleotide level.[5] Ehrlich ascites tumor cells rapidly phosphorylated 1, in vitro, with the 5'-triphosphate being the major product.[5] Formycin (1) was a less effective inhibitor of de novo purine synthesis in a subline of Ehrlich ascites which lacked adenosine kinase.[5] Studies[5] have substantiated that 1 is a good substrate for the adenosine kinase in human tumor cells. Investigations[5,58] of the effects of 1 on purine metabolism in Ehrlich ascites tumor cells, showed that 1 depressed the synthesis of intermediates (FGAR and PRPP) in the purine de novo synthetic pathway and that 1 was an effective, in vitro, inhibitor of hypoxanthine phosphoribosyl transferase. In synchronized HeLa cell cultures, 1 inhibited an important factor in early DNA synthesis phases.[5]

Along with a number of other modified nucleosides, 1 has been used as a base modified, model adenosine analog in a study[59] of the biphasic inhibitory and stimulatory affects of adenosine on adenylate cyclase. In another study,[60] formycin (1) inhibited the synthesis of low molecular weight RNA and nucleolar processing in HeLa cells, while not affecting the synthesis of either heterogeneous, nuclear RNA or 45-S pre-ribosomal RNA. The observed, selective inhibition of low molecular weight RNA synthesis by 1, led to the suggestion[60] that a separate polymerase was responsible for the synthesis of such r-RNAs.

Formycin B (6) was not a substrate for human erythrocytic nucleoside phosphorylase.[5] In fact, formycin B (6) has been found to be a competitive inhibitor of human erythrocytic purine nucleoside phosphorylase (PNPase).[57] In the previously cited study[51] on the morphogenic effect of 6 on D. discordeum, the principal mode of action of 6 was found to be competitive inhibition of PNPase. The inhibition of X. oryzae by 6 is the result of interference with purine and pyrimidine metabolism by blocking the entry of exogenously supplied nucleosides into the cell.[5] Competitive inhibition of the uptake of uridine by human erythrocytes has also been shown for 6.[61]

Detailing the pathway(s) of the biosynthesis of formycin (1) and formycin B (6) has been the subject of several investigations. The finding[28] that hadacidin, an inhibitor of adenylosuccinate synthetase, blocked the amination of 6, suggested that 1 was formed from 6 in a

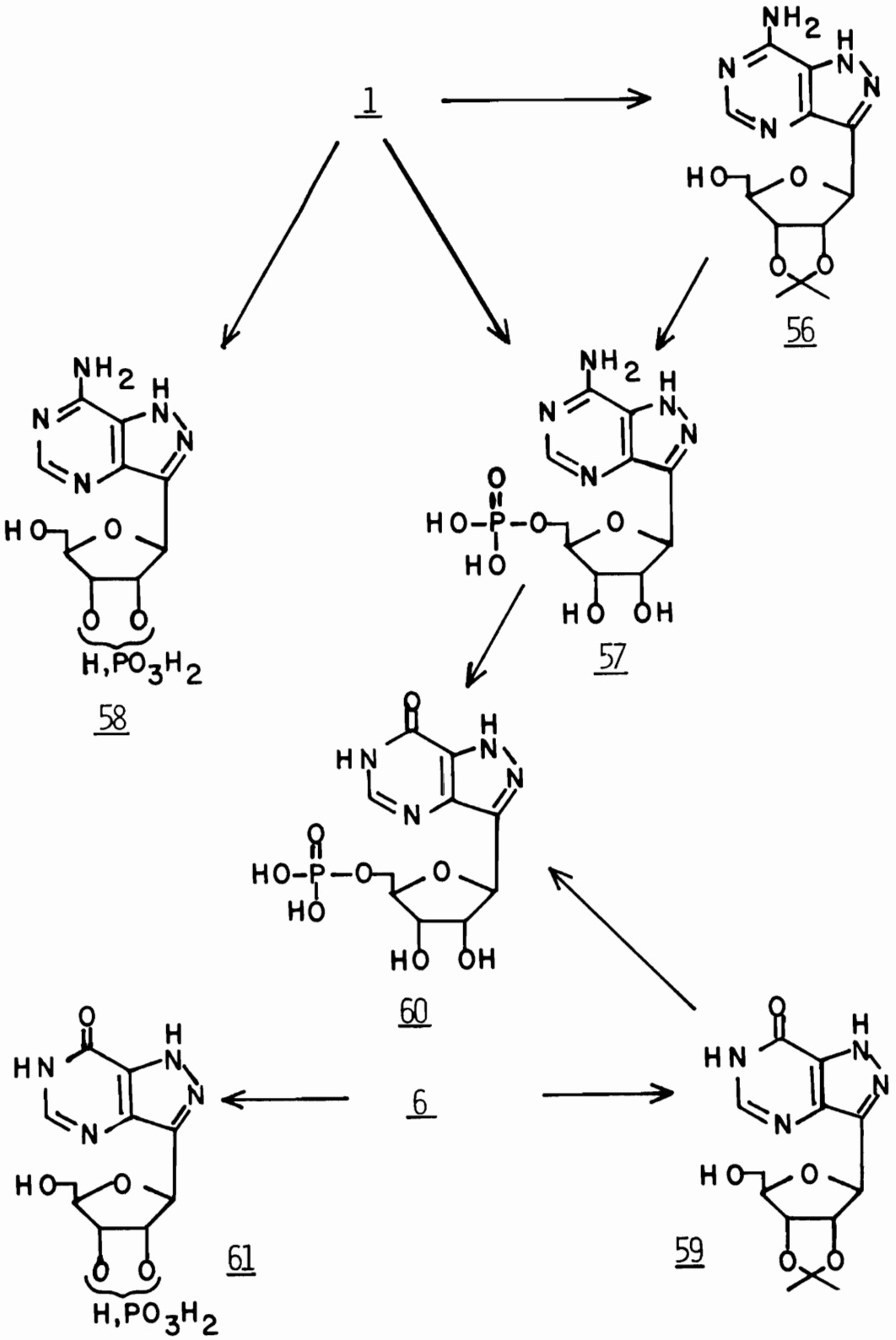
manner similar to the biosynthesis of adenosine. Thus, it was proposed[28] that formycin B-5'-phosphate (from an unknown source) was converted to formycin-5'-phosphate *via* a succinate and then dephosphorylated to give 1. A later study found[62] that adenine, adenosine, pool adenylic acid and RNA-adenine were not direct precursors of 1, concluding that the biosynthesis of 1 was not closely related to purine metabolism. Recent studies[63-65] using *Streptomyces sp.* MA-406-A-1, which quantitatively converts formycin B into formycin, have further delineated the biosynthetic pathway. One of the carbons of the aglycon, both of the nitrogens in the pyrazole ring and one of the nitrogens in the pyrimidine ring of 1 come from lysine.[65] Glutamate was incorporated into 1 through a pathway other than that of degradation to γ-aminobutyrate.[65] When radiolabelled ammonium chloride was added to the *Streptomyces* medium, two nitrogens of the aminopyrimidine part of 1 were derived from this source.[65] A novel enzyme(s) was found[64] to be responsible for the amination of 6, utilizing aspartate, giving 1 and fumarate. 7-Aminopyrazolo[4,3-d]-pyrimidine (the aglycon of 1) inhibited the formation of 1 from 6, but not the action of adenylosuccinate synthetase or adenylosuccinate lyase.[64] Additionally, auxotrophs of *Streptomyces sp.* MA-406-A-1 lacking either adenylosuccinate synthetase of lyase readily converted 6 into 1.[64] It was noted, that in the presence of GTP, the adenylosuccinate enzymes did utilize aspartate to convert formycin B-5'-phosphate (FBMP) to formycin-5'-phosphate (FMP) at approximately ten-percent of the rate observed for the synthesis of inosinic acid.[64]

Isono and Suhadolnik have reported[66] that sedoheptulose-7-phosphate, derived from the hexose monphosphate oxidative pathway, was the immediate precursor of the ribose moiety of the C-nucleoside, minimycin (2), and have suggested that this carbohydrate might similarly be a precursor for other C-nucleosides. The studies in S. sp. MA-406-A-1[63-65] indicated, however, that ribose is a more likely precursor to the carbohydrate moeity of 1. The aglycon of 1 was not converted to 1 via a salvage pathway[65] and was, in addition to pyrazofurin (5), an inhibitor of the biosynthesis of 1 in S. sp. MA-406-A-1.[65]

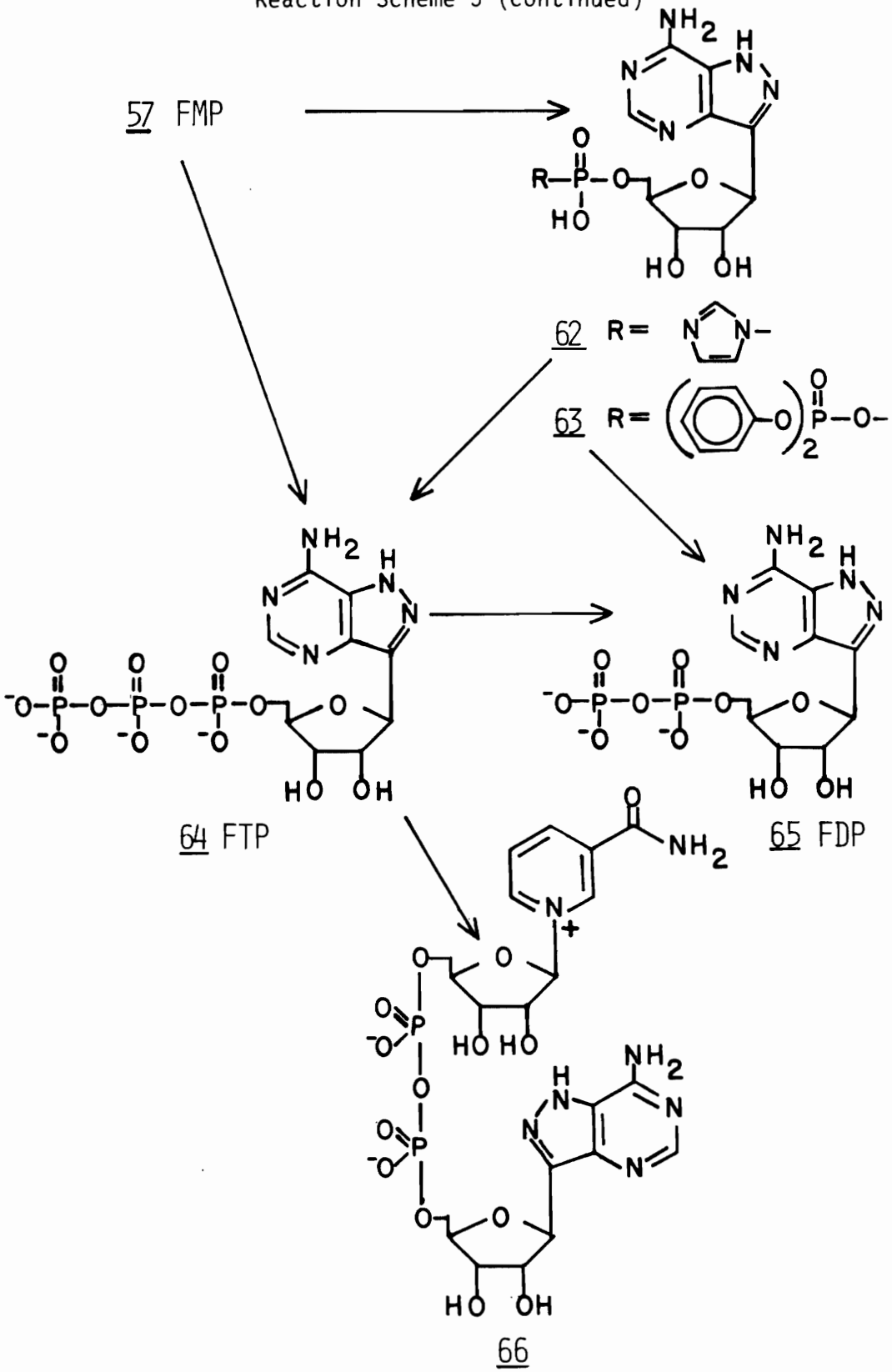
Derivatives of Formycin, Formycin B and Oxoformycin

The nucleotides of formycin (1) and formycin B (6) have been prepared by a variety of chemical and enzymatic methods. Formycin-5'-phosphate (FMP, 57) has been synthesized by conversion of 1 to the 2',3'-O-isopropylidine, 56,[6,12,13] and subsequent phosphorylation with phosphoryl chloride.[5] FMP has also been synthesized by direct phosphorylation of 1 with phosphoryl chloride in trialkylphosphate[67] or acetone,[16] and by enzymatic means using either a strain of Serratia marcescens[6] or wheat-shoot phosphotransferase.[68] Both of the enzymatic syntheses utilized p-nitrophenylphosphate as the phosphate donor. When 1 was added, along with p-nitrophenylphosphate, to a strain of Proteus mirabilis, formycin-2'(or 3')-phosphate (58) was the product.[6] Formycin B-5'-phosphate (FBMP, 60) has been synthesized by phosphorylation of the 2',3'-O-

Reaction Scheme 5



Reaction Scheme 5 (continued)



isopropylidine, 59,[6] and by enzymatic deamination of FMP (57).[6] It was found[6] that Serratia marcesens would not phosphorylate 6 but that Proteus mirabilis, with p-nitrophenylphosphate as the donor, did convert 6 into formycin B-2'(or 3')-phosphate (61).

Formycin-5'-triphosphate (FTP, 64) has been obtained from the reaction of pyrophosphate with the phosphorimidazolidate, 62,[67] and from the reaction of an amine salt of 57 with orthophosphoric acid.[16,69] FTP (64) has also been obtained by incubation of 57 with myokinase and phospho-enol-pyruvate kinase.[16] Formycin-5'-diphosphate (FDP, 65) was formed from the reaction of phosphate with the diphenyl-pyrophosphate, 63,[16] and also by incubation of 64 with hexokinase.[16]

FMP (57) has shown activity equivalent to 1 against Yoshida sarcoma, in vitro, and X. oryzae, but FBMP (60) only showed equivalent activity against X. oryzae.[70] Neither 57 nor 60 showed any detectable activity against influenza virus.[70] Compound 58 showed one-tenth of the activity observed for 1 against Yoshida sarcoma and one one-hundredth of the observed activity of 1 against X. oryzae.[70] The formycin B analog, 61, showed equivalent activity only against the latter organism and neither 58 nor 61 was active against influenza.[70] 2',3'-O-Isopropylidineformycin B (59) was tested against Yoshida sarcoma, X. oryzae and influenza and was found inactive.[70]

FMP (57) has been deaminated by rabbit muscle 5'-adenylic acid aminohydrolase.[71] The incorporation of formycin nucleotides into t-RNA has also been reported.[5,67,72] Formycin can replace adenosine

at the 3' end of t-RNA and efficiently transfer amino acids into peptides.[5,16] Formycin was incorporated into the 3-'terminus of yeast phenylalanine t-RNA and then oxidized with periodate.[67] The resulting dione was reduced (borohydride) to furnish a competitive inhibitor of the aminoacylation reaction.[67] Formycin nucleotides have been found to be effective replacements for adenosine nucleotides as substrates for polymerases.[5,16] Several studies of oligonucleotides and polynucleotides containing formycin have been reported.[5,16] The unexpected behavior of certain of these formycin containing polymers, in particular poly-F, has led to theories relating the conformation about the glycosidic bond of 1 with observed behavior.[5,16] The fluorescence spectrum of polymers containing formycin nucleotides has been the subject of other studies.[5,67]

FTP (64) is not a substrate for NAD synthetase[16] but is a substrate for NAD pyrophosphorylase.[16,73] Suhadolnik, et al.[73] prepared nicotinamide formycin dinucleotide (NFD, 66) from 64 and nicotinamide mononucleotide (NMN) by the enzyme (NAD pyrophosphorylase) catalyzed reaction. This fraudulent NAD was one of several NAD analogs used to study the structural requirements of the coenzyme binding site in four different dehydrogeneases.[73] This study found[73] that there was a tighter binding of NFD, when compared to NAD, to horse liver dehydrogenase and lactate dehydrogenase, but that catalysis was slowed.

That a formycin nucleotide is a substrate for a nucleotide reductase can be assumed since the appearance of formycin in the DNA

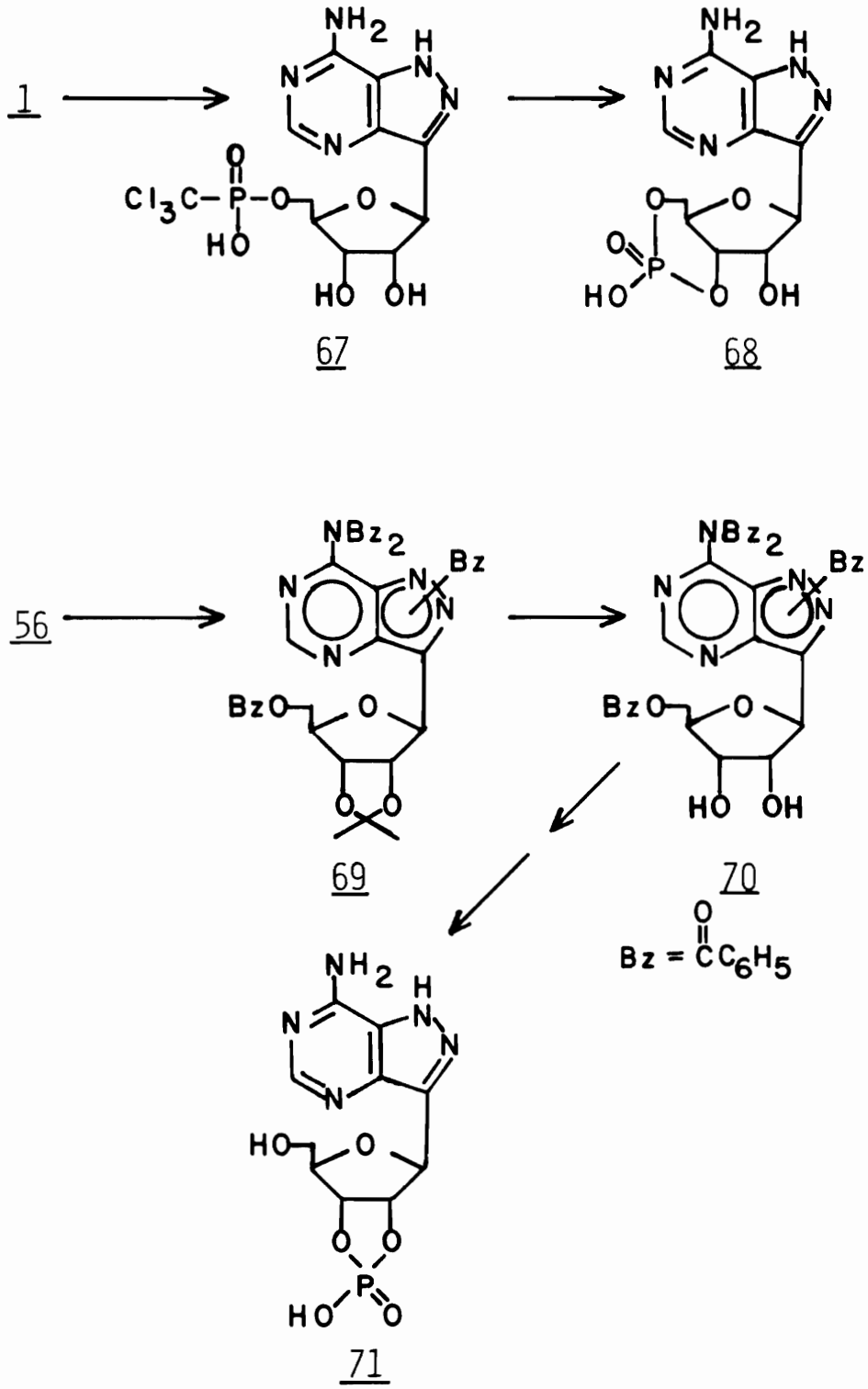
of L-cells has been reported.[16] The regulatory site of the nucleoside triphosphate reductase of L. leichmannii did not, however, accomodate FTP (64) since 64 had little effect on the reduction of CTP by this enzyme.[74] Curiously, this latter study[74] did not comment on the acceptability of 64 as a substrate for the L. leichmannii reductase.

Formycin cyclic-phosphates have only recently been described.[75] Treatment of 1 with trichloromethylphosphonic acid dichloride (TPAD)[76] in triethyl phosphate gave formycin-5'-trichloromethylphosphonate (67).[75] Hydrolysis of 67 gave formycin-3',5'-cyclicphosphate (68, 3',5'-cFMP).[75] The 2',3'-cyclicphosphate of 1, has also been prepared.[75] 2',3'-O-Isopropylidineformycin (56) was perbenzoylated to give 69, which was treated with formic acid to produce 70.[75] Compound 70 was phosphorylated with phosphoryl chloride, then debenzoylated to give formycin-2',3'-cyclic phosphate (71, 2',3'-cFMP).[75]

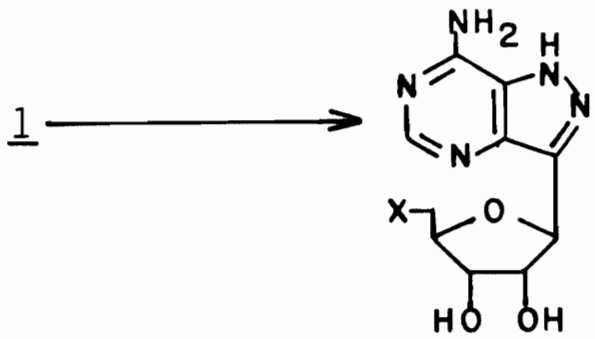
Takadiastase adenosine deaminase, deaminated the cyclic phosphates 68 and 71, but much more slowly than it deaminated formycin.[75] The cyclic phosphates were not substrates for calf intestinal mucosa adenosine deaminase.[75] 3',5'-cFMP (68) inhibited rat brain c-AMP phosphodiesterase with an ID_{50} similar to that observed for theophylline.[75] The ID_{50} for 68 against L-1210 cell cultures was approximately six fold greater than the ID_{50} for formycin,[75] however, 68 was ineffective against L-1210 in vivo.[75]

A B_{12} coenzyme analog incorporating 1 has been synthesized and studied.[77] Formycin (1) was treated with thionyl chloride in

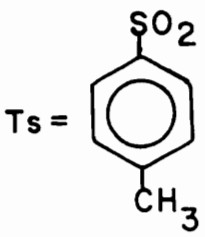
Reaction Scheme 6

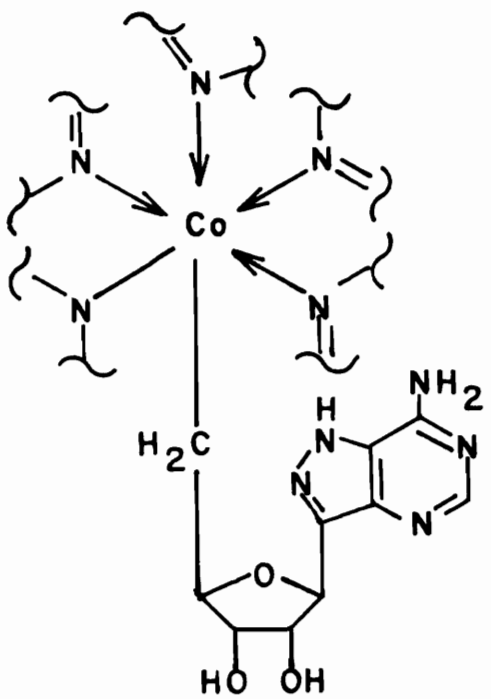


Reaction Scheme 7



72 X = Cl
73 X = NH_2
74 X = OTs





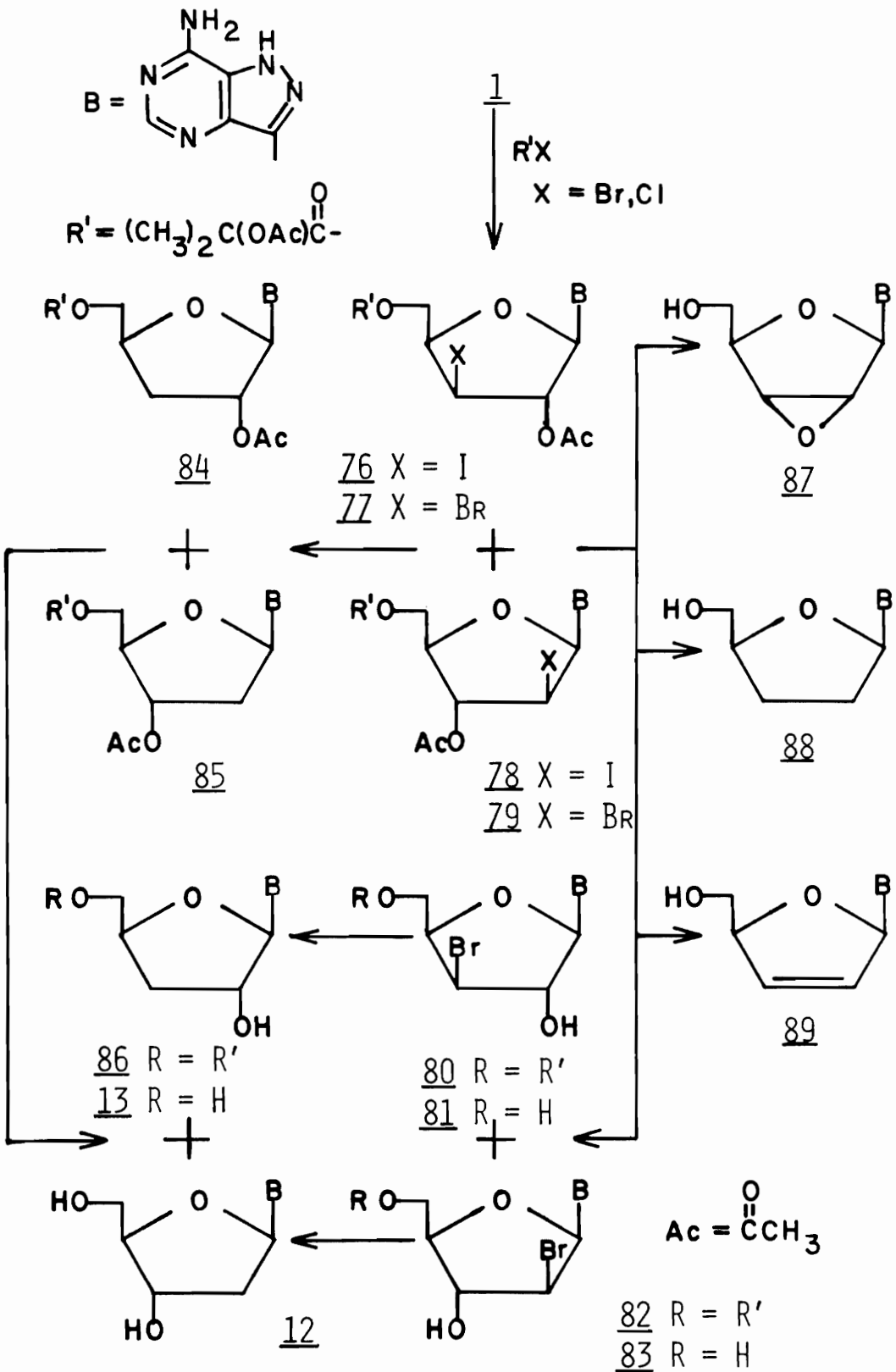
75

hexamethylphosphoramide to obtain 5'-chloro-5'-deoxyformycin (72),[77] which was subsequently reacted with cob(I)alamine to form 5'-deoxy-5'-formycinylcobalamine (F-Cbl, 75).[77] The ability of this coenzyme analog to replace 5'-deoxy-5'-adenosylcobalamine (Ado-Cbl) as a cofactor in the B_{12} requiring nucleoside triphosphate reductase of L. leichmannii has been studied.[77] A fifty percent decrease in the activity of the enzyme was produced by 75 at a concentration of 2.5 μM.[77] The analog 75 was also found to be a competitive inhibitor (K_i = 10^{-6} M) of the reductase enzyme.[77] As a coenzyme, 75 had approximately six percent of the activity observed for Ado-Cbl.[77] F-Cbl also caused some inhibition of the transport of cyanocobalamine into L-1210.[77]

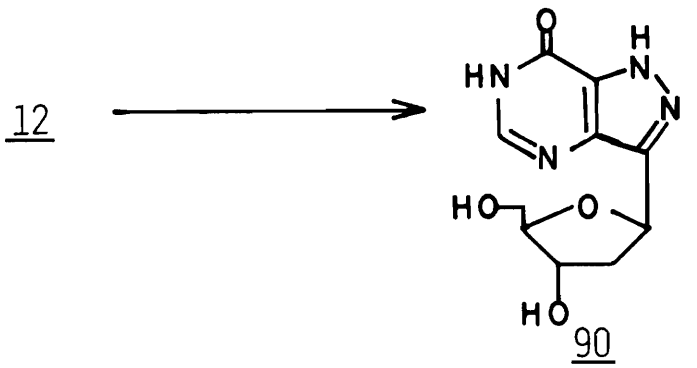
5'-Amino-5'-deoxyformycin (73) and 5'-deoxy-5'-O-(p-toluenesulfonyl)formycin (74) have been found to be inactive against Yoshida sarcoma (in vitro), X. oryzae and influenze.[70] The methods of syntheses for 73 and 74 used in this study were not disclosed.

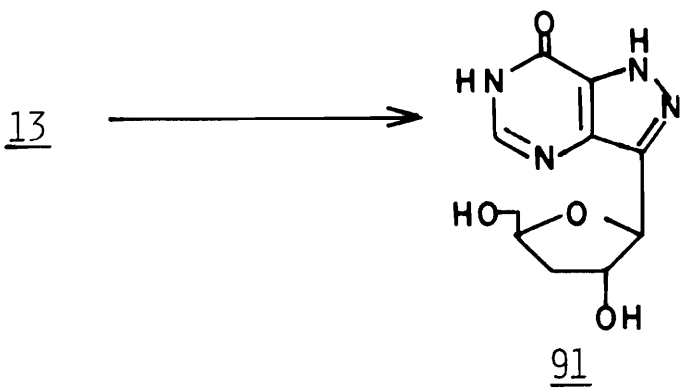
The synthesis of 2'-deoxyformycin (12) and 3'-deoxyformycin (13) were reported in two publications[17,18] which appeared at about the same time. Robins and coworkers[17] reacted 1 with a mixture of α-acetoxyisobutyryl chloride and sodium iodide to obtain a mixture of the 5'-O-(α-acetoxyisobutyryl)-2'-O-acetyl-3'-deoxy-3'-iodo-β-D-xylofuranosyl (76) and 5'-O-(α-acetoxyisobutyryl)-3'-O-acetyl-2'-deoxy-2'-iodo-β-D-arabinofuranosyl (78) derivatives. The mixture of 76 and 78 was not separated, but catalytically reduced to obtain

Reaction Scheme 8



Reaction Scheme 8 (continued)





a mixture of 84 and 85, which was subsequently deacylated with methanolic ammonia to give a mixture of 2'-deoxyformycin (12) and 3'-deoxyformycin (13).[17] The mixture was obtained (after chromatography on alumina) in a thirty percent yield and in a three to two ratio in favor of 13.[17] Compounds 12 and 13 were separated from the mixture by anion exchange chromatography, recovering twenty-five percent of 12 and fifty-six percent of 13.[17] The structures for 12 and 13, as well as those of the intermediates, were confirmed by elemental analyses and by ultraviolet, mass and proton magnetic spectral analyses.[17]

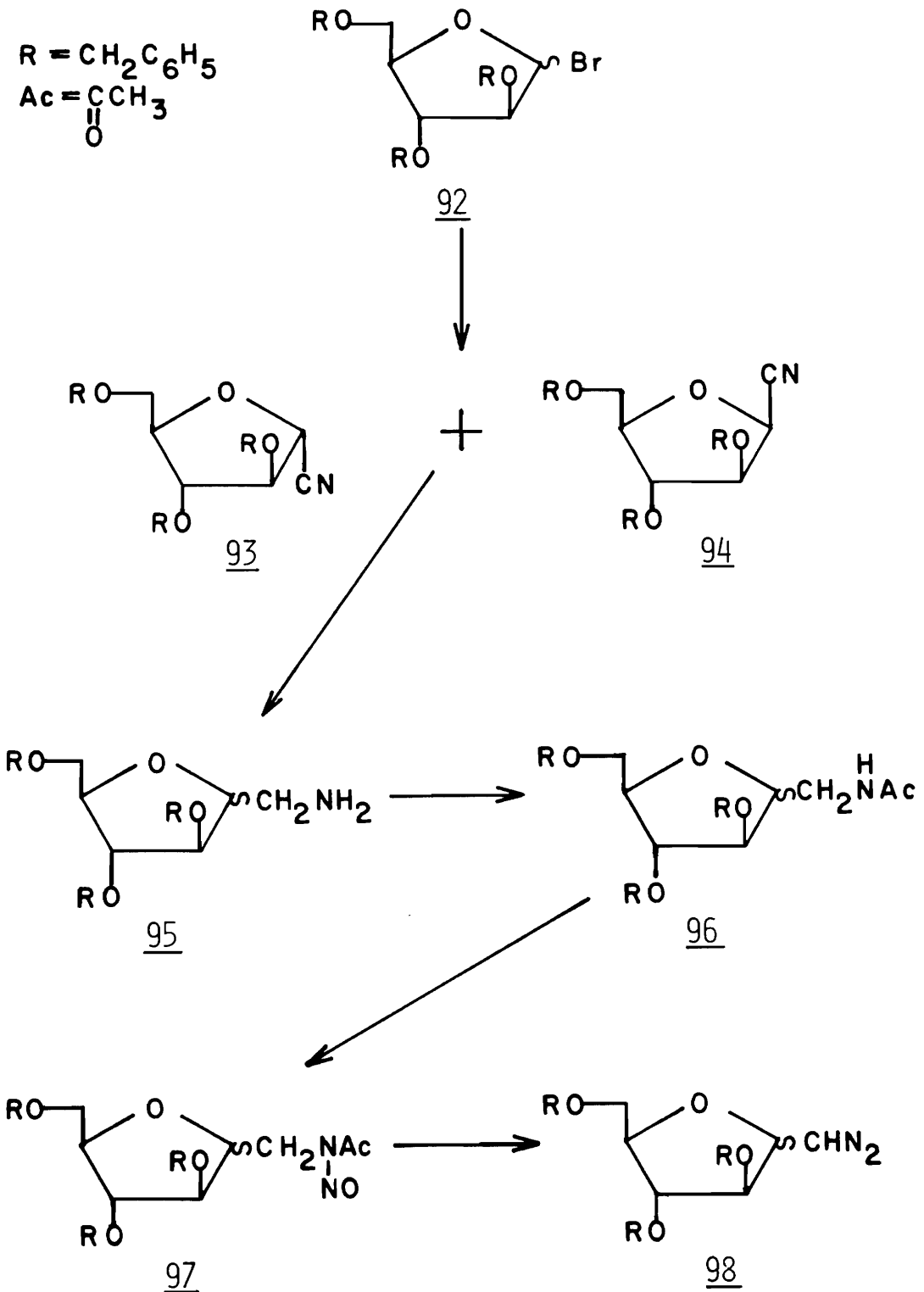
Jain, et al.,[18] used a similar approach for the synthesis of 12 and 13. The reaction of 1 with α-acetoxyisobutyryl bromide gave an inseparable mixture of 77 and 79.[18] The mixture was partially deacylated and then separated by preparative thin layer chromatography into 80 (sixty-one percent), 82 (twenty-six percent) and a mixture of 81 and 83 (nine percent).[18] Further treatment of the mixture of 80 and 82 with methanolic ammonia gave 81 and 83, which were separated by preparative thin layer chromatography in fifty-seven and eighteen percent, respectively, along with eight percent of 87.[18] Catalytic dehalogenation of 80 produced 86, which was then deacylated to 13.[18] Compounds 81 and 83 were reductively dehalogenated to yield 13 and 12.[18] The structures assigned to 12 and 13, and the intermediates, were, again, corroborated by elemental and spectral analysis.[18] The stereochemical relationship of 77 and 79 was established by conversion of the mixture of 77 and 79 into 87 by treatment with

methoxide.[18] Also, the mixture of 77 and 79 was treated with palladium in a hydrogen atmosphere and the product subsequently deacylated to give 2',3'-dideoxyformycin (88).[18]

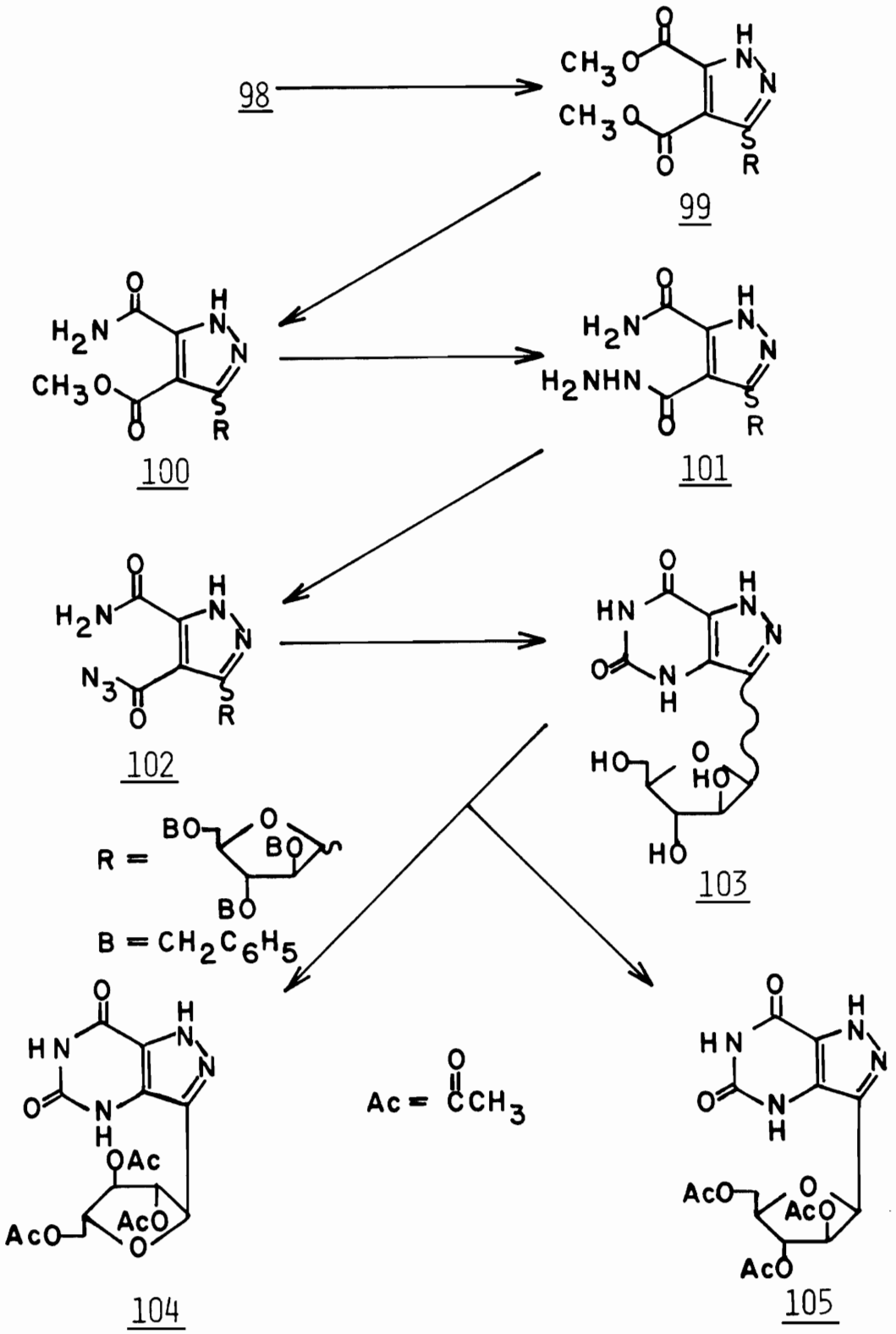
The mixture of 76 and 78 has been subjected to electrolysis and, following removal of the protecting groups, the 2',3'-unsaturated derivative 89 was obtained.[78] 2'-Deoxyformycin B (90) and 3'-deoxyformycin B (91) have been prepared by enzymic deamination (ADA) of 12 and 13.[17] No data regarding the biological activities of the deoxy derivatives was presented.

Acton, et al.,[79] have synthesized the arabinofuranosyl derivative of oxoformycin (14) by a method parallel to their synthesis of 14. 2,3,5-Tri-O-benzyl-D-arabinofuranosyl bromide was reacted with mercuric cyanide to give a mixture of the nitriles 93 and 94 in a four to one ratio, in favor of the latter.[79] The mixture of 93 and 94 was reduced with borane, to give the anomeric mixture 95, which was then acetylated to produce 96.[79] The anomeric mixture 96 was reacted with dinitrogen tetroxide and the resulting N-nitroso 97 was then treated with base to give an anomeric mixture of the diazomethylarabinose 98.[79] When 98 was added to dimethyl acetylenedicarboxylate, 99 resulted and was subsequently aminated to give 100.[79] The anomers of 100 were separated, obtaining a fifty percent yield of the β-anomer and an eleven percent yield of the α-anomer.[79] It was of some interest that the signal for the anomeric proton ($H_{1'}$), in the pmr spectra, for both anomers of 100, appeared as doublets with identical chemical shifts and coupling constants.[79]

Reaction Scheme 9



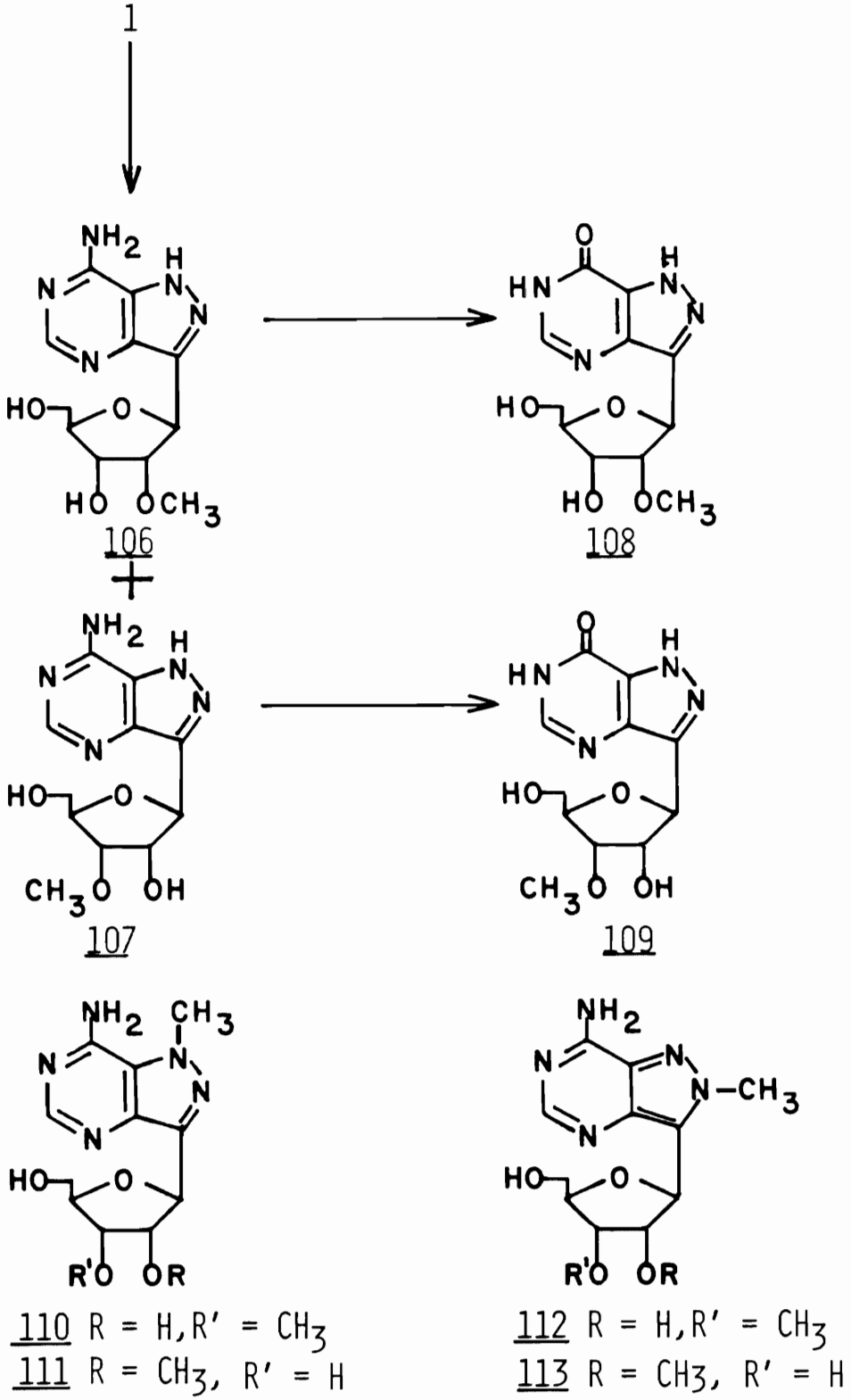
Reaction Scheme 9 (continued)



The anomers of 100 were, however, distinguished by differences in the chemical shifts of the protons of the methyl ester (α, OCH_3, s, δ3.78 and β, OCH_3, s, δ3.63).[79] Continuing the reaction sequence with the anomeric mixture, 100, was found to be a superior method since the β-anomer of the acetylated final product, 105, fractionally crystallized from the mixture.[79] The anomeric mixture, 100, was converted to the hydrazide, 101, then 101 was converted to the azide, 102, which underwent a Curtius reaction upon heating.[79] The product from the Curtius reaction was debenzylated to give a mixture of the α- and β-anomers of oxoformycin (103).[79] Acetylation of 103 gave a mixture of 104 and 105, from which the β-anomer, 105, selectively crystallized.[79] The pure β-anomer of 103 was then obtained by deacetylation of 105.[79] Ara-oxoformycin (103, β-anomer) has been found to be inactive and non-toxic when tested against L-1210 in mice.[79] The arabinosyl derivatives of formycin (1) and formycin B (6) have not been reported.

Robins, et al.,[80] have shown that treatment of formycin (1) with diazomethane, in the presence of stannous chloride, gives 2'-O-methylformycin (106, 17%) and 3'-O-methylformycin (107, 50%). Robins[80] also reported that other products, possibly dimethylated derivatives, were present in the reaction mixture and that the amount of these other products depended on the quantity of diazomethane used in the reaction. These other products were not, however, isolated or identified.[80] The reaction of 1 with diazomethane, in the presence of stannous chloride, was later

Reaction Scheme 10

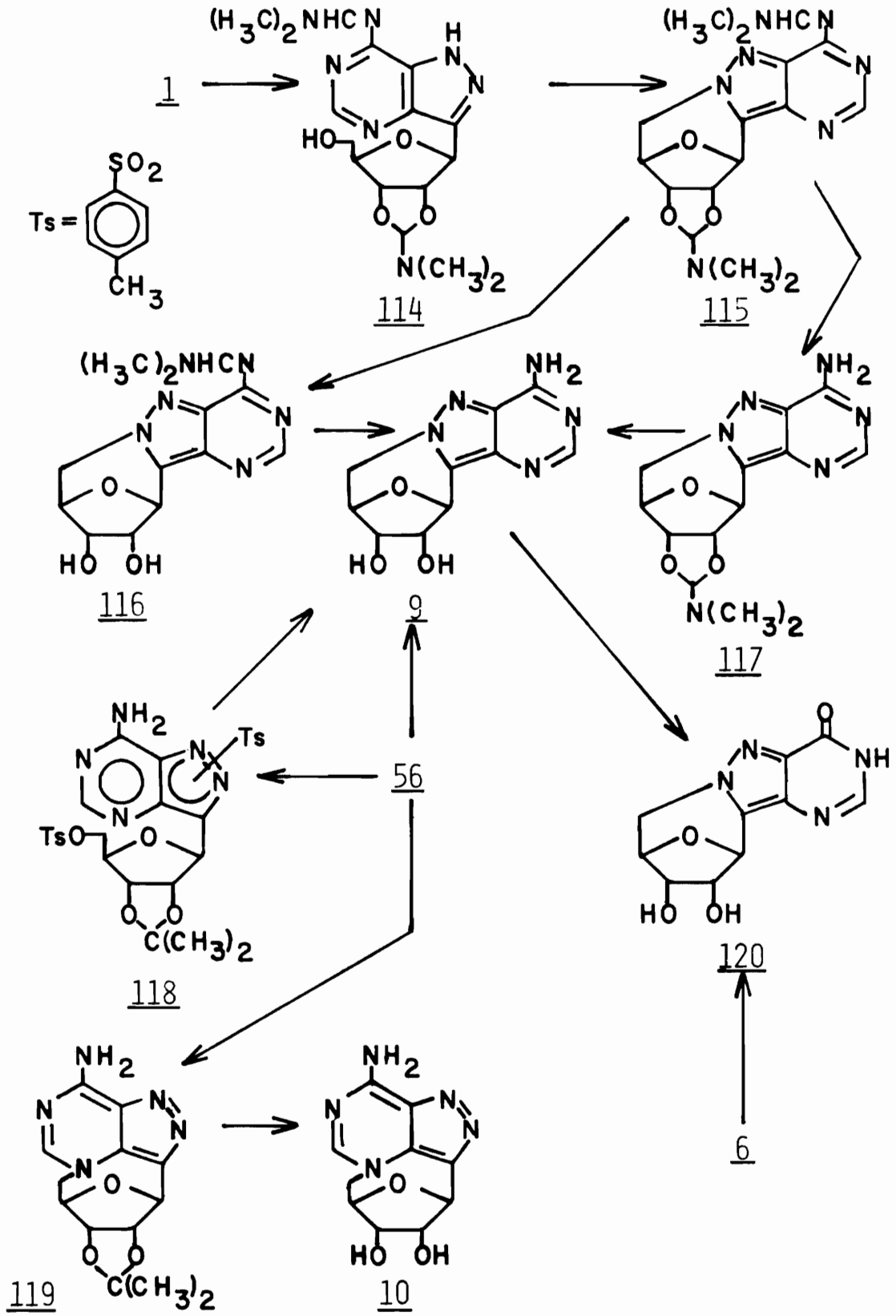


reinvestigated by Shugar and coworkers,[81] who found that, by altering the conditions, four other products could be obtained in addition to 106 and 107. These other products were identified as 3'-O,N_1-dimethylformycin (110, 4.5%), 2'-O-,N_1-dimethylformycin (111, 4%), 3'-O,N_2-dimethylformycin (112, 16%) and 2'-O,N_2-dimethylformycin (113, 11%) by elemental, chromatographic, spectral and enzymatic analysis.[81] The enzymatic (ADA) deamination of 106 and 107 to the corresponding formycin B derivatives, 108 and 109 has also been reported.[14]

2'-O-Methyl-(106) and 3'-O-methylformycin (107) were found to be inactive antiviral agents when tested against vaccinia, herpes simplex, and vesicular stomatitis virus.[14] Because of the inactivity observed for 106 and 107, the dimethylated derivatives, 110-113, were not biologically evaluated.[14]

The syntheses of the anhydronucleosides, 9 and 10, have been described in two independent reports.[12,13] Formycin was reacted with N,N-dimethylformamide dineopentyl acetal to form 114, which, upon further treatment with the same reagent, at elevated temperatures, gave 115.[12] The dimethylaminomethylene groups were selectively removed from 115; by treatment with water to give 116 or by treatment with ammonium hydroxide to give 117.[12] 2,5'-Anhydroformycin (9) was obtained by either treating 116 with ammonium hydroxide or 117 with water.[12] The anhydronucleoside, 9, was also obtained from 1 without isolation of the intermediates.[12] When 2',3'-O-isopropylidineformycin (56)[6,12,13] was treated with three equivalents of

Reaction Scheme 11



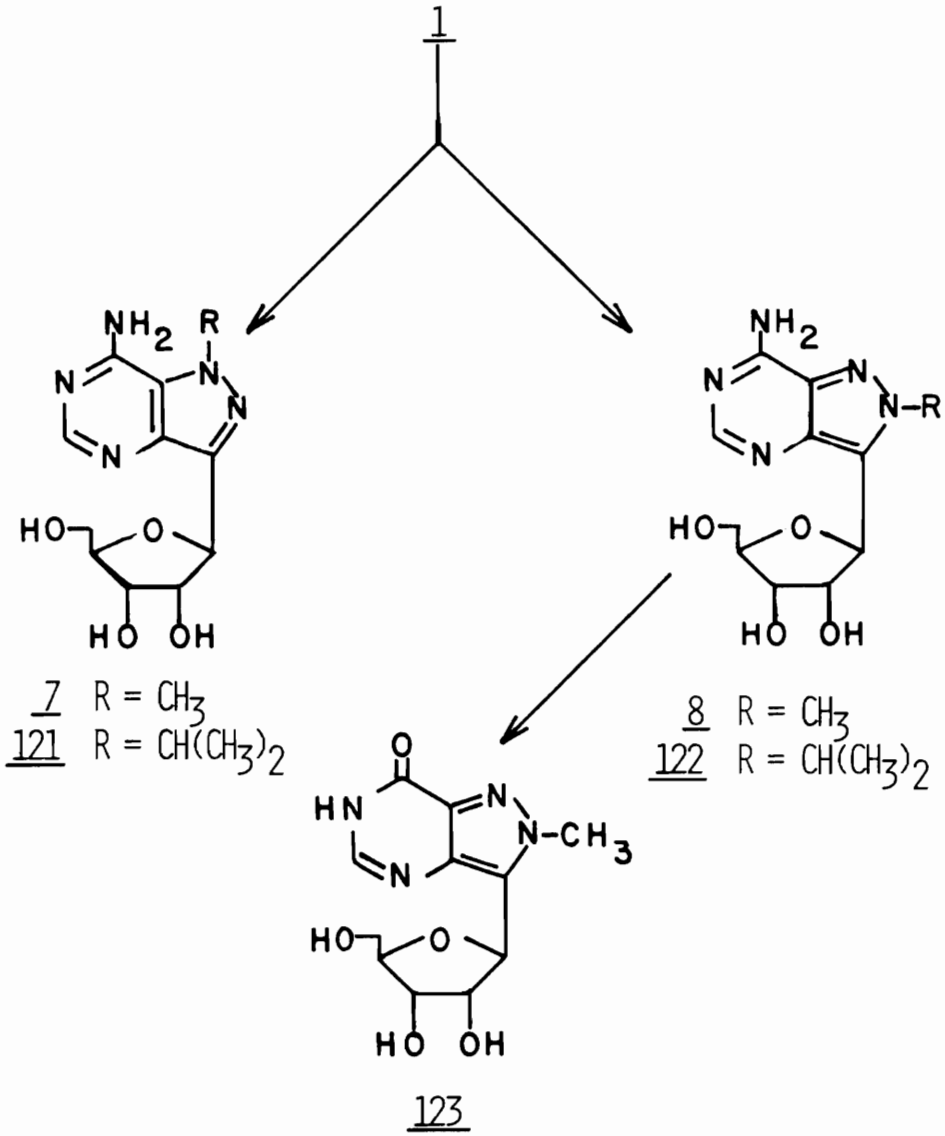
p-toluenesulfonyl chloride, the ditosyl derivative, 118, resulted.[13] Refluxing in dry dioxane converted 118 into an anhydronucleoside, which was subsequently treated with acid to produce 9.[13] Dehydration of 56 with a mixture of triphenylphosphine and diethyl azodicarboxylate gave 119, which was deblocked to provide 4,5'-anhydroformycin (10).[13] Makabe, et al.,[13] reported that treatment of 56 with only one equivalent of p-toluenesulfonyl chloride produced only a N_1 (or N_2) tosyl derivative, which when treated with ammonia reverted to 56. However, Zemlicka[12] treated 56 with one equivalent of p-toluenesulfonyl chloride to obtain a mixture of the 2',3',-O-isopropylidine derivatives of 9 and 10. Removal of the ketal and chromatographic separation afforded 9 and 10.[12] 2,5'-Anhydroformycin B (120) has been synthesized by treatment of 6 with dimethylformamide dineopentyl acetal in an identical manner as described for the synthesis of 9.[12] Enzymic deamination (ADA) of 9 also produced 120.[12] The structures of 9, 10 and 120 were established by elemental and spectral analysis.[12,13] Fluorescent and circular dichroic spectral studies[12,13,82] have attempted to correlate the rotameric conformation of 9 and 10 to their spectral characteristics.

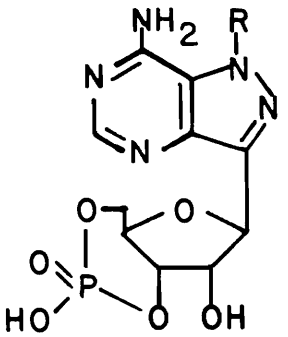
The anhydroformycins, 9 and 10, were inactive against Ehrlich ascites tumor and L-1210 in vivo.[13] These two derivatives were designed as models of the anti and syn conformers of formycin. The anti model, 9, was deaminated by both calf intestinal mucosa adenosine deaminase[12,13] and Takadiastase adenosine deaminase,[13] while the syn model, 10, was not a substrate for either enzyme.[12,13] Ogilvie[8] had previously suggested that only adenosine derivatives

which had an anti conformation about the glycosidic bond were substrates for adenosine deaminase. The results from the deamination studies of 9 and 10, thus, led to the extension of this postulate to the adenosine analog, formycin.[12,13] The rate of deamination of 9 by calf intestinal mucosa adenosine deaminase was found to be slower than the deamination of 1 by the same enzyme.[13] In contrast, 9 was a better substrate than 1 for the Takadiastase enzyme.[13]

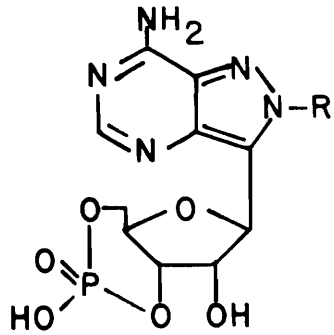
The first study dealing with alkylation in the aglycon of formycin (1) was published by Townsend, et al.[9] Under basic conditions, treatment of 1 with methyl iodide produced 1-methylformycin (7) and 2-methylformycin (8),[9] with the latter isomer predominating. The structures of 7 and 8 were established by spectral comparisons to the model compounds 7-amino-1,3-dimethylpyrazolo[4,3-d]pyrimidine and 7-amino-2,3-dimethylpyrazolo[4,3-d]pyrimidine, which were synthesized by unambiguous routes.[9] Treatment of 1 with diazomethane, without a Lewis acid catalyst, also has produced 7 and 8, with the 2-methylisomer again being predominant.[81] A nearly quantitative conversion of 1 to 7 was achieved from the treatment of 1 with N,N-dimethylformamide dimethyl acetal.[12,83] 2-Methylformycin has been converted into 2-methylformycin B (123) by chemical[9] and enzymatic (ADA)[81] deamination. A recent report[75] described the synthesis of 1-iso-propylformycin (121) and 2-iso-propylformycin (122) as well as the 3',5'- and 2',3'-cyclicphosphates of 7, 8, 121 and 122. The iso-propylformycins, 121 and 122, were prepared by reacting 1 with iso-propyl iodide under basic conditions.[75] The cyclicphosphates, 124 to 131, were prepared by methods similar to

Reaction Scheme 12

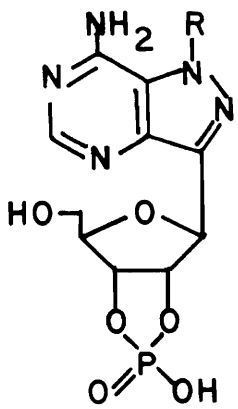




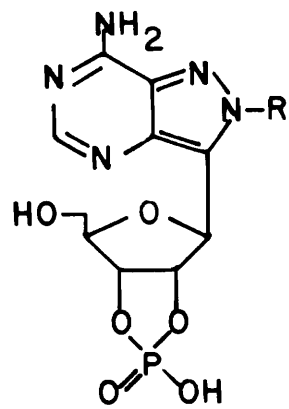
124 R = CH_3
125 R = $CH(CH_3)_2$



126 R = CH_3
127 R = $CH(CH_3)_2$



128 R = CH_3
129 R = $CH(CH_3)_2$



130 R = CH_3
131 R = $CH(CH_3)_2$

those described previously for the synthesis of 3',5'-cFMP (68) and 2',3'-cFMP (71).[75]

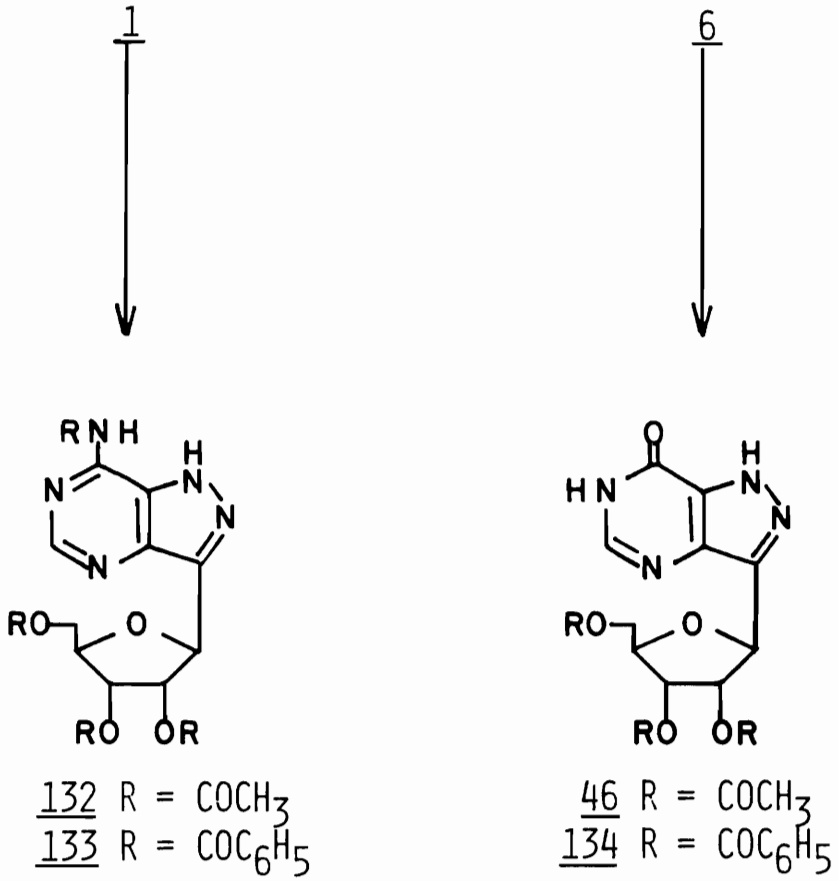
Townsend, et al.,[9] pointed out that, unlike the methylation of adenosine, N-methylation of 1 does not result in quaternization of the ring system and that methylation of the 2-position of 1 might restrict the rotation of the sugar moiety, resulting in a syn nucleoside. X-ray analysis[10] of 8, in addition to confirming the site of methylation, also confirmed the high degree of syn character for this nucleoside, at least, in the solid state. Several studies[14,15,75] have shown that 8 was a substrate for calf intestinal mucosa,[14,75] human erythrocytic[15] and Takadiastase[75] adenosine deaminase (ADA), but that 7 was a substrate only for the latter enzyme.[75] These findings seemed in direct contradiction to the findings from the studies[12,13] of the syn and anti models, 9 and 10, which led to the suggestion[81] that 8 might adopt an anti conformation upon interaction with adenosine deaminase. The iso-propylformycins, 121 and 122 were not substrates for either Takadiastase adenosine deaminase nor calf intestinal adenosine deaminase.[75] Also, none of the alkylated cyclicphosphates (124-131) were found to be substrates for either enzyme.[75]

A biological study[14] of 7 showed that it did not inhibit vaccinia, herpes simplex or vesicular stomatitis virus and also did not effect DNA or RNA synthesis in host PRK cell cultures. The same study[14] found, however, that 8 inhibited vaccinia virus at a concentration far below that which affected DNA or RNA synthesis in host

PRK cells. 2-Methylformycin (8) also exhibited some inhibitory effect against herpes simplex virus, but was inactive against vesicular stomatitis virus.[14] Compounds 7 and 8 had nearly identical therapeutic indexes against L-1210 *in vivo*.[11] The N^2-alkyl-3',5'-cyclic phosphates, 126 and 127, showed less inhibition of cAMP phosphodiesterase than did 3',5'-cFMP (68).[75]

Peracylation of formycin (1) produces tetra-acyl derivatives. Treatment of 1 with acetic anhydride in pyridine gave 132.[45] When 1 was treated with acetic anhydride in methanol, a diacetyl derivative was obtained and when 132 was refluxed with water, a monoacetyl derivative resulted.[45] These partially acetylated derivatives of 1 have not been further characterized. The reaction of 1 with benzoyl chloride in pyridine similarly afforded the tetrabenzoyl derivative 133.[45] The synthesis of N^1(or N^2), N^7, N^7, $O^{5'}$-tetrabenzoyl -2',3'-O-isopropylidineformycin (69), N^1(or N^2), N^7, N^7, $O^{5'}$-tetrabenzoylformycin (70) and analogous derivatives of the N^1 and N^2 alkylated formycins was previously discussed as these compounds were intermediates in the preparation of the respective 2',3'-cyclic phosphate derivatives, 71 and 128-131.[75] Also, as discussed previously, the acetylation (acetic anhydride in pyridine) of 6 afforded 46.[44,45] Similarly, 2',3',5'-tri-O-benzoylformycin B (134) was obtained from the reaction of 6 with benzoyl chloride in pyridine solution.[45] Oxoformycin (14) reacted with acetic anhydride in pyridine to give 2',3',5'-tri-O-acetyloxoformycin (135).[21a]

Reaction Scheme 13



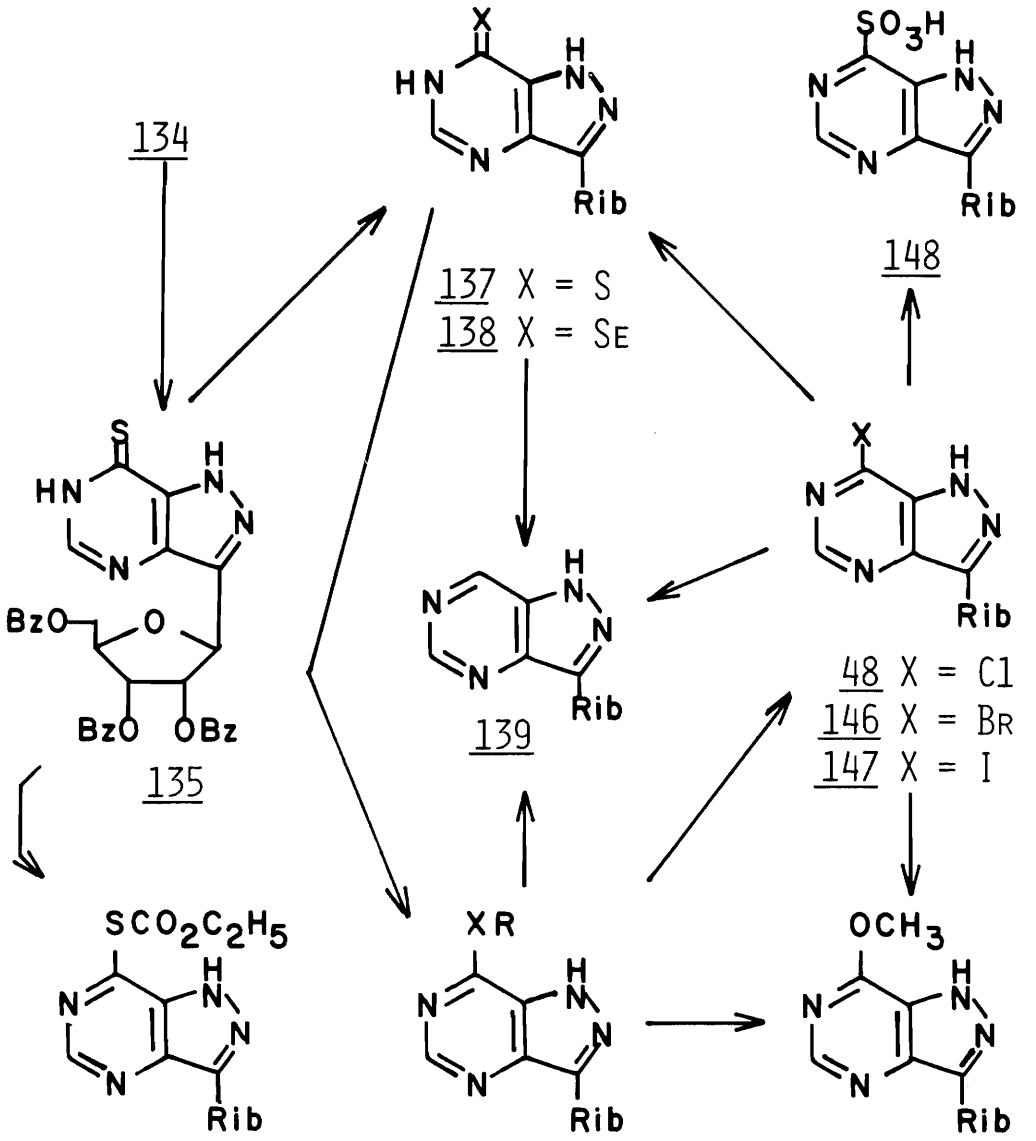
The partially acetylated formycins were found to have the same inhibitory effect against X. oryzae as did 1, however, the toxicity to rice plants was lower.[45] The fully acylated derivatives of 1, 6 and 14 were primarily prepared as intermediates in the synthesis of other derivatives.[21a,44,45,75]

The nucleoside 135 resulted from the treatment of 2',3',5'-tri-O-benzoylformycin B (134) with phosphorous pentasulfide.[45] When 135 was reacted with ethyl chloroformate and the product then debenzoylated, 7-ethoxycarbonylthio-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]-pyrimidine (136) was produced.[45] Debenzoylation of 135 by treatment with methoxide gave 3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidin-7-thione (137)[45] which has also been prepared from the reaction of 48 with thiourea.[44] Raney nickel dethiation of 137 afforded the nebularine analog, 139.[45] Alkylation of 137 with methyl iodide[44,45] and with allyl bromide[44] produced 140 and 141, respectively.

Compounds 137 and 140 were initially reported[45] to have approximately the same inhibitory effect on X. oryzae as formycin, however, with lower toxicity to rice plants. A later study[70] found that 140 was ineffective against X. oryzae and that 137 had one-tenth of the activity of 1 against this organism. Compounds 137 and 140 were slightly inhibitory to influenza virus[70], slightly active against Yoshida sarcoma, in vitro[70], but inactive against Ehrlich ascites.[45] The minimum inhibitory concentrations of 137 and 140 for St. aureus 209P and Erwinea aroideae have been reported.[45]

Milne and Townsend[84] used the versitile chloronucleoside 48 to prepare 3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidin-7-selenone

Reaction Scheme 14



(selenoformycin B, 138). The reaction of 48 with selenourea in refluxing ethanol afforded 138, which was found to be a surprisingly stable compound.[84] Alkylation, under basic conditions, of 138 with methyl iodide, benzyl bromide and p-nitrobenzyl bromide gave 142, 143 and 144, respectively.[84] Deselenation of 142 and dechlorination of 48 produced the same compound, 139, which corroborated the site of alkylation.[84] In a study of the differences in the reactivity of 142 and 48, both were converted into 145.[84] However, 142 yielded 145 only after treatment with methoxide at reflux temperature, whereas, 145 was obtained from 48 by treatment with refluxing methanol.[84] The seleno nucleosides, 138 and 142-144 did not exhibit any significant activity against L-1210 *in vivo*.[85]

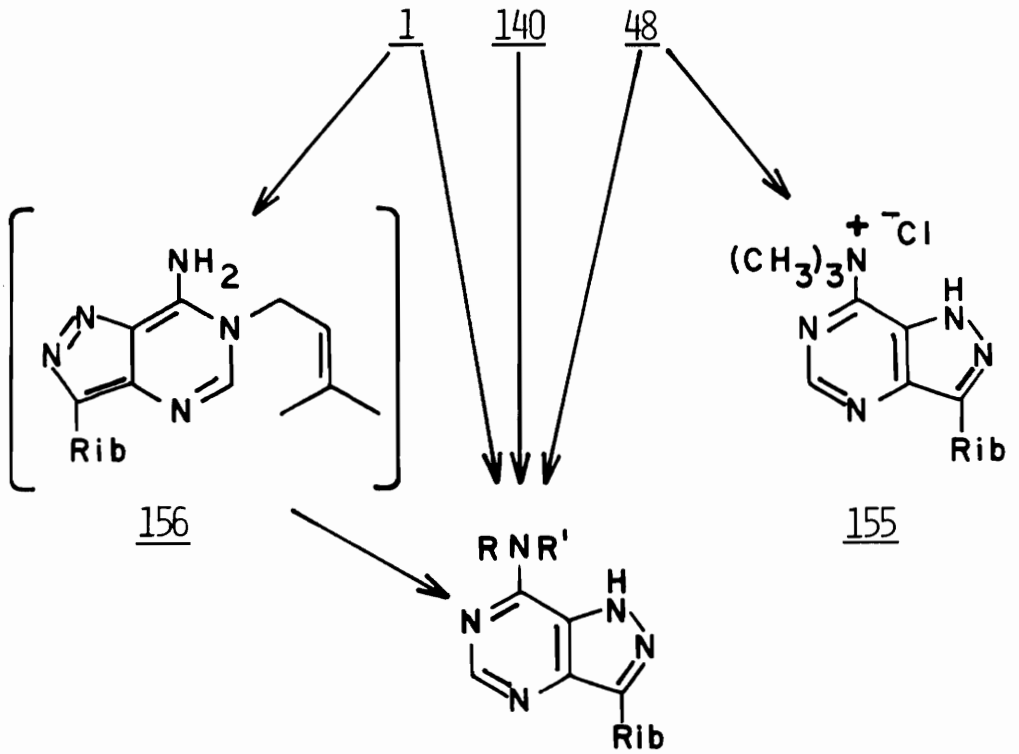
The reaction of 140 with chlorine, bromine and iodine has been reported[45] to afford 48, 146 and 147, respectively. The synthesis of 48 from 2',3',5'-tri-O-acetylformycin B[44] has been previously discussed. The inhibitory effect of the 7-halonucleosides against *X. oryzae* increased in the order 147>148>48 and the toxicity to rice plants decreased in the order 1>48>146>147.[45] The 7-halo compounds were ineffective against Ehrlich ascites[45], and influenza virus.[70] Compound 48 was slightly active against Yoshida sarcoma, *in vitro* and the activity was increased when coformycin (25) was present.[70]

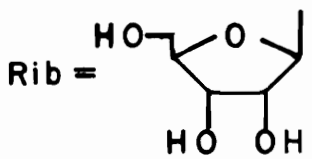
The 7-chloronucleoside, 48, was treated with sodium sulfite to afford the sulfonic acid, 148.[45] No biological data for 148 was reported.[45]

A variety of N^7-substituted formycins have been prepared.[44,45,86-88] The methylthio-nucleoside 140 was reacted in

alcohol solution, by heating in a sealed tube, with methylamine, dimethylamine, morpholine, hydrazine and hydroxylamine to obtain the corresponding formycin derivatives, 150-154, respectively.[45] The spectral data given for 153 and 154, however, indicated that the structures of these two nucleosides might be other than that reported. Long, et al.,[44] found the 7-chloro nucleoside, 48, to be a superior intermediate for the synthesis of N^7-substituted formycins. Treatment of 48, in alcoholic solution with hydrazine, hydroxylamine, and trimethylamine gave 153, 154 and 155, respectively.[44] The ultraviolet spectral data obtained for 153 and 154 prepared by this latter method[44] did not agree with the uv data obtained for the same compounds prepared by the former procedure.[45] The uv spectral characteristics of 153 and 154 as noted by Long, et al.,[44] resembled the uv spectral characteristics observed for 150-152, whereas, the earlier reported[45] data did not. Hecht, et al.,[86] first reported the synthesis of N^7-γ,γ-dimethylallylformycin (157) from alkylation of 1 at the 6-position with 3-methyl-2-butenyl bromide and subsequent rearrangement (Dimroth) of the intermediate, 156. Robins and Trip[87] reinvestigated this reaction and found 156 to be a minor component in a complex reaction mixture. Robins and Trip[87] prepared 157 and 158 from 48 by nucleophilic displacement of the chlorine with the requisite alkylamine. Hecht and co-workers[88] have reported that nucleosides 159, 160 and 161 were obtained from 1 by application of the exchange amination procedure of Whitehead and Traverso.[89]

Reaction Scheme 15





150 R = H, R′ = CH_3
151 R = R′ = CH_3
152 R,R′ = $-(CH_2)_2O(CH_2)_2-$
153 R = H, R′ = NH_2
154 R = H, R′ = OH
157 R = H, R′ = $CH_2CHC(CH_3)_2$
158 R = H, R′ = $CH_2C_6H_5$
159 R = H, R′ = $(CH_2)_4CH_3$
160 R = H, R′ = $(CH_2)_5CH_3$
161 R = H, R′ = $CH(CH_2)_4$

The compounds 150-154 were found[45] to be less effective against X. oryzae than 1, but did show some interesting variety in their antibacterial activities.[45] The minimum inhibitory concentrations of 150-154 against St. aureus, and either Erwinea aroidae, Mycobacterium 607, Mycobacterium tuberculosis BCG, Candida albicans or Sacchromyces cervisae have been reported.[45] Compounds 150 and 151 were inactive against Yoshida sarcoma in vitro and influenza virus.[70] Nucleoside 154 was reported[70] to be as active as 1 against Yoshida sarcoma and the activity was synergistic with coformycin (25). The activities reported for 153[45] and 154[45,70] must, however, be suspect as there is some doubt about the exact structure of the compounds tested. Of the derivatives prepared by Long, et al.,[44] 153, 154 and 155, only 7-hydrazino-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]-pyrimidine (153) has shown significant activity against L-1210 in vivo.[11] When 153 was administered daily for nine days, at a concentration of 100 or 150 mg/kg, beginning on the first day after inoculation of the test animals with tumor cells, a therapeutic index (T/C) range of 130 to 157 was obtained by repeated testing.[11]

N^7-Methylformycin (150) was designed[70] as an effective improvement on formycin (1) as it was assumed that 150 would not be deaminated nearly as readily as 1. Since 150 was found to be inactive against Yoshida sarcoma cells and influenza virus[70], the suggestion was made[5] that N^7-methylformycin (150) might not be a substrate for a kinase.

N^7-γ,γ-Dimethylallylformycin (157), an analog of the natural cytokinin, isopentenyladenosine, has been found[86,87] to have weak

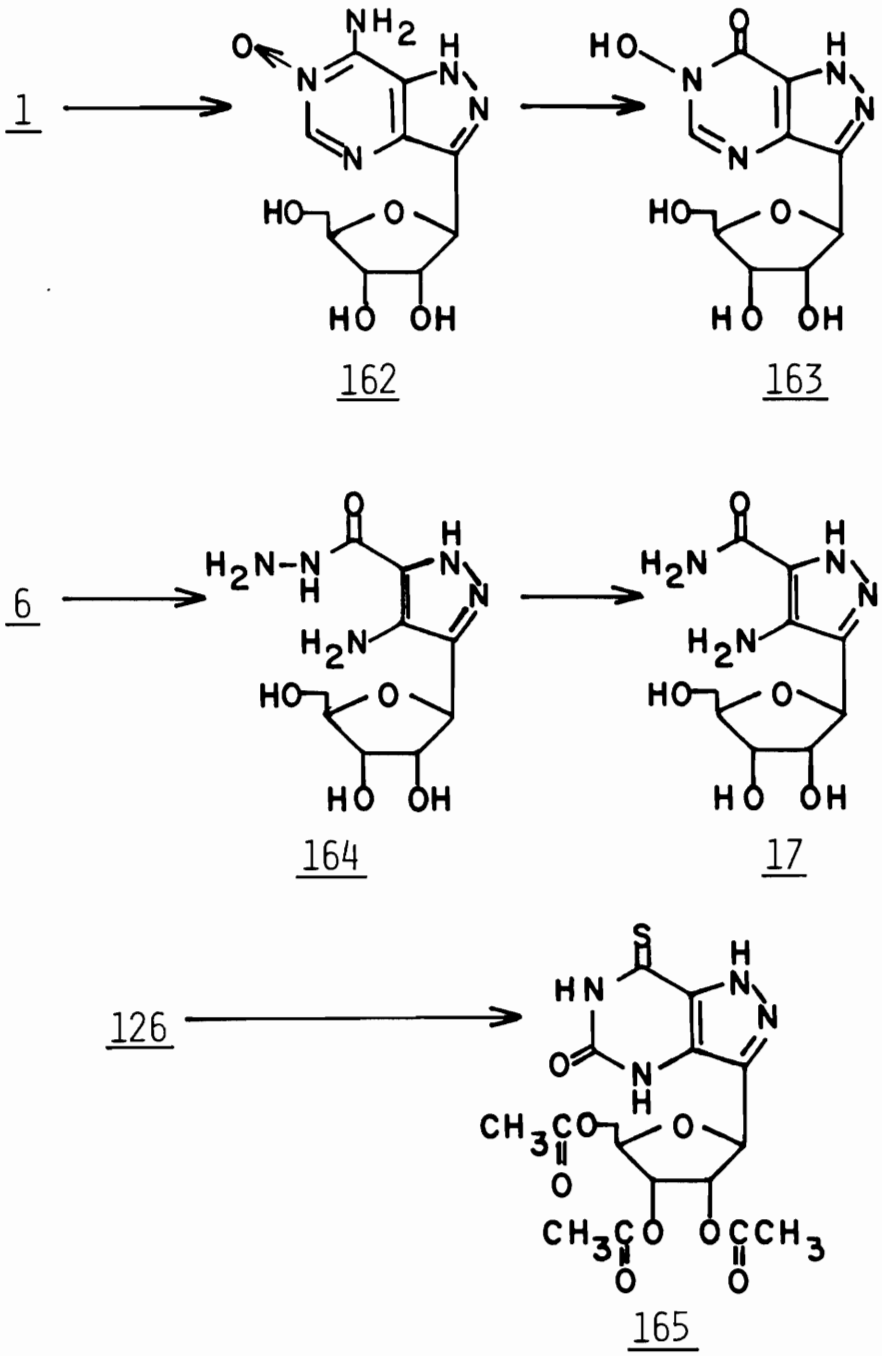
cytokinin activity. The compounds, 159, 160 and 161 were found[88] to have anticytokinin activities approximately equal to formycin. The slow deamination of 157 and 158 by adenosine deaminase has also been reported.[87]

Formycin (1) has been oxidized (m-chloroperbenzoic acid) to produce the N^6-oxide, 162.[21a] The site of oxidation was established[21a] by the similarity of the uv spectra of 162 to the uv spectra of 7-amino-3-methylpyrazolo[4,3-d]pyrimidine-N^6-oxide, which was obtained by an unambiguous synthesis.[90] Formycin-N^6-oxide was chemically deaminated into N^6-hydroxyformycin B (163)[21a]. Biological studies on 162 and 163 have not been disclosed.

Formycin B (6) undergoes pyrimidine ring fission when treated with hydrazine hydrate.[21] The product of this reaction has been established[21] as 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxhydrazide (164) by uv spectral comparisons to 4-amino-3-methylpyrazole-5-carboxhydrazide.[90] The carboxhydrazide, 164, was catalytically reduced[21] to give a low yield of the amide 17. 4-Amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide (17, ΨAPCA-riboside) is an isomer of an important intermediate in the purine-nucleotide *de novo* pathway, 5-amino-1-(β-D-ribofuranosyl)imidazole-4-carboxamide-5'-phosphate (AICAR) and can also be viewed as the amino analog of pyrazofurin (pyrazomycin, 5).

The only reported example of an oxoformycin derivative modified in the aglycon is nucleoside 165.[21a] This derivative was obtained from thiation (phosphorous pentasulfide) of 2',3',5'-tri-O-acetyl-oxoformycin (126)[21a]. The structure of 165 was established by the

Reaction Scheme 16



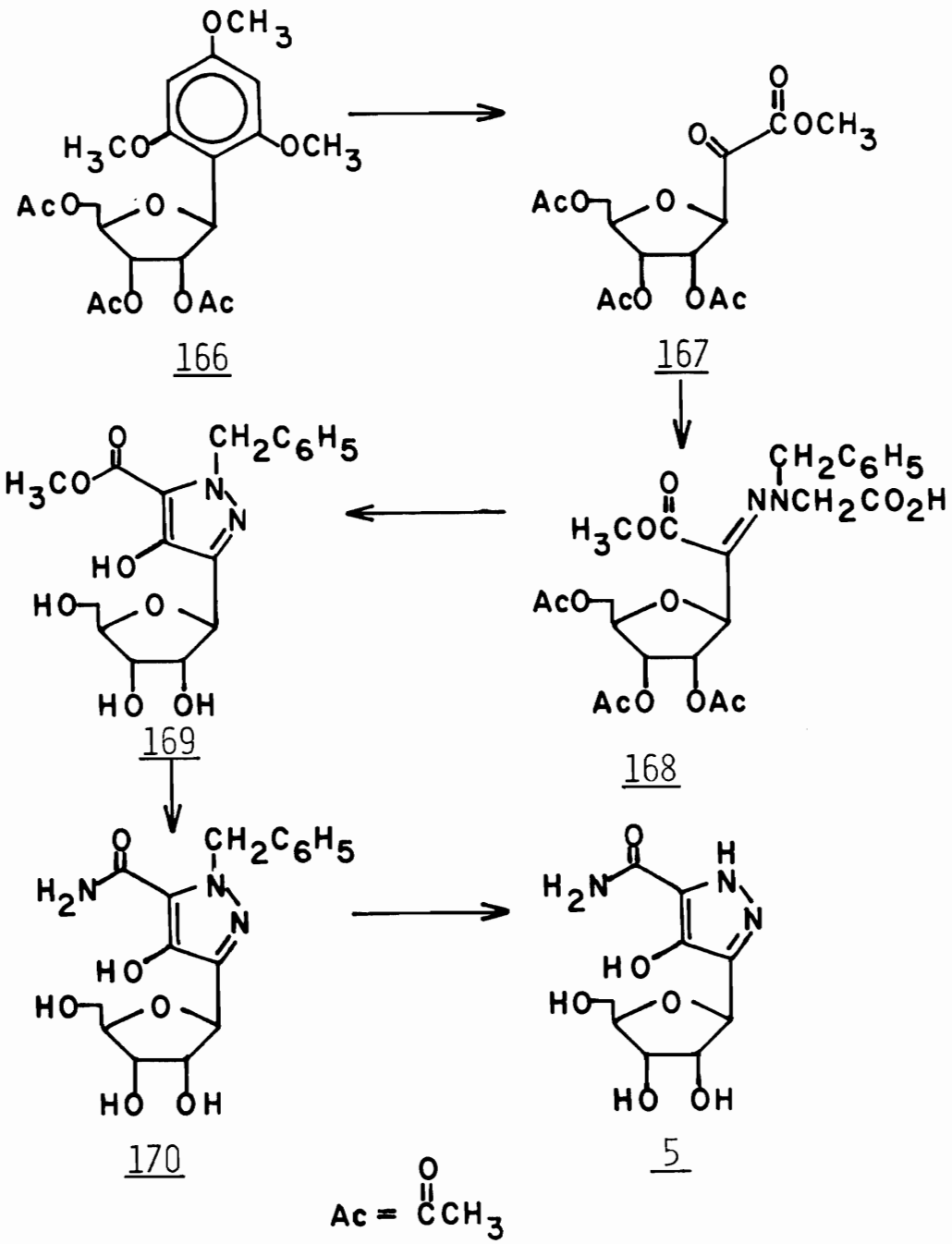
comparison of its spectra with the spectra of 3-methylpyrazolo[4,3-d]pyrimidin-7-one-5-thione[90] and 3-methylpyrazolo[4,3-d]pyrimidin-5-one-7-thione.[90]

Pyrazofurin

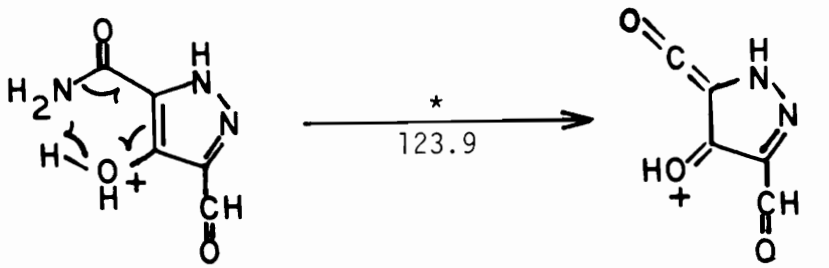
Pyrazofurin (pyrazomycin, 5), a naturally occurring pyrazole-C-nucleoside, is one of three C-nucleosides which have been isolated from a strain of Streptomyces candidas.[22] The structure of pyrazofurin has been established as 3-(β-D-ribofuranosyl)-4-hydroxypyrazole-5-carboxamide.[22] Pyrazofurin (5) has shown only limited antifungal activity but good antiviral activity against many virus.[22] In initial studies, pyrazofurin (5) was ineffective against L-1210, but has shown activity against a form of Walker 256 and 9, 10-dimethyl-1,2-benzanthracene (DMBA) induced mammary carcinoma.[22] The 5'-mono- and 5'-triphosphate of 5 as well as several acylated derivatives have been prepared and studied.[22] Pyrazofurin-5'-phosphate has been shown to be a potent inhibitor of orotidylic acid decarboxylase.[5]

The synthesis of pyrazofurin has been accomplished.[91] 2,4,6-Trimethoxy-1-(β-D-ribofuranosyl)benzene (166)[92] was ozonized to afford the α-ketoester 167.[91] Compound 167 was converted to the hydrazone 168, which, after esterification, underwent a Dieckman reaction[91] when treated with methoxide to produce the pyrazole-C-nucleoside 169.[91] The amide 170 was obtained by treatment of 169 with methanolic ammonia and was then debenzylated to give pyrazofurin (5).[91]

Reaction Scheme 17



The ultraviolet spectrum of 5 is unique in that a maximum at 307 nm is observed for 5 in basic (pH 12) solution.[4,5] By contrast, Long[21a] reported that the 4-amino analog of pyrazofurin, 17, exhibited a maxima at 283 nm in the uv spectrum (pH 11). Another interesting feature of 5 was noted upon examination of its mass spectrum.[93] As discussed previously, existence of a b+30 ion in the mass spectrum is a diagnostic feature of C-nucleosides. A high intensity ion at m/e 139 was also observed in the mass spectrum of 5.[93] This ion resulted from the expulsion of ammonia from the b+30 ion, as shown, and is a diagnostic feature of the o-hydroxy-carboxamide grouping in 5.[93]



m/e 156.0406 m/e 139.0139

CHAPTER 3

STUDIES ON THE ISOMERIC N-METHYLFORMYCINS*

Background

To determine the biological effects attendant upon the changes arising from specific alkylation of the base moiety of formycin (1) in contrast to the *syn*, *anti* conformational relationships, the synthesis of 4-methylformycin (11) was investigated. Examination of CPK molecular models indicated that the *anti* conformation would be highly favored, sterically, for 11. The synthesis of 4-methylformycin would thus provide a formycin derivative similar, with respect to alkylation of the aglycon, to 4,5'-anhydroformycin (10) but with a predominately opposite rotameric conformation. A similar parallel (i.e., the same site of alkylation but an opposite, predominate conformation) exists between 2-methylformycin (8) and 2,5'-anhydroformycin (9).

The methylation of the formycin isomer, adenosine has been thoroughly investigated.[94,95] Methylation of adenosine under non-basic conditions (methyl *p*-toluenesulfonate in N,N-dimethylacetamide)

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* 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.

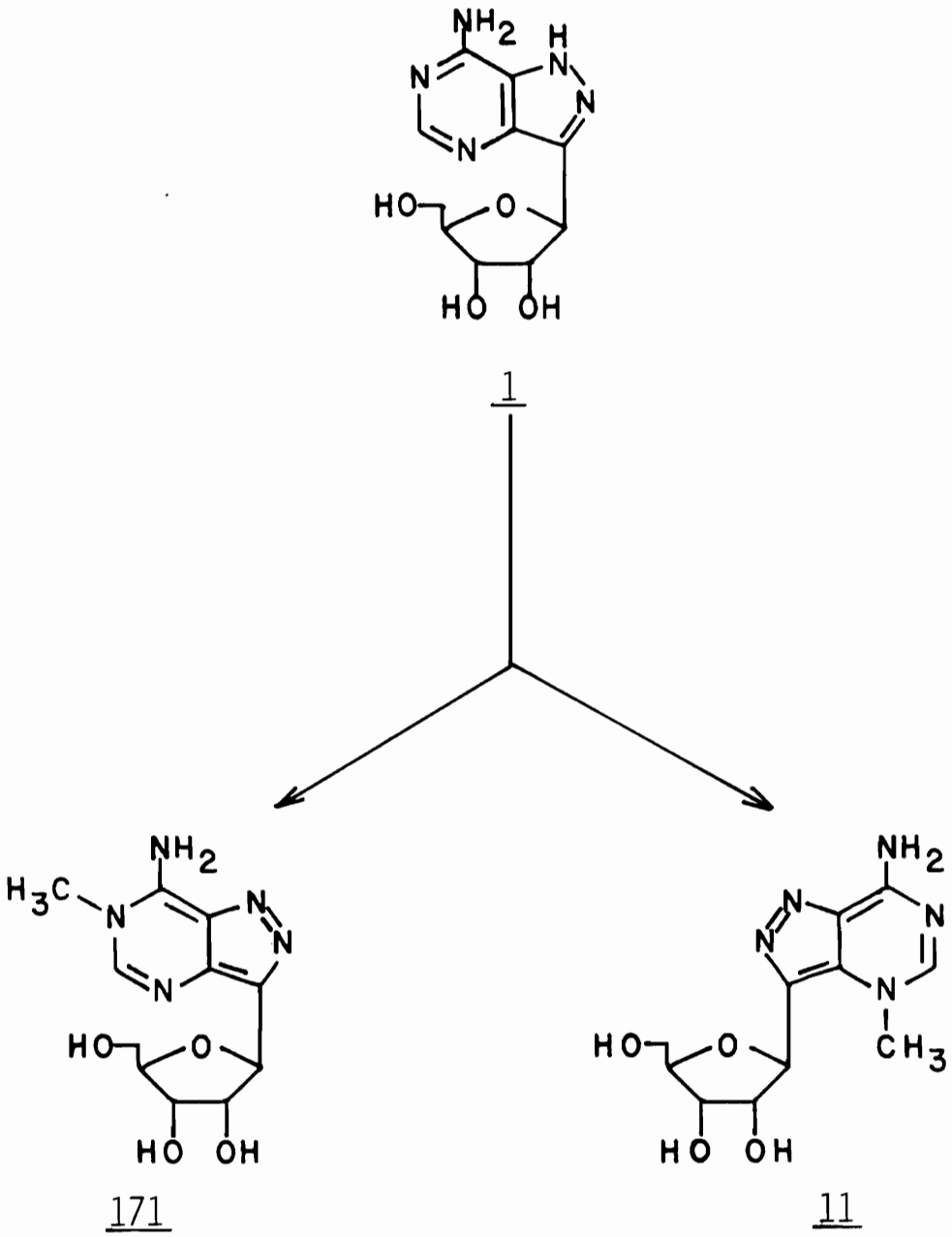
proceeds to give 1-methyladenosine.[94] Although, formycin is sterically akin to adenosine, the aglycon of 1 more nearly compares to adenine rather than the aglycon of adenosine. Unlike the methylation of adenosine, N-methylation of formycin does not result in quaternization of the ring system nor require the existence of the imino tautomer. The alkylation of adenine has also been thoroughly studied.[96,97] Adenine, under nonbasic conditions (methyl p-toluenesulfonate in N,N-dimethylacetamide), methylates in the 3-position.[96] Fujii, et al.,[97] have investigated the nonbasic alkylation of adenine with a wide variety of alkyl halides. This study found[97] that the 3-alkyl isomer predominated, but that 1-alkyl and/or 9-alkyl adenines were also obtained. Fujii, et al.,[97] pointed out that the predominate product resulted from the transition state which provided the best charge distribution and that delocalization of positive charge in the pyrimidine ring was most likely. The site of alkylation in the pyrimidine ring was also dictated by steric considerations. Solvents that favored the ionic character in the transition state (dimethylacetamide and dimethylformamide) were found to be most effective for alkylation in the pyrimidine ring.[97] Methylation (methyl iodide in dimethylacetamide) of the adenine isomer, 4-aminopyrazolo[3,4-d]pyrimidine (4APP) has been reported[98] to produce a mixture of the 5- and 7-methyl isomers. Early studies[33,99] of the 7-aminopyrazolo[4,3-d]pyrimidine system concluded that the nitrogen at the 4-position was most basic, and on this basis, methylation of 1 under nonbasic conditions was expected to give 4-methylformycin.

4-Methyl- (11) and 6-Methylformycin (171): Synthesis and Structure Elucidation

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° dec) was obtained in 50% yield, and the minor product (mp 268-269° dec) in 17% yield based on the assumption that they were mono-methylformycin derivatives. The ratio of the products as determined by uv analysis[100] of the reaction mixture was found to be 2:1 (low melting:high melting).

Pmr analysis confirmed that both products were mono-methylated 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- (7) (δ N-CH_3 = 4.28) nor 2-methylformycin (8) (δ N-CH_3 = 4.18) and chromatographic comparisons confirmed this fact. In the adenines, methylation of the exocyclic amine (i.e., 6-methylaminopurine) gives a methyl signal, in the pmr, which is upfield from the methyl signals observed for ring nitrogen methylated derivatives.[101] 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 150. A previous report[45] on the

Reaction Scheme 18

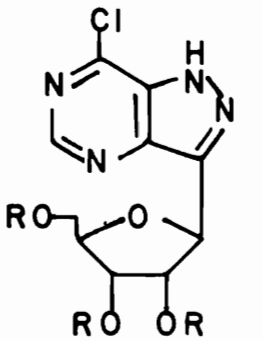


synthesis of 150 did not include pmr data which prompted the re-synthesis of 150 for this study.

This resynthesis of N^7-methylformycin (150) 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 (48)[44,45] with ethanolic methylamine. Treatment of 47[44] with ethanolic methylamine gave a mixture of products. Ultraviolet analysis of the reaction mixture showed that the 7-chloro group had been displaced. Ultraviolet analysis of the individual products showed that incomplete deacetylation had resulted. When the reaction mixture was treated with aqueous methylamine, 150 was the sole product. This latter procedure was a superior method for the synthesis of 150 as the overall yield was improved and required one less synthetic step. The nucleoside 150 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. This spectral data and chromatographic comparisons eliminated N^7-methylformycin (150) as the major isomer. Thus, the two products obtained were assumed to be 4-methyl- (11) and 6-methylformycin (171).

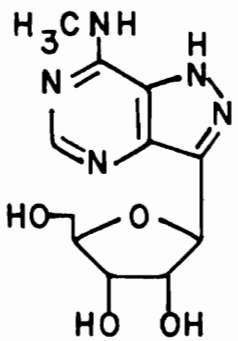
A comparison of the uv spectral characteristics of the five isomers (Table 1), with the published data for the cyclonucleosides 9 and 10, allowed a structural assignment to be made for the minor isomer obtained in the methylation reaction as 4-methylformycin (11). This isomer is the only N-methyl isomer to show a definite long wavelength maximum in the 310-315 nm region of the pH 11 spectrum

Reaction Scheme 19



<u>47</u> R = C-CH_3 (=O)

<u>48</u> R = H



<u>150</u>

Table 1.

Ultraviolet Absorption Spectra
of the N-Methylformycins

Compound	pH 1		λ_{max} (nm) ($\varepsilon \times 10^{-3}$)		
1(a) (pK=4.4,9.7)			295 (10.1)		232.5 (8.28)
7(b) (pK=4.0) (c)		302 (6.32)			236 (7.03)
8(b) (pK=4.8) (c)		305 (11.2)	270 (5.9)	260 (6.05)	231 (10.9)
9(d)		304 (11.2)		260 (5.63)	231 (13.7)
10(d)		304 (14.3)			245 (7.93)
11 (pK=6.4)	325 sh (4.08)	307 (10.8)	300 (11.0)		242 (5.91)
171 (pK=6.8,10.3)			293 (7.78)		229 (14.5)
150	324 sh (5.69)	307 (15.9)	297.5 (17.1)		241.5 sh (10.6)

(a) Data from Reference 4.
(b) Data from Reference 9.
(c) Data from Reference 81.
(d) Data from Reference 13.

Table 1 (continued)

Compound	pH 11			λ_{max} (nm) ($\varepsilon_{max} \times 10^{-3}$)	
1(a) (pK=4.4,9.7)		302 (7.9)			234 (17.9)
7(b) (pK=4.0) (c)	314 sh (3.93)	301 (6.46)	293 sh (6.19)		232 (6.51)
8(b) (pK=4.8) (c)	317 sh (8.45)	305 (12.9)	295 sh (11.2)		237 (5.61)
9(d)		305 (12.5)			234 sh (9.7)
10(d)	317 (7.99)			272 (9.78)	253 (9.86)
11 (pK=6.4)	314 (6.47)			270 (9.7)	246 (10.5)
171 (pK=6.8,10.3)		303 sh (7.03)	288 (11.1)	278.5 sh (10.3)	238.5 (15.7)
150	317 sh (7.63)	300.5 (12.9)			240 (14.5)

(a) Data from Reference 4.
(b) Data from Reference 9.
(c) Data from Reference 81.
(d) Data from Reference 13.

and is comparable in this regard to 4,5'-anhydroformycin (10). Thus, by default, the major isomer obtained in the methylation reaction was assumed to be 6-methylformycin (171).

These structural assignments were also confirmed by cmr analysis. The coupled cmr spectrum of the major and minor isomers showed a doublet for the C_5, each half being further split (quartet) by 3-bond coupling[102] to methyl protons. This confirmed that the N-methyl groups for both isomers were residing in the pyrimidine ring. The C_7 of the minor isomer exhibited only a doublet from a 3-bond coupling to the C_5-proton which corroborated the assignment of this isomer as 4-methylformycin (11). The C_7 of the major isomer, although partly obscured, showed a multiplet as a result of 3-bond coupling to both the C_5-proton and the methyl protons. This observation confirmed the initial structural assignment of 6-methylformycin (171) to this isomer.

A Comparison of Some of the Physico-Chemical Properties of the Isomeric N-Methylformycins

The synthesis of 4-methylformycin (11) and 6-methylformycin (171) provided the two remaining mono-N-methyl isomers of formycin and a comparison of some of the physico-chemical properties of the five isomers was possible. For this study, 1-methylformycin (7) was prepared by the method described by Zemlicka[83] and 2-methylformycin (8) was prepared by the method described by Townsend, et al.[9] Although 11 was the desired compound for biological studies, 171 proved to have the more intriguing chemical and

spectroscopic properties.

The pKa's of 11 and 171 were determined spectrophotometrically.[103] 4-Methylformycin (11) has a basic pK of 6.4 while 6-methylformycin (171) has a basic pK of 6.8 and an acidic pK of 10.3. A comparison of these values with the published[81] pK values for 1-methyl- (7) (4.02) and 2-methylformycin (8) (4.87) indicates that methylation in the pyrimidine ring significantly increases the basicity of formycin (1) (pK = 4.4, 9.7).[4] On the other hand, 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. An acidic pK for the other ring methylated isomers was not observed. This same pattern has been noted in certain methylated adenines[104] and methylated 4-aminopyrazolo[3,4-d]pyrimidines[98] in that only isomers wherein the methyl group resides on a nitrogen ortho to the exocyclic amino group (i.e., 1-methyladenine) displayed acidic pKa's. The demonstration of an acidic pK for 171 would tend to provide additional proof for the structural assignment. Initial attempts to obtain pK's for 171 were unsuccessful since only variable data was recorded. 6-Methylformycin was subsequently found to be unstable in hot aqueous solutions and, therefore, the preparation of solutions of 171 for pK determinations required that 171 be dissolved in a large volume of water to avoid all but minimum warming of the solution followed by rapid acquisition of data thereafter. The pK data from samples of 171 prepared in this manner were reliably consistent.

The ultraviolet absorption spectral data for the five N-methyl isomers are in Table 1. The isomers 7, 8 and 11 exhibited the expected[105] bathochromic shift, when compared to the spectra for 1, in both the acidic (pH 1) and basic (pH 11) solution spectra. The 6-methyl isomer, 171, showed little change from 1 in its pH 1 spectra and in this regard was comparable to similar acidic spectra obtained for 1-methyladenine and adenine.[105] The hypsochromic shift of the low energy absorption maximum in the pH 11 spectrum of 171, when compared to 1, was not, however, expected from a consideration of the basic solution spectra of 1-methyladenine and adenine.[105] The possibility that the observed uv spectra for 171 is not the true spectrum due to the instability of 171 in aqueous solution, is unlikely, but has not been conclusively ruled out.

Selected pmr spectral data for the five isomeric N-methylated formycins are in Table 2. The signal for the anomeric ($H_{1'}$) proton of the two isomers 8 and 11 wherein rotation about the glycosidic bond (C_3-$C_{1'}$) is restricted are observed approximately 0.2 ppm downfield from the signal for the anomeric proton of the other three methyl isomers. A downfield shift of the $H_{1'}$ proton of 2-alkylformycins when compared to 1-alkylformycins, has also been noted by Makabe, et al.[75] The upfield shift observed for the H_5 and the methylprotons of 6-methylformycin (171) was unexpected. Townsend[101] has reported that the introduction of an alkyl on a nitrogen in the pyrimidine ring of adenine resulted in an apparent π-deficient pyrimidine ring and a π-excessive imidazole ring. If a like situation were obtained upon alkylation in the pyrimidine ring of 1, the H_5

Table 2

Selected Pmr Spectral Data for the N-Methylformycins (a)

	$N\text{-}CH_3$	H_5	$H_{1'}$
Formycin (1)		8.21	5.03
1-Methylformycin (7)	4.28	8.12	4.96
2-Methylformycin (8)	4.18	8.09	5.20
4-Methylformycin (11)	4.04	8.21	5.20
6-Methylformycin (171)	3.47	7.88	4.90
N^7-Methylformycin (150)	3.07	8.20	4.98

(a) DMSO-d_6-D_2O was used as a solvent and chemical shifts are in ppm from an internal standard (DSS).

pmr signal of 11 and 171 should have appeared downfield from the H_5 signal of 1. The H_5 signals observed for 11 and 171 were not downfield from the H_5 signal for formycin (1). In the case of 6-methylformycin (171) the H_5 signal was significantly upfield.

Selected cmr data for the five N-methyl isomers are compiled in Table 3. As was observed in the pmr spectra, the signal for the N-methyl group of 171 was upfield from the corresponding signals for the other ring-methylated isomers, 7, 8 and 11. A significant upfield shift of the C_5 signals was noted for the two isomers methylated in the pyrimidine ring, 11 and 171.

Table 3

Selected Cmr Data for the N-Methylformycins (a)

	$N\text{-}CH_3$	C_3	C_{3a}	C_5	C_7	C_{7a}	$C_{1'}$
1 (b)		143.2	138.2	151.4	151.6	123.4	78.2
7 (b)	39.0	142.1 (-1.1)	140.2 (+1.9)	150.1 (-1.3)	151.3 (-0.3)	122.1 (-1.3)	78.2
8 (b)	39.0	133.4 (-9.8)	136.1 (-2.2)	152.1 (+0.7)	155.8 (+4.2)	129.9 (+6.5)	75.9 (-2.3)
11	39.2	136.9 (-6.3)	126.7 (-11.6)	146.0 (-5.4)	155.7 (+4.1)	130.3 (+6.9)	78.6 (+0.4)
171	34.3	141.3 (-1.9)	130.5 (-7.8)	146.1 (-5.3)	150.2 (-1.4)	130.1 (+6.7)	77.2 (-1.0)
150	27.0	143.6 (+0.4)	137.4 (-0.9)	151.3 (-0.1)	151.3 (-0.3)	123.3 (-0.1)	77.8 (-0.4)

(a) Spectra were recorded in d_6-DMSO 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. Chemical shifts are in ppm with respect to Me_4Si. Numbers in () are $\Delta\delta$ from formycin (1).

(b) Data from Reference 102.

Table 4

Mass Spectral Data for the Isomeric N-Methylformycins

Compound	7	8	11	171	150
M^+ (relative abundance in %)	281 (14)	281 (25)	281 (6)	281 (17)	281 (20)
m/e of the 6 most abundant ions (relative abundance in %)	193 (25)	192 (100)	192 (73)	192 (80)	192 (87)
	192 (100)	190 (31)	178 (100)	179 (47)	179 (46)
	179 (51)	179 (70)	163 (22)	178 (100)	178 (100)
	178 (100)	178 (100)	44 (52)	57 (18)	163 (24)
	162 (43)	162 (51)	42 (44)	44 (21)	57 (33)
	42 (26)	42 (26)	36 (38)	42 (100)	42 (22)

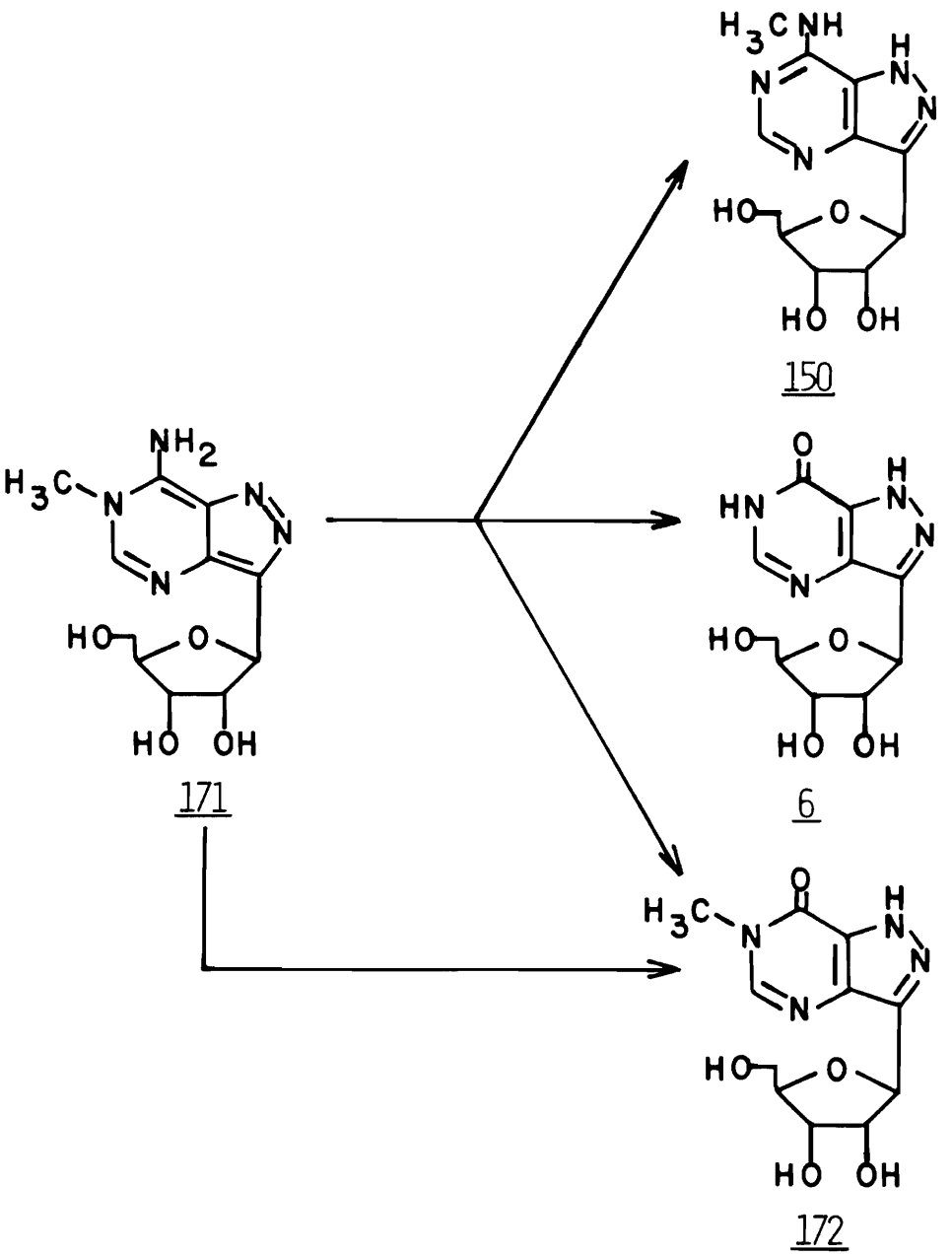
Mass spectral abundance for the six most predominant ions from fragmentation of the isomeric N-methylformycins are compiled in Table 4. Although some differences in the fragmentation patterns were observed, these differences were not sufficient to delineate structural features of a particular isomer. The presence of a predominate b+30 ion at m/e 178 in all isomers confirmed that all isomers were methylated in the aglycon rather than in the carbohydrate moiety.

A Study of the Water Hydrolysis of 6-Methylformycin (171)

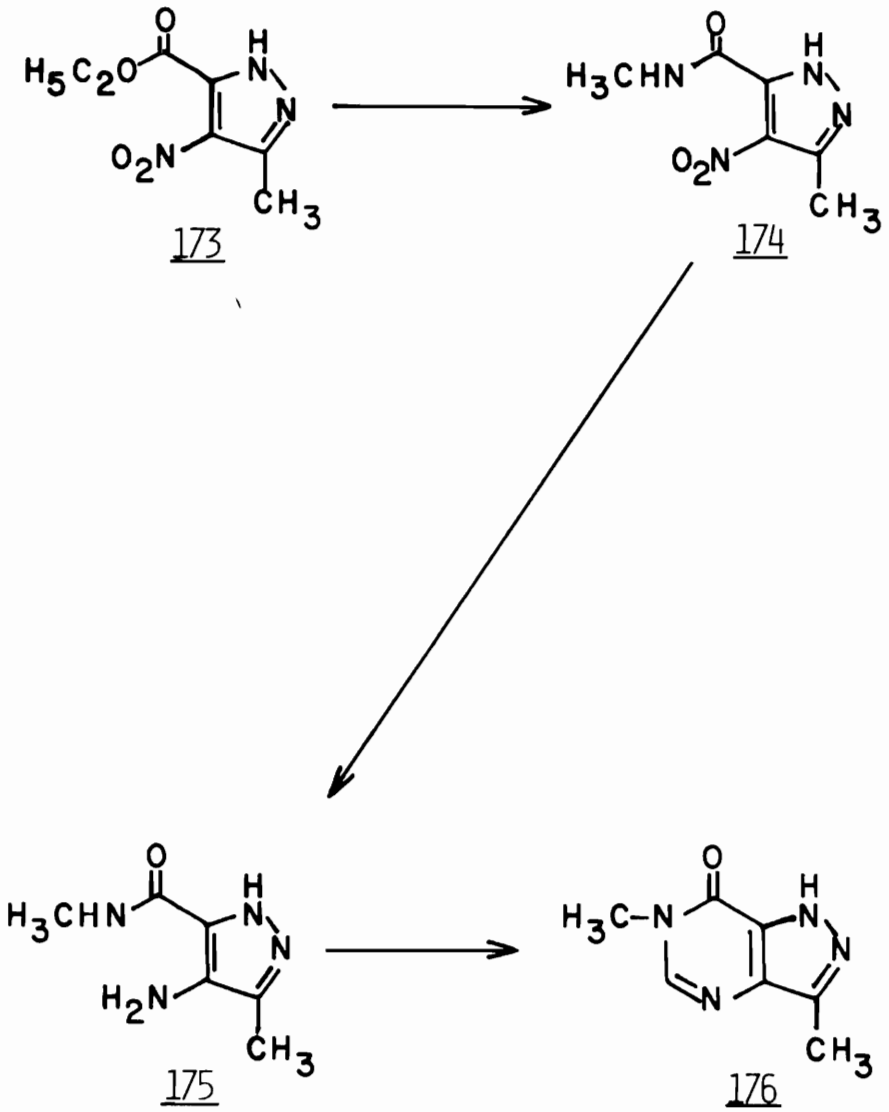
Reverse phase HPLC analysis of aqueous solutions of 171 always showed the presence of three additional products. Since samples of 171 for HPLC analysis were prepared by dissolution in hot water, a study was designed to establish the structure of the products formed from 171 in water. Boiling water (4-6 hours) converted 171 into three products. The three compounds were separated and isolated by column chromatography. Two of the products were identified as N^7-methylformycin (150) and formycin B (6) by spectroscopic (uv) and chromatographic (tlc) comparisons with authentic samples of 150 and 6. The third product had a uv λ_{max} (pH 1) of 272 nm and λ_{max} (pH 11) of 280 nm. A mass spectrum of this third product showed M^+ = m/e 282 indicating it was a mono-methylated formycin B derivative. The structure of 6-methylformycin B (172) was assigned to this product from mechanistic considerations.

To confirm that the third product from the water hydrolysis of 6-methylformycin (171) was indeed the corresponding formycin B

Reaction Scheme 20



Reaction Scheme 21



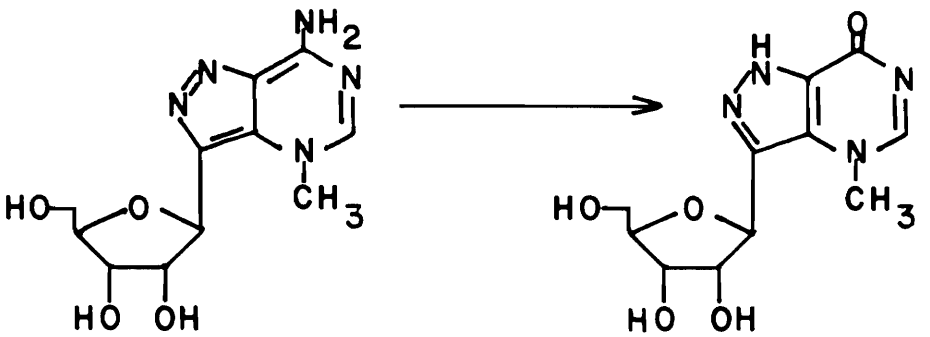
derivative 172, an unambiguous synthesis of a model compound, 3,6-dimethylpyrazolo[4,3-d]pyrimidin-7-one (176) was accomplished for spectroscopic comparisons. The reaction of ethyl 3-methyl-4-nitropyrazole-5-carboxylate (173)[106] with aqueous methylamine gave 3-methyl-4-nitropyrazole-5-(N-methyl)carboxamide (174). Catalytic reduction of 174 afforded the o-aminoamide 175, which was treated with either formamide or diethoxymethyl acetate[107] to produce 3,6-dimethylpyrazolo[4,3-d]pyrimidin-7-one (176). 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 (176) failed to confirm this assignment since 176 did not exhibit an acid-base shift in its uv spectra [λ_{max} (pH 1) = 281.5 nm, λ_{max} (pH 11) = 281.5 nm]. This prompted the preparation of 6-methylformycin B (172) by an alternate route so a direct comparison with the hydrolysis product could be accomplished.

The Synthesis of 6-Methylformycin B (172), 4-Methylformycin B (177) and 1-Methylformycin B (178)

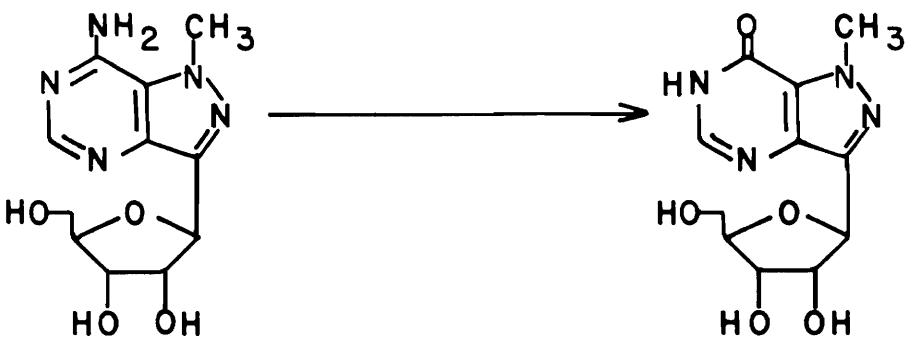
6-Methylformycin B (172) was obtained from a deamination of 171 with nitrosyl chloride by the method described[108] for the preparation of isoinosine. Although the uv maxima were not precisely coincidental, the nucleoside prepared by this method was chromatographically identical to the third product obtained from the water hydrolysis of 171 and confirmed our initial assignment of structure 172 to that product.

4-Methylformycin (11) and 1-methylformycin (7) were similarly

Reaction Scheme 22



11 177



7 178

deaminated to give 4-methylformycin B (177) and 1-methylformycin B (178), respectively. 2-Methylformycin B (123) has been previously prepared by a chemical[9] and an enzymatic[81] deamination of 8. The synthesis of 172, 177 and 178 thus completed the synthesis of the monomeric N-methyl derivatives of formycin B.

As was observed with the methylformycins, the pmr methyl signal for 172 (δ 3.52) is upfield from the methyl signals for 177 (δ 3.93), 178 (δ 4.14) and 2-methylformycin B (123) (δ 4.11).[109] Similarly, the $H_{1'}$ signals of the isomers wherein rotation about the glycosidic (C_3-$C_{1'}$) bond is restricted, 177 (δ 5.03) and 2-methylformycin B (123) (δ 5.12)[109] are downfield from the isomers with unrestricted rotation, 172 (δ 4.93) and 178 (δ 4.87).

Biological Evaluation and Some Biochemical Studies of the Isomeric N-Methylformycins

One of the primary goals of this investigation was to prepare a formycin derivative which would not undergo enzymatic deamination as readily as formycin yet retain therapeutic value. A collaborative arrangement with R. E. Parks, Jr. and coworkers, G. W. Crabtree and R. P. Agarwal, at Brown University was initiated in order to study some of the biochemical properties of the N-methylformycins. In particular, a determination of the abilities of the five methyl isomers to act as substrates for adenosine deaminase and/or a kinase were two parameters of major interest. A study of the cytotoxicities of the methylformycins to L-1210 cell cultures was completed at The University of Utah in collaboration with L. L. Wotring. The results

Table 5

Biological Data for the N-Methylformycins

	Erythrocytic ADA Kinetic Parameters		*In Vitro* L-1210 Cytotoxicity
	Km(mM)	relative V_{max}	ID_{50} (μM)
Adenosine	0.025	100	80
Formycin (1)	1.0	750-850	1
1-Methylformycin (7)	- -	- -	0.8
2-Methylformycin (8)	6.1	396	0.5
4-Methylformycin (11)	- -	- -	No effect at 100 μM
6-Methylformycin (171)	- -	- -	130
N^7-Methylformycin (150)	0.1	3	2

of these studies are partially summarized in Table 5 and are the subject of a recent publication.[110]

Of the five isomers only 2-methyl- (8) and N^7-methylformycin (150) showed substrate activity with adenosine deaminase (ADA). 4-Methyl- (11) and 6-methylformycin (171) failed to form nucleotides when incubated with human erythrocytes, whereas, large amounts of the corresponding mono-, di- and triphosphates were formed from 1-methyl- (7) and 2-methylformycin (8). N^7-Methylformycin (150) also formed nucleotides but to a lesser extent than that observed for 7 and 8. The incorporation of 8 and 150 into erythrocytic nucleotide pools was enhanced by pretreatment with the adenosine deaminase (ADA) inhibitor, 2'-deoxycoformycin. Pretreatment with 2'-deoxycoformycin had no effect on the incorporation of 1-methylformycin (7) into erythrocytic nucleotides. The conversion of 1-methyl- (7) and 2-methylformycin (8) into nucleotides was almost complete after eighteen hours of incubation (in the presence of 2'-deoxycoformycin for the case of 8) whereas the conversion of N^7-methylformycin into erythrocytic nucleotides was only partially complete. Transient accumulation of the monophosphates prior to the conversion to the triphosphates was observed for 7 and 150. 1-Methylformycin (7), 2-methylformycin (8) and N^7-methylformycin (150), which were the only isomers incorporated into erythrocytic nucleotide pools, were also the only isomers to show significant cytotoxicity to L-1210 cells in culture. 6-Methylformycin (171) displayed very weak cytotoxicity to L-1210, but the fact that this cytotoxicity may be due to a rearrangement of 171 into 150 under

the test conditions has not been ruled out.

Following communications[15,111] of the preliminary results of these studies, another report[75] on the enzymatic deamination of certain 1- and 2-alkylformycins has appeared in the literature. This latest report showed[75] that both 1-methyl- (7) and 2-methylformycin (8) were deaminated by Takadiastase ADA and calf intestinal mucosa ADA. The observed rate of deamination of 8, with both enzymes, was found[75] to be approximately equal to the observed rate of deamination of formycin (1). By contrast, the rate of deamination of 7, by both enzymes, was shown[75] to be very much slower than the rate of deamination of 1 and 8. In fact, no detectable deamination of 1-methylformycin (7) was observed at enzyme concentrations which resulted in complete deamination of 1 and 8.[75] This recent study[75] also reported that neither 1-iso-propyl- (121) nor 2-iso-propylformycin (122) was a substrate for either deaminase. The results concerning the deamination of 121 and 122 are not relative 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 (C_3-$C_{1'}$) bond, must also be considered: 1) electronic changes resulting from alkylation of the aglycon, 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 nitrogen of 1 eliminates tautomerism and provides molecules with more fixed bond character. Methylation in the pyrimidine ring, as in 11 and 171, significantly increases the basicity of the system. Neither 11 or 171 is a substrate for ADA and significantly neither is incorporated into nucleotide pools. Methylation in the pyrazole ring, as in 7 and 8, causes a comparatively much smaller increase in the basicity of the system. Both 7 and 8 are phosphorylated but only 8 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 7 and 171, respectively. It is pertinent to point out, however, that 171 is neither a substrate for ADA nor a kinase, whereas methylation at the 1-position only inhibits deamination.

Isomers 7, 171 and 150 apparently have unrestricted rotation about the C_3-$C_{1'}$ bond. A methyl group on the exocyclic amine, as in nucleoside 150, does not present the same degree of steric inhibition for enzyme binding as discussed previously for 7 and 171. In fact, the nucleoside 150 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 C_3-$C_{1'}$ bond, the more basic, pyrimidine methylated isomer 11 is neither deaminated nor

phosphorylated, and the less basic, pyrazole methylated compound 8 is both a substrate for ADA and a kinase.

This finding that the syn nucleoside 8 is deaminated while 7 is not deaminated parallels another study[14] using calf intestinal ADA, one of the enzymes used in studies[12,13] involving the cyclonucleosides 9 and 10. For consistency with the syn-anti postulates from the studies of 9 and 10, the deamination of 8 has been explained[81] by assuming that 8 can adopt an anti conformation on interaction with ADA. Examination of CPK models show that an anti conformation for 8 is possible, although sterically unfavorable, and that the anti conformation for 11 is highly favored. Thus, the fact that 11 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 do not establish that formycins with a syn relationship are deaminated and anti nucleosides are resistant to deamination. Further, this study does not conclusively invalidate the assumptions advanced from the deamination studies of 9 and 10 since deamination of these anhydronucleosides by human erythrocytic ADA has not been investigated. However, it appears from the findings of this study,[110] that the electronic effects attendant upon alkylation of the 4-position of the base to produce the syn model 10 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 (150) and those of an earlier study.[70] Kunimoto, et al.,[70] reported that 150 was inactive against Yoshida sarcoma cells in vitro, which led to the speculation[5] that perhaps di- and triphosphates of 150 were not biosynthesized. This present study has shown[110] that 150 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, N^7-methylformycin (150) has an ID_{50} against L-1210 cell cultures only twice that observed for formycin (1).[110]

CHAPTER 4

DEOXYFORMYCIN: AN ENZYMATIC SYNTHESIS

Background

The methods of synthesis of 2'-deoxyformycin (12)[17,18] have a common problem in that a mixture of 2'-deoxy- and 3'-deoxyformycin (13) are obtained with the latter isomer being predominant. A possible alternate method for the synthesis of 2'-deoxyformycin (12) was suggested by the availability of nucleoside triphosphate reductase from Lactobacillus leichmannii. In fact, at the time this method was suggested, a description of 2'-deoxyformycin had not been published.

Ribonucleotide reductase is the enzyme responsible for de novo synthesis of cellular deoxynucleotides.[112] Two classes of ribonucleotide reductase occur in biological systems. Type I, ribonucleoside diphosphate reductase, has been found primarily in eukaryotes and, as its name implies, requires ribonucleoside diphosphates as substrates.[112] Type II reductase, has been found primarily in prokaryotes and is a ribonucleoside triphosphate reductase.[112]

The ribonucleotide reductase found in L. leichmannii is a triphosphate reductase (Type II) and has been extensively studied by Blakley and colleagues.[113,114] Methods for the isolation and purification of substantial quantities of this enzyme have been

disclosed.[115,116] The nucleotide reductase from L. leichmannii has been found[114] to be a monomeric, allosteric enzyme with a single polypeptide chain and has a molecular weight of approximately 76,000. The peptide chain contains about 689 amino acid residues with 9 one-half cystines.[114] The reductase has an absolute requirement for 5'-deoxyadenosylcobalamine.[113] The nucleoside triphosphates are reduced by this enzyme at a much higher rate than the diphosphates; the monophosphates are not reduced.[113] The physiological mechanism of reduction by the L. leichmannii reductase is illustrated in Figure 1.

The in vitro reduction of ribonucleotides with ribonucleoside triphosphate reductase has also been extensively studied.[113] The mechanism of in vitro reduction is outlined in Figure 2. Several factors determine the practicability of an in vitro enzymatic reduction of ribonucleotides on a preparative scale. Most importantly, the nucleotide must be accepted as a substrate by the enzyme. The extent of in vitro enzymic reduction achievable is dependent upon the rate of reduction, the optimum concentrations of reaction components, the effective recovery and reutilization of the enzyme, the ease of separation of the desired product from the reaction mixture and the efficiency of conversion of the resulting 2'-deoxynucleotide into the 2'-deoxynucleoside. To establish conditions for the optimum production of 2'-deoxyformycin via an enzymic reduction, a collaborative effort was initiated with R. L. Blakley of the University of Iowa. The initial results of this collaboration have been recently communicated.[117]

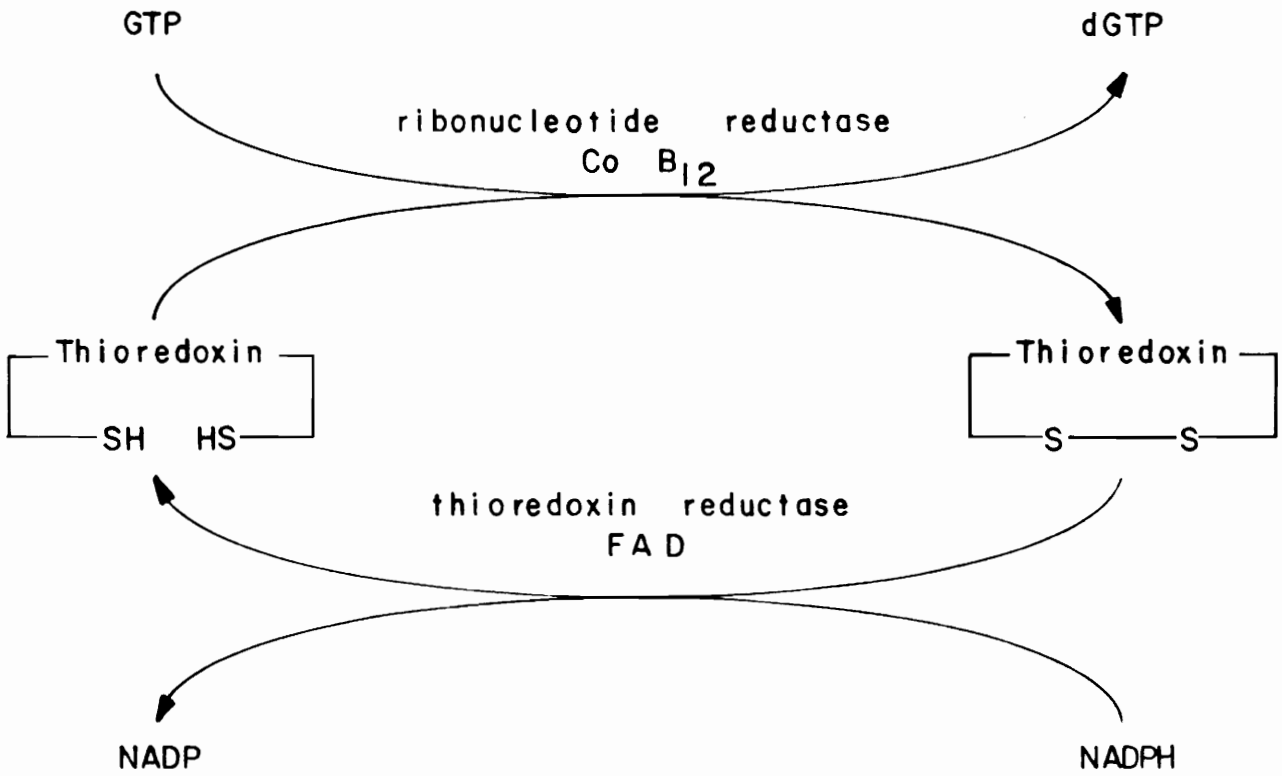


Figure 1

In Vivo Reduction System

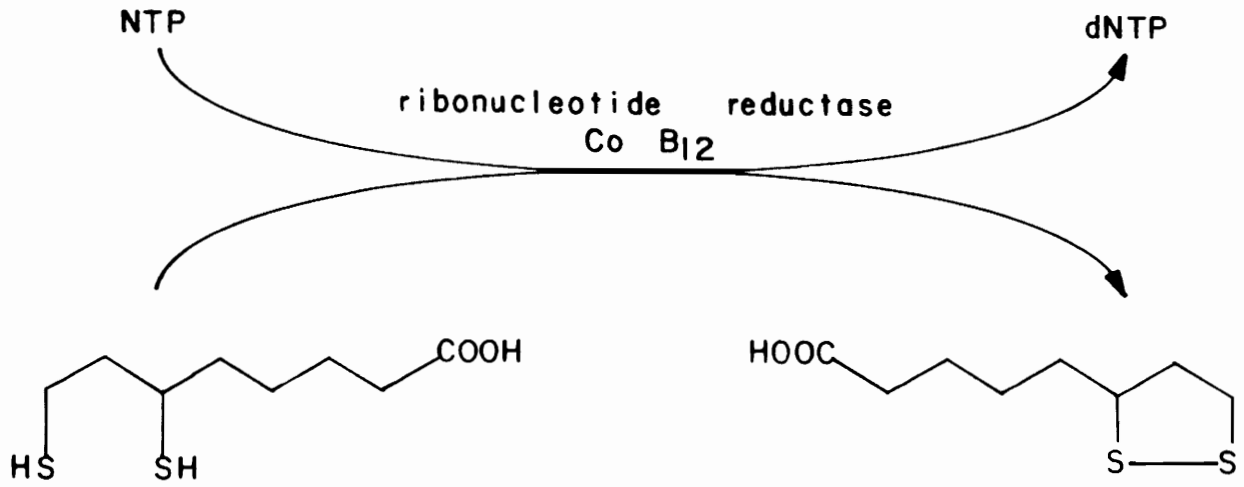


Figure 2

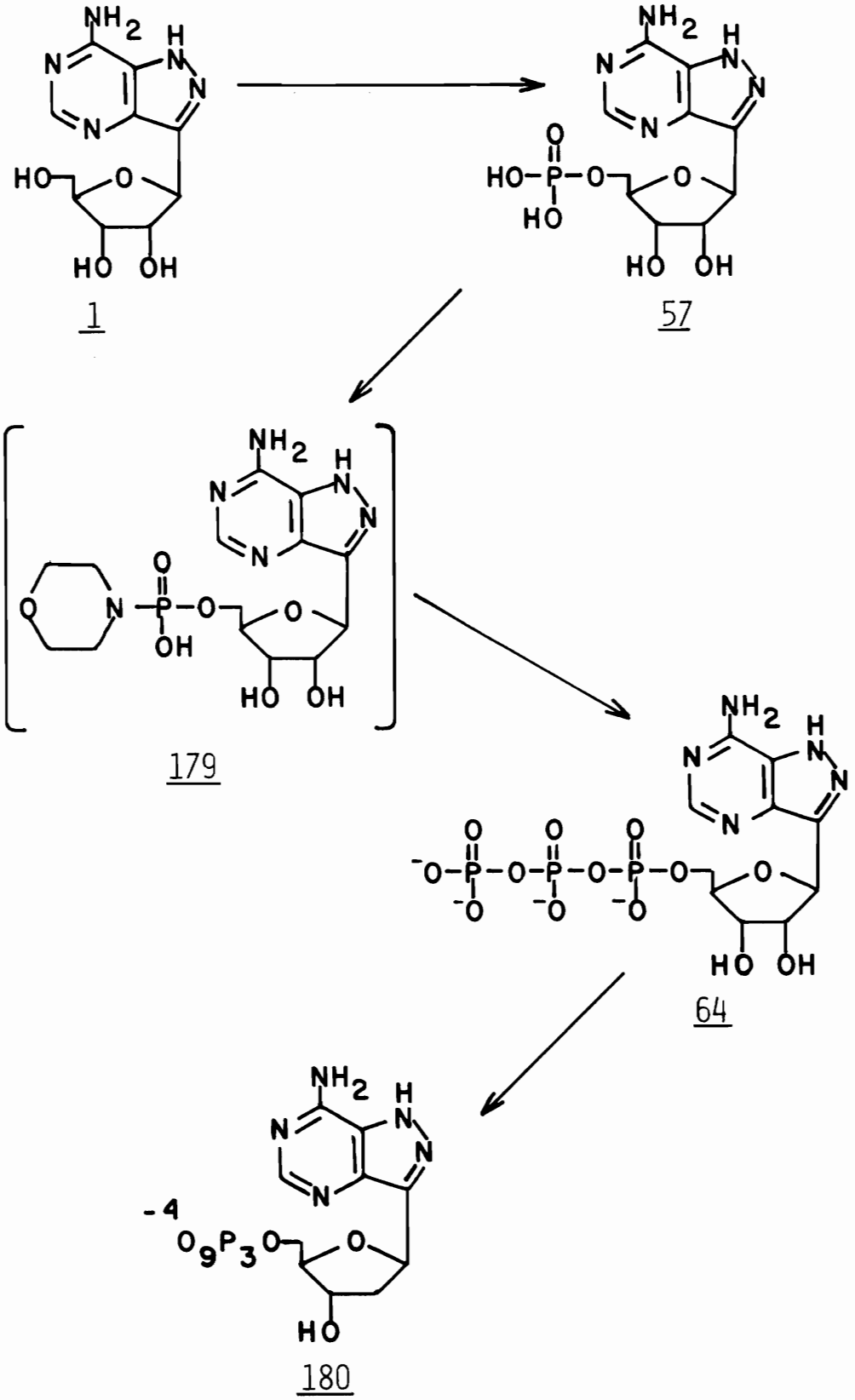
In Vitro Reduction System

The Synthesis of Formycin-5'-Triphosphate (FTP, 64)

The first requirement for an evaluation of the enzymic production of 2'-deoxyformycin (12) was to obtain the requisite substrate, formycin-5'-triphosphate. The methods of synthesis of formycin nucleotides were discussed in Chapter 2. To obtain formycin-5'-phosphate (FMP, 57) the phosphorylation method of Yoshikawa, Kato and Takenishi[118] was chosen. This procedure utilizes an unprotected nucleoside and thus, was the most direct method for obtaining FMP (57). This procedure has been employed by others[67] for the synthesis of FMP (57), however, no experimental details were included in that report. Some limitations to the procedure of Yoshikawa, et al., have been reported by Perini and Hampton[119] and Dawson, Cargill and Dunlap.[120]

Formycin (1) was reacted with phosphoryl chloride in a trimethylphosphate solution containing one equivalent of water. The resulting formycin-5'-phosphate was isolated from the reaction mixture by a method similar to that used by Imai, et al.,[121] for the isolation of adenosine-5'-phosphate (AMP). FMP (57) was obtained in an approximate seventy-five percent yield and was homogeneous on tlc. Dawson, et al.,[120] have pointed out that thin layer or paper chromatographic methods are inadequate to detect the occurrence of phosphorylation of secondary hydroxyl groups. The presence of small amounts of 3' (or 2') phosphate in the FMP samples was not determined. However, the presence of small amounts of a 3' (or 2') phosphate should only affect the observed rate of reduction and not the overall

Reaction Scheme 23



evaluation of formycin-5'-triphosphate (FTP) as a substrate for the reductase.

Several preliminary investigations limited the method of synthesis and isolation of formycin-5'-triphosphate (FTP, 64). The method of Hoard and Ott[122] has been applied to the synthesis of FTP 64, however, earlier experience with this method had resulted in an alteration of the aglycon of certain nucleosides. This method was, thus, adjudged problematical for the conversion of FMP to FTP. Separation of triphosphates from other reaction products by gradient elution from Dowex 1 (Cl^-) with lithium chloride/hydrochloric acid solutions was found unsuitable as removal of chloride salts from the triphosphates was incomplete. The reductase from L. leichmannii has been shown[113] to be inhibited by high concentrations of chloride. Considering the purpose of this study, this method of isolation was not used.

Formycin-5'-triphosphate (FTP, 64) was prepared from FMP (57) following the method developed by Moffatt and Khorana.[123] Formycin-5'-phosphate (FMP, 57) was converted to the 5'-phosphomorpholidate (179), which was reacted with pyrophosphate to give a mixture of nucleotides. FTP (64) was isolated from the mixture by gradient elution of the reaction products from DEAE-cellulose (HCO_3^-) with a linear gradient of triethylammonium bicarbonate. An elution profile of the components of the reaction mixture was plotted from estimations of the concentration of uv absorbing material in the chromatography fractions. Fractions containing only FTP (64) were pooled and the product converted to the sodium salt following the

instructions of Hoard and Ott.[122] The FTP prepared in this manner was chromatographically and electrophoretically homogeneous. The conversion of FMP (57) to FTP (64) was approximately thirty-two percent.

The preparation and isolation of FTP did not require a cold temperature. Further, it should be possible to convert the side products, recovered during the isolation of FTP, to FMP (57) and reutilize this FMP (57) in the synthesis of FTP (64).

The Enzymatic Reduction of FTP

The reduction of formycin-5'-triphosphate (64) by nucleoside triphosphate reductase was studied by R. L. Blakley and coworkers, S. A. Brinkley, W. J. Critz and L. L. Witt of The University of Iowa. The initial results[117] of this study indicated that FTP (64) was not a substrate for the reductase enzyme from L. leichmannii. However, it was apparent that FTP was bound at the catalytic site of the reductase enzyme since FTP was found[117] to be a linear competitive inhibitor of the reduction of ATP, GTP and UTP. FTP was not an inhibitor of CTP reduction.[117]

Extension of these studies showed[117] that FTP was reduced when the positive allosteric effector 2'-deoxyguanosine-5'-triphosphate (dGTP) was added to the reaction. In the presence of 1 mM dGTP, FTP was reduced to 2'-deoxyformycin-5'-triphosphate (dFTP, 180) at a rate comparable to the rate of reduction of ATP; FTP:ATP = 0.91.[117] The absolute requirement for dGTP as an effector to achieve reduction of FTP was a limiting factor in the enzymic preparation of dFTP (180). The dFTP (180) produced in the reductase reaction was

separated from unreacted FTP by chromatography on dihydroxyboryl substituted cellulose.[117] Insignificant interferences, by the added dGTP, to the isolation of dFTP was noted when the concentration of dGTP did not exceed 0.5 mM[117] Under this restriction, the reduction of FTP was only forty-eight percent complete in the standard reaction time (1.5 hr) and the dFTP (180) isolated by dihydroxyboryl substituted cellulose chromatography contained 1.8% of dGTP.[117] Pure dFTP (180) was obtained by further chromatography on DEAE-cellulose using a linear gradient of triethylammonium bicarbonate as the eluting buffer.[117] Pure 180 was obtained in an approximate thirty percent yield from FTP.[117]

These preliminary studies have demonstrated the feasibility of using the L. leichmannii ribonucleotide reductase as a "reagent" for producing dFTP (180) and subsequently 2'-deoxyformycin (12). All factors that might limit the size of enzymatic reactions have not been fully investigated. However, the present data have shown that from a single commercial fermentation of L. leichmannii, sufficient enzyme[116] is obtained to reduce six millimoles of FTP. Blakley and coworkers are presently investigating the behavior of the reductase enzyme which has been insolubilized by attachment to Sepharose. Preliminary indications are that the insolubilized enzyme can be repeatedly used[117] and, thus, a potential production of fifty millimoles (approx. 25 g) of deoxynucleotide from a single enzyme preparation is feasible.

Allosteric Effects of dFTP (180)

Blakley and colleagues[117] found that dFTP (180) mimics dATP as

a positive effector for the reduction of CTP by the L. leichmannii reductase. Suhadolnik, Finkel and Chassy[74] studied the effects of FTP (64) on the reduction of CTP by L. leichmannii reductase and found that there was no stimulation of reduction. These authors[74] additionally observed that tubercidin-5'-triphosphate (TuTP) and toyocamycin-5'-triphosphate (ToTP) did stimulate the reduction of CTP but could not rule out that this stimulation might be caused by the deoxynucleotides since both were also found[74] to be substrates for the enzyme. Based on these results Suhadolnik, et al.,[74] concluded that the carbon atom 8 and the nitrogen atom 9 of the purine base portion of nucleosides are required for interaction at the regulatory site in nucleoside triphosphate reductase. This conclusion has certainly been invalidated by the finding that dFTP markedly stimulated the reduction of CTP.[117]

Blakley[124] has demonstrated that ribonucleoside triphosphates can weakly bind at the regulatory site in L. leichmannii reductase. If, contrary to the findings of Suhadolnik, et al.,[74] FTP was binding at the regulatory site and was thus, stimulating the reduction of CTP, this effect could offset inhibition of CTP reduction by FTP. A biphasic effect of this type would explain why FTP (64) had no net effect on the observed reduction of CTP, whereas FTP competitively inhibited the reduction of ATP, GTP and UTP.[117]

CHAPTER 5

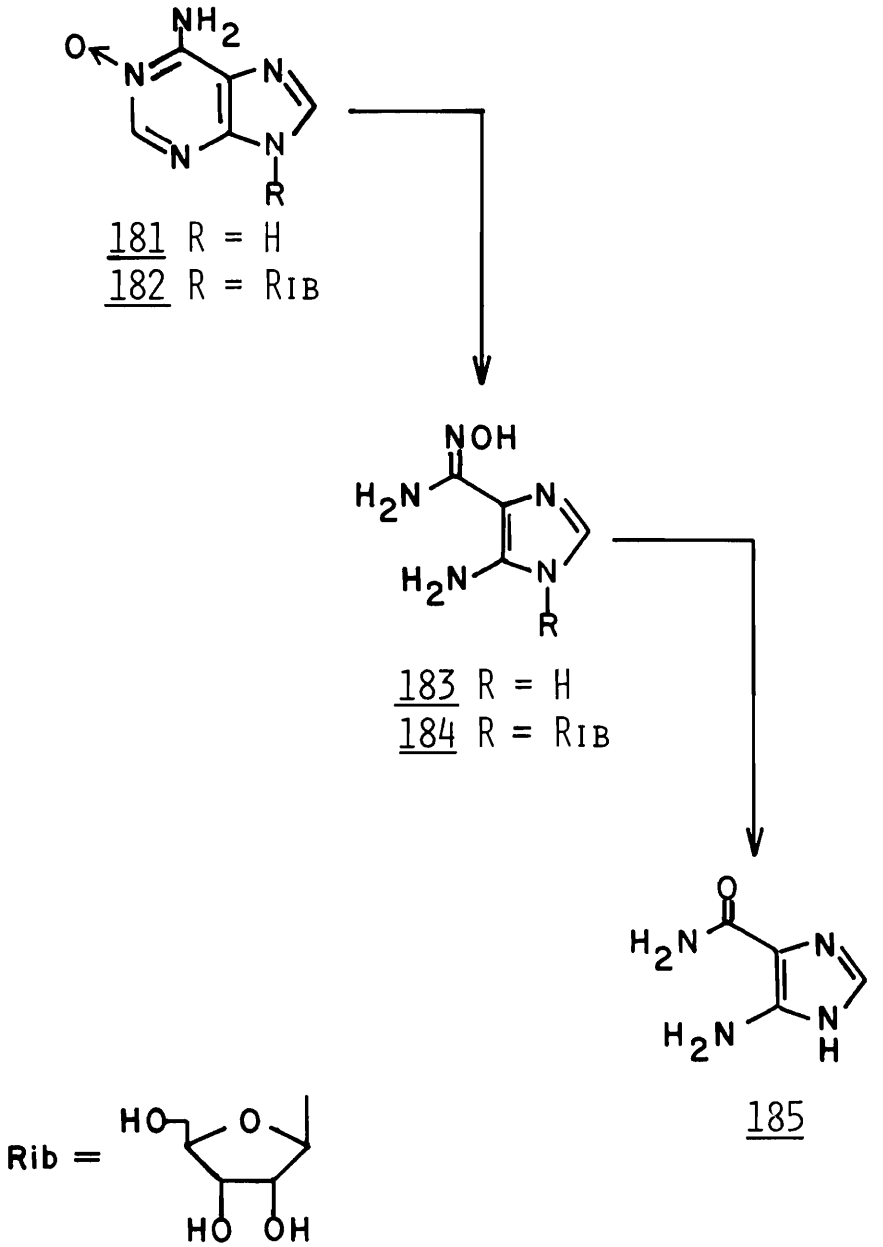
ΨAPCA-RIBOSIDE: AN IMPROVED SYNTHESIS

Background

The o-aminopyrazolecarboxamides, 15 and 16, have proven to be valuable intermediates for the synthesis of 5,7-disubstituted pyrazolo[4,3-d]pyrimidines.[19,20] 4-Amino-3-(β-D-ribofuranosyl)pyrazolo-[4,3-d]pyrimidine-5-carboxamide (ΨAPCA-riboside, 17) could similarly provide ready access to derivatives of formycin (1) and formycin B (6) substituted in the 5-position. The synthesis of 17 has been described by Long[21a] (Chapter 2). However, the low yield of 17 obtained by this method[21a] prompted the investigation of alternate routes of synthesis.

Brown and coworkers[125-127] have disclosed that adenine-N^1-oxide (181) and adenosine-N^1-oxide (182) undergo pyrimidine ring fission when treated with acid or base. The amidoxime 183 was found to be the product from treatment of either 181 or 182 with dilute acid.[125,126] The nucleoside 184 could only be obtained from treatment of 182 with dilute base.[126] 4-Aminoimidazole-5-carboxamide (AICA, 185) was obtained from 183 by reduction with Raney nickel followed by treatment of the resulting amidine with base or by prolonged acid treatment.[125] These studies[125-127] suggested a possible alternative route to ΨAPCA-riboside (17).

Reaction Scheme 24



181 R = H
182 R = RIB

183 R = H
184 R = RIB

185

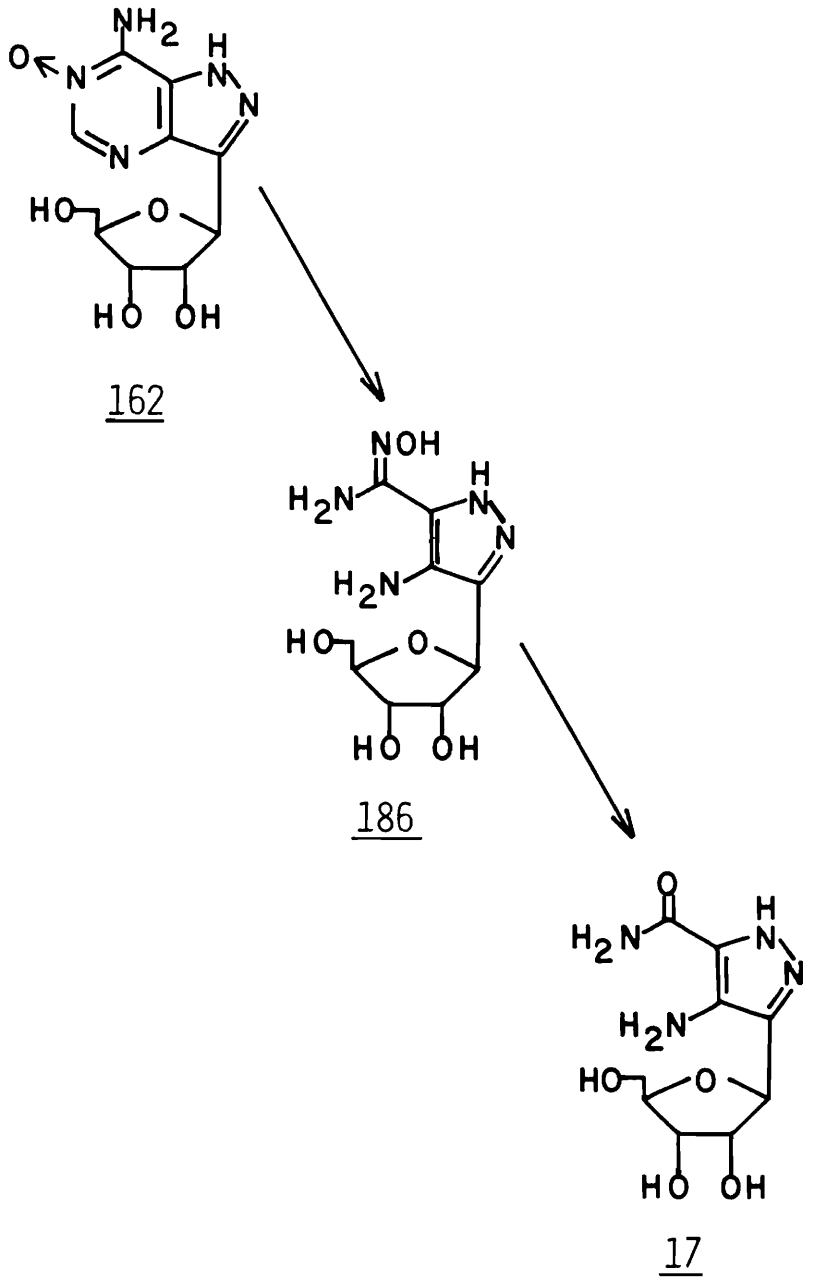
Rib = HO O HO OH

The Synthesis of ΨAPCA-Riboside via Ring Fission of Formycin-N^6-Oxide

Formycin-N^6-oxide (162) was prepared by the method described by Long[21a] (Chapter 2). A study of the stability of 162 showed that it rapidly reacted with dilute hydrochloric acid, even at ambient temperature. After two hours, a solution of 162 in 1 N hydrochloric acid exhibited only high intensity end absorption in its uv spectrum (pH 11). Ultraviolet analysis of a 2 N hydrochloric acid solution of 162 at the end of one hour similarly showed only high intensity end absorption (pH 11). However, within the same time period, a 3 N hydrochloric acid solution of 162 displayed a shoulder at approximately 270 nm (pH 11) and a maximum at 234 nm (pH 11), while a 4 N hydrochloric acid solution of 162 exhibited a definite maximum at 267 nm (pH 11). A time course study of the reaction, by uv analyses, showed that the initial rapid loss of the long wavelength absorption of 162 was followed by the slow development of a maximum at 267 nm (pH 11).

The product of the reaction of formycin-N^6-oxide (162) with acid was assumed to be the amidoxime 186 as the uv maxima (pH 11) exhibited by the reaction mixture was comparable to the maxima observed for 4-amino-3-methylpyrazole-5-carboxamidoxime ($\lambda_{max}^{pH\ 11}$ = 268, 235.5 nm).[90] This assumption was supported by the finding that 7-amino-3-methylpyrazolo[4,3-d]pyrimidine-N^6-oxide[90] reacted with acid (4 N hydrochloric acid, room temperature, 1.5 hours) to give a product which was chromatographically identical to

Reaction Scheme 25



162

186

17

7-amino-3-methylpyrazole-5-carboxamidoxime prepared by the method of Long, et al.[90]

Surprisingly, formycin-N^6-oxide (162) was found to be relatively stable to basic environments. Ultraviolet analysis of a 1 N sodium hydroxide solution of 162 showed no change after eleven days at room temperature. In fact, 162 was only slowly changed by sodium hydroxide solutions of higher normality or elevated temperatures.

Attempts to isolate 186 from reactions of 162 with dilute acid were unrewarded. A variety of conditions with regard to acid strength (1 N to 6 N hydrochloric acid), temperature (ambient to boiling) and time (10 min to 24 hr) were investigated. The reaction mixtures were routinely monitored by tlc analysis. Thin layer chromatography on neutral silica gel using alcoholic or aqueous solvents invariably showed single elongated spots. However, chromatography on acidic silica gel clearly indicated that several products resulted from the reaction of 162 with dilute acid. Eventually, 0.1 M ammonium chloride-acetonitrile mixtures were found to be excellent solvents for developing neutral silica gel chromatograms of these reaction mixtures.

The extreme ease of pyrimidine ring fission resulting from treatment of formycin-N^6-oxide (162) with acids, suggested the use of an acidic ion-exchange resin as a mild acid reagent. When an aqueous solution of formycin-N^6-oxide (162) was stirred with Dowex 50 (H^+) for several hours, fission of the pyrimidine ring was achieved. Conveniently, the product of this reaction was tightly held by the resin which allowed filtration and removal

of some side products by thorough washing. A crude product which gave a uv spectra expected for compound 186 was obtained from extraction of the cation exchange resin with dilute ammonium hydroxide. The crude product was stirred with ethanol to produce crystalline 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamidoxime (186). Tlc analysis of the ethanol filtrate showed that a minor second product was also obtained. This second product was not identified. The carboxamidoxime (186) was identified by spectral comparisons with 4-amino-3-methylpyrazole-5-carboxamidoxime[90] and by its elemental composition. The reaction time for several preparations of 186 varied from thirteen to twenty-four hours with little effect upon the product yield.

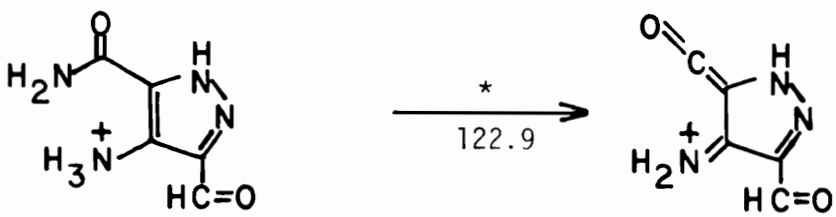
Experience with the oftentimes difficult isolation, and resultant diminished yields, of highly functionalized pyrazole-C-nucleosides, suggested that 17 might be more advantageously synthesized by concurrent reduction and hydrolysis of the amidoxime function of 186. In a preliminary experiment, 4-amino-3-methylpyrazole-5-carboxamidoxime[90] was dissolved in dilute sodium hydroxide and, after the addition of Raney nickel, the mixture was shaken under hydrogen for several days. Analysis of the reaction mixture by uv spectroscopy and by direct tlc comparisons to authentic 16[20] confirmed that 4-amino-3-methylpyrazole-5-carboxamide (16) was the major product resulting from this reaction. This investigation also showed that the conversion of the amidoxime into the amide was a very slow reaction. In a subsequent pilot investigation, 186 was similarly treated with base, Raney nickel

and hydrogen and the course of the reaction monitored by tlc (SilicAR-7GF, 0.1 M NH_4Cl/CH_3CN, 2/9, v/v). At a reaction time of twenty-four hours, three components were observed in the reaction mixture; unreacted 186, a much slower migrating component and a third component which had only a slightly slower mobility (R_{186} = 0.86) than 186. Following a reaction time of forty-eight hours, only the two components with mobilities less than 186 were present and after ninety-two hours the component with R_{186} = 0.86 was predominate with only traces of the slowest migrating component discernable.

Subsequent to these pilot experiments, formycin-N^6-oxide (162) was treated with Dowex 50 (H^+) to give 186, which, without purification, was then subjected to basic, Raney nickel catalyzed hydrogenation for four days. An approximate seventeen percent yield (from 162) of 17 was obtained from this reaction after dry column chromatography. The 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide (17) obtained in this manner was homogeneous on thin layer chromatograms. However, high pressure liquid chromatographic analysis indicated that 17 had been obtained in about ninety percent purity. Pure 17 was obtained only by preparative, reverse phase, high pressure liquid chromatography (HPLC) with a recovery of approximately fifty percent. Thus, 17 was obtained in only eight percent yield from 162; six percent from formycin (1). Three minor components were separated during the purification of 17 but were not characterized.

The structure assigned to nucleoside 17 was confirmed by uv spectral comparisons to the uv spectral data reported[90] for the known 4-amino-3-methylpyrazole-5-carboxamide (16), by the pmr spectra and by mass spectral analysis. As noted previously (Chapter 2), the b+30 ion derived from pyrazofurin (5) readily eliminates ammonia to give a predominate ion at m/e 139 which is a diagnostic feature of the o-hydroxycarboxamide grouping of 5.[93] The appearance of a predominate ion at m/e 138 in the mass spectrum of ΨAPCA-riboside (17) further supported the structural assignment.

A more productive preparation of 17 was achieved by purification of the intermediate amixodime, 186, prior to conversion into 17. When purified 186 was used in the basic hydrogenation reaction, nucleoside 17, which was sufficiently pure for use in subsequent syntheses, was obtained in thirty-one percent (from 186) yield (19% from 162, 15.5% from 1), without extensive chromatography.



m/e 155 m/e 138

A Modification of the Synthetic Procedure for Formycin-N^6-Oxide (162)

The facile, acid catalyzed, pyrimidine ring fission of formycin-N^6-oxide (162) caused some concern with regard to the procedure for the synthesis of 162. The published[21a] procedure for the preparation and isolation of 162 included exposure to hot acetic acid-dimethylformamide solution and evaporation of aqueous acetic acid solutions. To avoid some of these acidic conditions, a modified procedure was developed. Formycin (1) was treated with m-chloroperbenzoic acid in refluxing methanol and formycin-N^6-oxide (162) was isolated by pouring the reaction mixture into a large volume of ethyl acetate. Although the yield of 162 obtained was not improved by this modification, the isolation was greatly simplified and the evaporation of acidic solutions of 162 was avoided.

Biological Evaluation of ΨAPCA-Riboside (17)

The isolation and structural elucidation of pyrazofurin (pyrazomycin)[128] and bredinin[129] as 4-hydroxy-3-(β-D-ribofuranosyl)-pyrazole-5-carboxamide (5) and 5-hydroxy-1-(β-D-ribofuranosyl)-imidazole-4-carboxamide, respectively, have generated considerable interest due to their biological and chemotherapeutic activity.[4,22] The amino analog [5-amino-1-(β-D-ribofuranosyl)imidazole-4-carboxamide, AICA-riboside, 18] of bredinin also has been the subject of numerous biological investigations.[130] In fact, the 5'-phosphate derivative of 18 (AICAR) has been firmly established[130] as a very

important intermediate in the de novo pathway of purine biosynthesis. Thus, the biological evaluation of ΨAPCA-riboside (17) was of considerable interest since 17 can be viewed as the amino analog of pyrazofurin (pyrazomycin, 5).

In a collaborative arrangement with L. L. Wotring at The University of Utah, the cytotoxicity of 17 to L-1210 cell cultures was evaluated in a study which also included pyrazofurin (5) and AICA-riboside (18). The findings of this study have been communicated.[21b]

Nucleosides 17 and 18, at a concentration of 10^{-4} M, had no effect on L-1210 cell growth. On the other hand, 5, at a concentration of 10^{-4} M, caused a total inhibition of cell growth after a delay of approximately 24 hrs, with an ID_{50} of 2×10^{-7}. Thus, a replacement of the 4-OH group of pyrazofurin (5) by an NH_2 group (17) resulted in an apparent loss of cytotoxicity. These data provide some support for the proposal[22] that the 4-OH group of pyrazofurin may be essential for its activity. The nucleoside 17 can be viewed as a C-nucleoside analog of AICA riboside with the distinction that it will most likely be resistant towards an enzymatic cleavage by a nucleoside phosphorylase, but may still function as a substrate for an appropriate kinase to form the analog of AICAR. It is tempting to postulate that the 5'-phosphate derivative of 17 may subsequently be established as a key intermediate in the biosynthetic pathway of certain C-nucleoside antibiotics, e.g., formycin (1) and formycin B (6).

CHAPTER 6

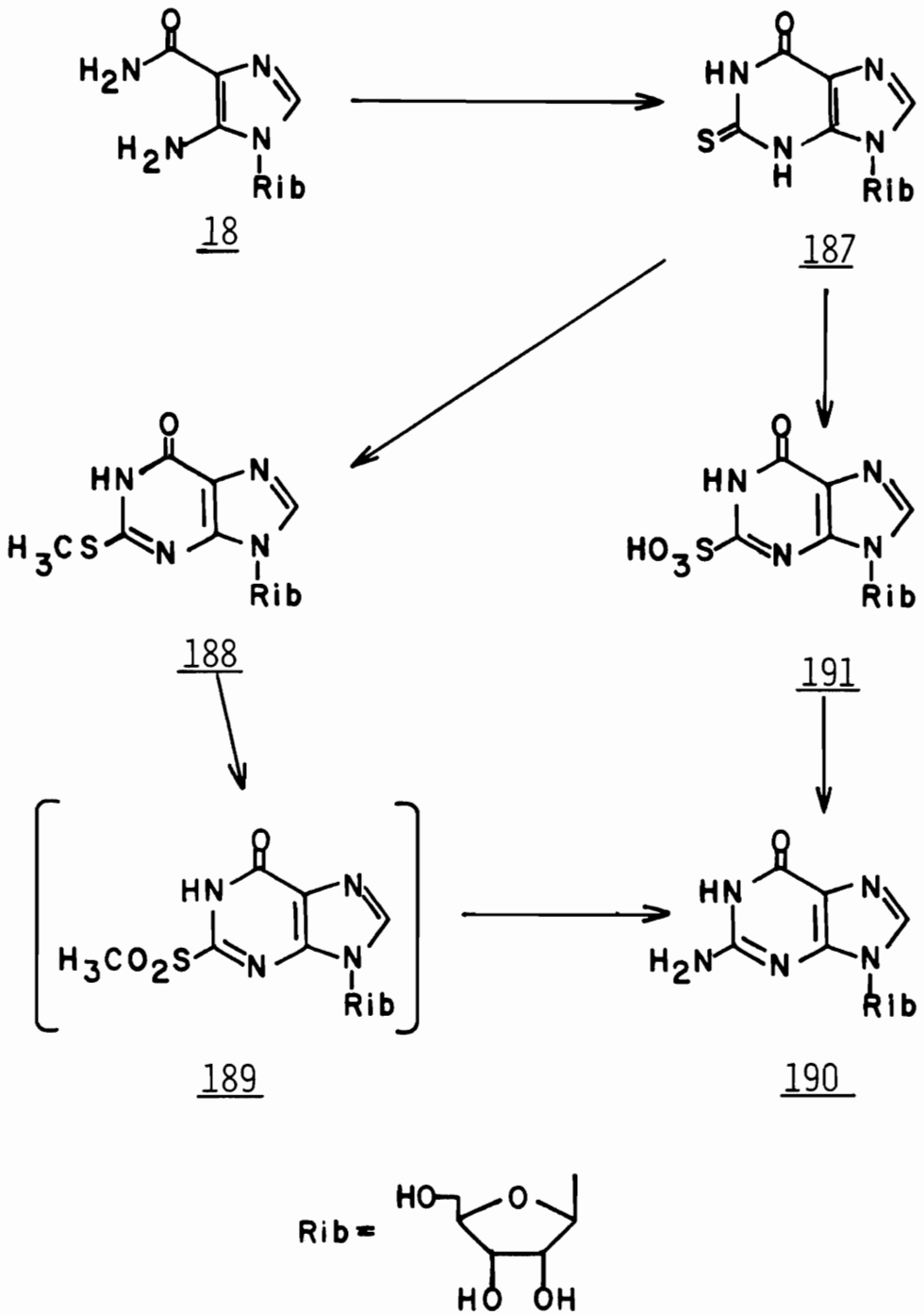
5-AMINO-3-(β-D-RIBOFURANOSYL)PYRAZOLO-[4,3-d]PYRIMIDIN-7-ONE: A GUANOSINE ANALOG

Background

Several methods[131-133] which were developed for the synthesis of 2-substituted adenines and hypoxanthines could possibly be applied to obtain 5,7-disubstituted pyrazolo[4,3-d]pyrimidines from formycin-N^6-oxide (162), 6-hydroxyformycin B (163) or 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamidoxime (186). However, the most direct route to the guanosine analog 19 is by ring annulation of the o-aminoamide 17.

Yamazaki and coworkers have described two methods for the synthesis of guanosine (190) from AICA-riboside (18).[134-137] AICA-riboside was treated with sodium methylxanthate at elevated temperatures to obtain 2-thioinosine (187).[135] Nucleoside 187 was then methylated to obtain 2-methylthioinosine (188).[135] The methylthio function of 188 was oxidized (NCS) and then displaced with ammonia to give guanosine (190).[135] The nucleoside 187 was also oxidized (peroxide) to the sulfonic acid 191, which when treated with ammonia also produced guanosine (190).[135] The reaction of 18 with sodium methylxanthate and the ammonia

Reaction Scheme 26



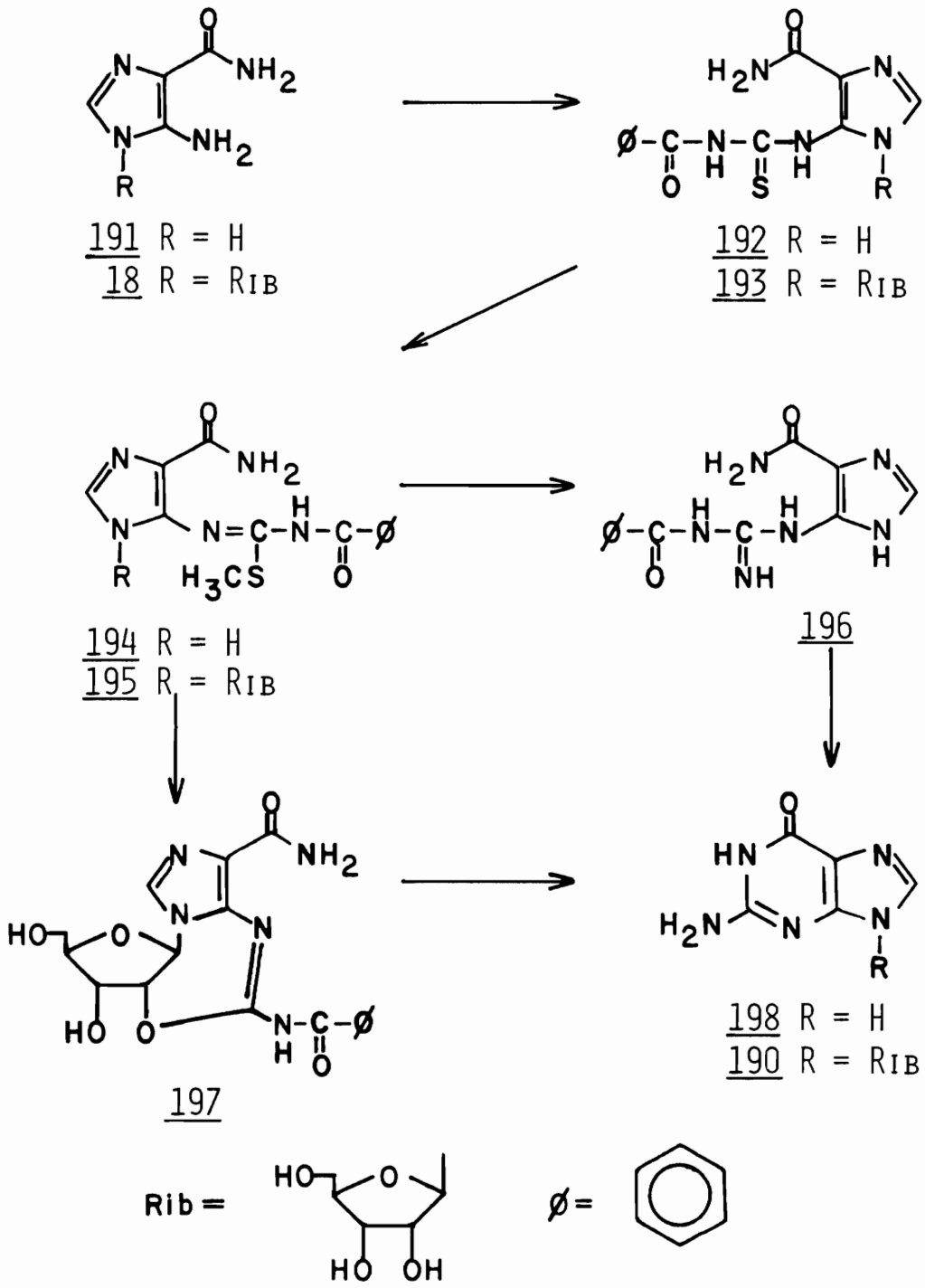
displacements required heating in an autoclave at temperatures of one-hundred and twenty to one-hundred and eighty degrees.[135]

The other method developed by Yamazaki and coworkers[134,136,137] for the synthesis of guanosine (190) avoided the necessity of high temperature autoclave reactions. AICA-riboside (18) was condensed with benzoylisothiocyanate to form the thiocarbamoyl derivative 193 which was then methylated to give 194.[137] Treatment of 194 with base resulted in the formation of the anhydronucleoside 197.[137] Compound 197 was readily attacked by a variety of nucleophiles to produce various 2-substituted inosines.[137] Treatment of 197 with ammonium hydroxide gave guanosine (190).[137] The avoidance of several high temperature autoclave reactions and the versatility of the anhydronucleoside 197, made this procedure most attractive as a means of obtaining the guanosine analog 5-aminoformycin B (19).

Two reports[138,139] have described the synthesis of 5-aminopyrazolo[4,3-d]pyrimidin-7-one. Neither of these two reports gave spectral data for this quanine analog. As a model for the synthesis of 5-aminoformycin B (19) the synthesis of 5-aminopyrazolo[4,3-d]pyrimidin-7-one (203) by the method of Yamazaki[134] was planned.

Yamazaki, et al.,[134] condensed AICA (191) with benzoylisothiocyanate to obtain the thiocarbamoyl derivative 192.[134] Compound 192 was methylated to form 194, which was then heated in an autoclave with two percent ammonia in dimethylformamide to give 5-N'-benzoylguanidinoimidazole-4-carboxamide (196).[134] When 196

Reaction Scheme 27



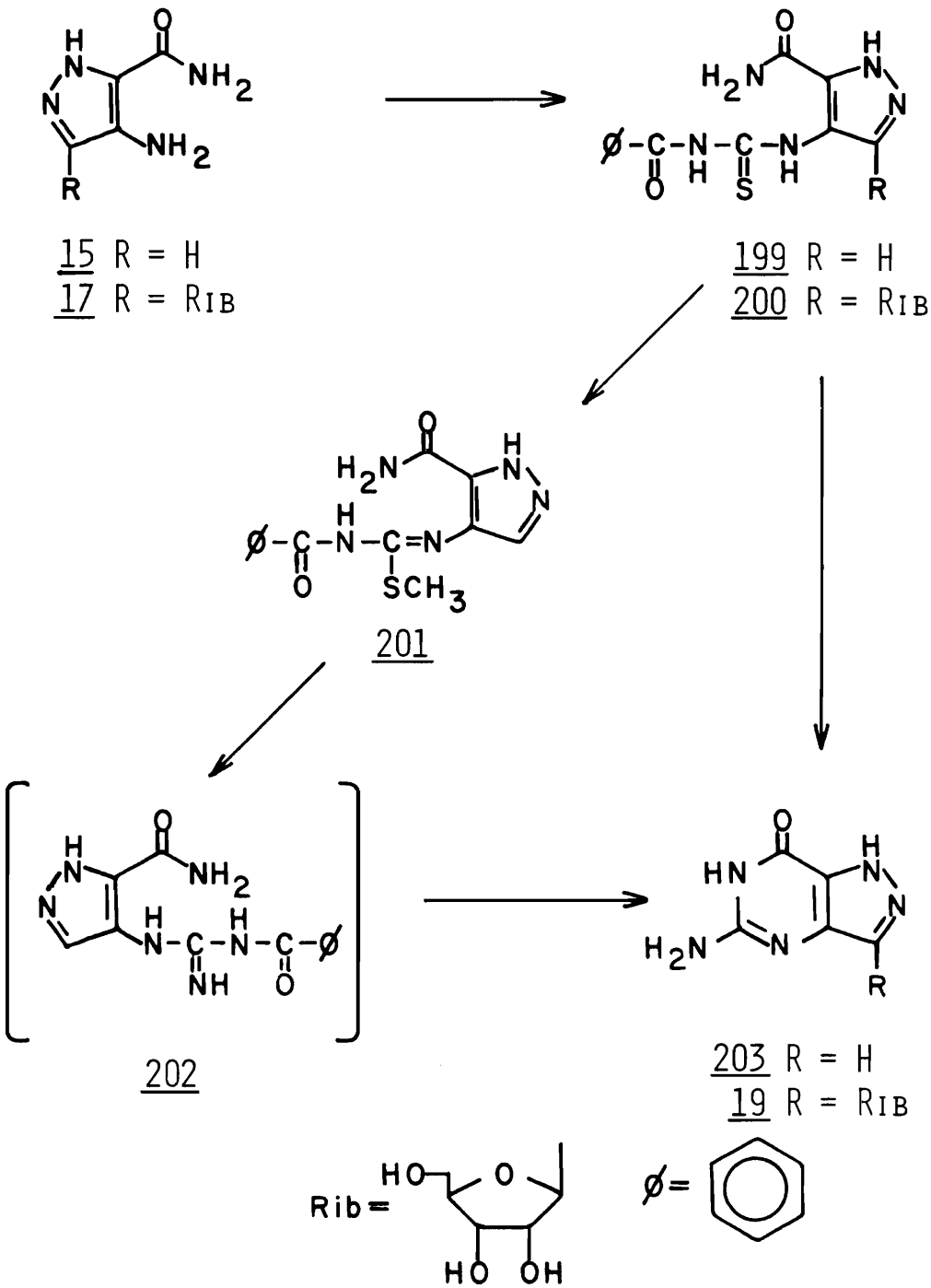
was boiled with dilute sodium hydroxide, guanine (198) resulted.[134]

The Synthesis of 5-Aminopyrazolo-[4,3-d]pyrimidin-7-one (203)

Condensation of 4-aminopyrazole-3-carboxamide (15)[19] with benzoylisothiocyanate resulted in the formation of 4-(N'-benzoyl-thiocarbamoyl)aminopyrazole-3-carboxamide (199) in fifty percent yield. The structure 199 was confirmed by examination of its pmr (DMSO-d_6) spectra. A pattern of three acidic, exchangeable protons was observed between δ11 and δ14 and no signal was observed between δ4 and δ7. Treatment of 199, under basic conditions, with methyl iodide gave 4-(N'-benzoyl-S-methylisothiocarbamoyl)aminopyrazole-3-carboxamide (201), which was obtained in ninty-five percent yield. The pmr (DMSO-d_6) spectrum of 201 established that methylation of the pyrazole ring had not resulted. The only signal attributable to a methyl group was observed at δ2.67, which indicated that only the sulfur atom of the thiocarbamoyl side chain of 199 had been methylated.

Striking differences were observed from comparisons of the uv and pmr spectra of compounds 199 and 201. Surprisingly, 199 exhibited, under all conditions, considerable low energy uv absorption at wavelengths above 300 nm. In contrast, 201 showed definite low energy absorption only in methanolic solution. In the pmr (DMSO-d_6) spectra of 199, the aromatic (H_3) proton was observed at δ9.14 whereas the corresponding signal for 201 (δ8.44) was considerably more upfield. The favored tautomeric form of 201 could not be determined from a comparison of the pmr (DMSO-d_6)

Reaction Scheme 28



spectra of 199 and 201. Presumably, 201 exists as a single tautomer, at least in dimethyl sulfoxide solution, as relatively sharp singlets appeared, in the pmr spectrum, for the acidic, exchangeable protons at δ10.95 and δ13.42.

Compound 201 was treated with a saturated solution of ammonia in dimethylformamide to obtain a mixture of two new products. This mixture was refluxed with dilute sodium hydroxide solution to produce a compound which was identified as 5-aminopyrazolo[4,3-d]-pyrimidin-7-one (203) by its pmr (DMSO-d_6) spectrum. The pmr spectrum of 203 exhibited a broad, two proton singlet at δ6.24 which disappeared on the addition of D_2O. Several recrystallizations and/or reprecipitations were required to obtain pure 203.

A reexamination of the mixture resulting from the treatment of 201 with ammonia revealed that one of the two products was 203 and presumably the other was the guanidino derivative 202. Thus, 203 was achieved, in part, directly from 201 by the use of a more concentrated ammonia solution than the solution used by Yamazaki, et al.,[134] for the conversion of 194 into 196.

The low yield of 203 obtained from the just described procedure, prompted the investigation of an alternate route which might afford 203 in a more substantial yield. Compound 15 was treated with O-methylisourea, at high temperatures, to obtain a product which had closely similar uv spectra to those observed previously for 203. The pmr spectrum for this product clearly indicated that 203 had not been obtained from this reaction since a signal for an aromatic amino group between δ6 and δ7 was absent. An aromatic proton (H_3)

singlet at δ7.54 and exchangeable singlets at δ10.87 (b,2H) and δ13.8 (b,2H) were the only signals observed in the pmr (DMSO-d_6) spectrum of this compound. The synthesis of 203 by this route was not further pursued.

The Synthesis of 5-Amino-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]-pyrimidin-7-one (19)

An excellent yield of 4-(N'-benzoylthiocarbamoyl)amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide (200) was obtained from the condensation of benzoylisothiocyanate with ΨAPCA-riboside (17). The pmr (DMSO-d_6) spectrum of 200 showed a similar pattern of acidic, exchangeable protons as had been observed in the spectrum of 199. Nucleoside 200 was treated, under basic conditions with methyl iodide. A solid product was not obtained from the aqueous reaction mixture. In fact, it was very difficult to detect, by tlc, that a reaction had occurred. Ultraviolet analysis of the reaction mixture substantiated that a reaction had occurred and it was assumed that a methylthiocarbamoyl derivative analogous to 201 had been formed. The residue, after evaporation of solvent, from this reaction was stirred with dilute sodium hydroxide for two days. Attempts to isolate, from this reaction, an anhydro-nucleoside analogous to 197 were unrewarded. Thin layer chromatographic analysis indicated that four or more components were present in this reaction mixture. The mixture was heated with concentrated ammonium hydroxide to obtain another mixture composed of two major and several minor products. Pure samples of the two

major products were obtained by silica gel column chromatography of the mixture. One of these products gave uv spectra which were closely similar to the spectra recorded for the guanine analog 203. The second major product was assigned the structure of 5-methylthio-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidin-7-one (5-methylthioformycin B) from the close similarity of its uv spectra to the spectra of 3-methyl-5-methylthiopyrazolo[4,3-d]-pyrimidin-7-one[20,90] and its mass spectrum; M^+ = 674 (M + 5 TMS), m/e = 355 (b+3O+2TMS). Thus, evidently a ring closure of the methylthiocarbamoyl intermediate competes, in basic solution, with the formation of an anhydronucleoside, if the latter is formed at all. This initial investigation also showed that silica gel chromatography was not an efficient means for the purification of the guanosine analog 19 as it eluted from the column in a very broad band.

In a subsequent experiment, nucleoside 200 was reacted with methyl iodide and the crude reaction products were treated with a saturated solution of ammonia in dimethylformamide. After evaporation of the solvent, the mixture was then refluxed with dilute sodium hydroxide. The resultant basic reaction mixture was applied to Dowex 1 (formate) ion exchange resin. The resin was washed with water, which removed a uv absorbing compound that had spectral (uv) and chromatographic (tlc) characteristics identical to 17. Elution of the ion exchange resin with the dilute formic acid gave a mixture of three products, none of which had the appearance

of 5-methylthioformycin B. Pure 19 was obtained from this mixture by chromatography on Dowex 1 (formate), followed by chromatography on a shallow silica gel bed and then a second gradient elution from Dowex 1 (formate). 5-Aminoformycin B (19) was identified by elemental analysis and spectral comparisons with the guanine analog 203.

As was the case with the synthesis of 203, 19 was also obtained directly from the reaction between ammonia and the methylthiocarbamoyl intermediate. Following methylation of nucleoside 200, the reaction products were treated with a saturated solution of ammonia in dimethylformamide. The solvent and excess ammonia was removed by evaporation and the reaction residue stirred with water to produce impure 19. Pure 19 was obtained by solvent (ethanol) extraction of the impure product followed by recrystallization. An approximate yield of seven percent (from 17) was achieved. However, considerable quantities of impure 19 were recovered from the solvent extraction and recrystallization filtrates.

Biological Evaluation and Biochemical Studies

The synthesis of the guanosine analog, 5-aminoformycin B (19) has only just been completed. Insufficient time has passed to obtain even preliminary data regarding the biological or biochemical properties of this potentially valuable analog.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The methylation of formycin (1), under nonbasic conditions, has afforded 4-methylformycin (11) and 6-methylformycin (171). The pyrimidine N-methylated isomers of 1 (i.e., 11 and 171) were contrasted with the pyrazole N-methylated isomers 7 and 8. Methylation in the pyrimidine ring of 1 significantly increased the basicity of the system, whereas, methylation in the pyrazole ring resulted in only small changes in the basicity of the parent. The pyrimidine methylated isomers were not cytotoxic to L-1210 cells in culture and they did not form nucleotides when incubated with human erythrocytes.[110] On the other hand, both pyrazole methylated isomers displayed cytotoxicity to L-1210 cell cultures comparable to formycin (1) and both were incorporated into erythrocytic nucleotide pools.[110] Of the ring methylated isomers only 2-methylformycin (8) was a substrate for human erythrocytic adenosine deaminase.[110]

Methylation at the 6-position of formycin (1) profoundly altered the electronic structure and reactivity of formycin (1). Several unexpected spectral characteristics were observed for 6-methylformycin (171) which had no parallel with the other ring methylated isomers 7, 8 and 11. Additionally, nucleoside 171

readily reacted with boiling water to give formycin B (6), N^7-methylformycin (150) and 6-methylformycin B (172).

The reaction of 6-methylformycin (171) with nitrosyl chloride also produced 6-methylformycin B (172). 4-Methylformycin B (177) and 1-methylformycin B (178) were similarly synthesized from 7 and 8. The syntheses of 11, 171, 172, 177 and 178 completed the preparation of all possible mono-N-methyl isomers of formycin (1) and formycin B (6).

2-Methylformycin (8) and 4-methylformycin (11) were used as *syn* and *anti* models of formycin (1) in a study with human erythrocytic adenosine deaminase.[110] The *syn* model, 2-methylformycin (8), was a substrate for this deaminase and the *anti* model, 4-methylformycin, was not deaminated.[110] These findings oppose the postulate[12,13] that only *anti* formycin nucleosides are substrates for adenosine deaminase. The results of this study[110] argue against the *a priori* acceptance of conformational requirements for the interaction of nucleosides with adenosine deaminase, especially when these requirements result from studies wherein the aglycon is modified at a ring nitrogen to force a particular conformational stereochemistry. These studies[110] also suggest that steric inhibition to enzyme binding at the purine 7-position may be more important in inhibiting deamination. This possibility requires further study and should not be taken as a conclusion from the present study.

An alternate method for the synthesis of N^7-methylformycin (150) has been developed. Studies of 150 showed[110] that this

mono-N-methyl isomer of formycin was moderately cytotoxic to L-1210 cells in culture, was incorporated into erythrocytic nucleotide pools and was a poor substrate for erythrocytic adenosine deaminase. These findings[110] are in sharp contrast to previous studies which showed that N^7-methylformycin was inactive to Yoshida sarcoma cells *in vitro*[70] and the suggestion[5] that 150 is not a substrate for a kinase.

A preliminary study of the enzymatic reduction of formycin-5-triphosphate (FTP, 64) by the ribonucleotide reductase from *L. leichmannii* has shown this to be a viable alternative method for the synthesis of 2'-deoxyformycin (12). For this study, formycin-5-phosphate (FMP, 57) and FTP (64) were prepared by standard methods of nucleotide synthesis. The methods used for these syntheses allow for the efficient recovery and reutilization of unreacted starting materials and side products.

Blakley and coworkers found[117] that FTP was not a substrate for the *L. leichmannii* reductase unless the positive effector dGTP was present. In the absence of dGTP, FTP was found to be a competitive inhibitor of the reduction of ATP, GTP and UTP.[117] The product of FTP reduction, 2'-deoxyformycin-5'-triphosphate (dFTP, 180) markedly stimulated the reduction of CTP by the *L. leichmannii* reductase.[117] This finding refutes the conclusion of Shuadolnik, *et al.*,[74] that the carbon at the 8-position, but not the nitrogen at the 7-position of purine nucleosides is necessary for interaction at the regulatory site in nucleoside triphosphate reductase.

A new route, resulting in the improved synthesis of

4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide (ψAPCA-riboside, 17), has been established. Formycin-N^6-oxide was found to undergo facile pyrimidine ring fission in acidic environments, resulting in the formation of 4-amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamidoxime (186). Nucleoside 186 was simultaneously reduced and hydrolyzed to provide ψAPCA-riboside (17). Pure 17 was difficult to achieve, requiring reverse phase, high pressure liquid chromatography. Substantial quantities of 17 were, however, obtained without chromatography and in sufficient purity for use in subsequent synthetic experiments.

ψAPCA-riboside (17), the amino analog of pyrazofurin (5), was inactive against L-1210 cells in culture.[21b] Pyrazofurin (5), on the other hand, inhibited the growth of L-1210 cells.[21b] These findings supported the proposal[22] that the 4-hydroxy group of pyrazofurin (5) was essential to its activity.

Annulation of ψAPCA-riboside (17) has resulted in the first synthesis of the guanosine analog, 5-amino-3-(β-D-ribofuranosyl)-pyrazolo[4,3-d]pyrimidin-7-one (5-aminoformycin B, 19). Recent completion of this synthesis has precluded biological or biochemical studies of 19. The greatest potential utility for this guanosine analog is as a probe and/or inhibitor in certain purine metabolic pathways.

CHAPTER 8

EXPERIMENTAL

Instrumental and Analytical Procedures

Elemental analyses were obtained from Heterocyclic Chemical Corp., Harrisonville, Mo. or M-H-W Labs., Phoenix, Az.

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected.

Ultraviolet spectra were recorded on a Beckman ACTA C-III spectrophotometer.

Pmr spectra were obtained for both DMSO-d_6 and DMSO-d_6/D_2O solutions of the compounds and were recorded on a Varian EM-390 instrument. Chemical shifts are expressed as δ values from an internal standard (DSS).

Cmr spectra were obtained by the techniques described in reference 102.

Mass spectra were recorded on a Varian MAT 112S instrument and are electron impact spectra.

The pKa data were obtained by the spectroscopic method described by Albert and Serjeant.[103] A concentration of 20 µg/ml was used to increase the absorption differences between the ionic species.

HPLC was conducted on a Waters Associates instrument.

Chromatographic Procedures and Techniques

Thin layer chromatography was performed on glass plates coated (0.25 mm) with SilicaAR-7GF (Mallinckrodt). The chromatogram components were visualized with a shortwave (254 nm) uv mineralight.

Column chromatography used SilicAR-CC-7 (Mallinckrodt) as the stationary phase.

Dry column chromatography used silica gel (J. T. Baker #5-3405) to which 0.5% of a phosphor (Dupont #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 allowed to elute from the column. The progress of the uv absorbing components were monitored with a shortwave (254 nm) uv mineralight.

HPLC analyses used a 4 x 300 mm μBondapak C_{18} (Waters) column. A 8 x 1800 mm Proasil B-C_{18} (Waters) column was used for the preparative separations. Water was used as the mobile phase.

Paper chromatography was conducted on Whatman #1.

Electrophoresis (2000v) was conducted on Whatman 3MM paper and the components were visualized with a short-wave (254 nm) uv mineralight or with Hanes-Isherwood reagent.[140]

Chromatography solvents used were:

A: water.

B: methanol.

C: ethyl acetate:water:n-propanol, 4:2:1, v/v/v, upper phase.

D: chloroform:methanol, 9:2, v/v

E: ethanol.

F: chloroform:methanol, 3:1, v/v.

G: acetonitrile:0.1 M ammonium chloride, 7:3, v/v.

H: acetonitrile:0.1 M ammonium chloride, 9:2, v/v.

I: iso-butyric acid:1 M ammonium hydroxide:0.1 M disodium ethylenediamine tetraacetic acid, 25:15:0.4, v/v/v.

J: 1 M ammonium formate (pH 3.8).

K: ethyl acetate.

Chemicals and Reagents

Activated charcoal. Barnaby-Cheney AU-4 charcoal was acid washed, then washed thoroughly with water and dried before use.

Dowex 1 X 8 (formate). Bio-Rad commercial grade Dowex 1 X 8 (chloride), 100-200 mesh, was converted to the formate form as described in the table of conversions in the Bio-Rad catalog.

Bis-(tri-n-butylammonium)pyrophosphate. Dowex 50W-X 4 (H^+), 100-200 mesh, (200 ml) was stirred with a 10% pyridine solution (1 L.) for 12-16 hr. The resin was filtered and washed well with water. The resin was then slurried in water and packed in a column. A solution of tetrasodium pyrophosphate decahydrate (5 g) in water (30 ml) was applied to the column. The column was eluted with water collecting 1 L. The water solution was evaporated in vacuo to near dryness, and the remaining material dissolved in 80% pyridine (50 ml). Tri-n-butylamine (5.4 ml) was added and the mixture evaporated in vacuo. The residue was coevaporated with pyridine (4 x 25 ml) and the final evaporation continued until a constant

weight was obtained. The pyrophosphate was not heated at a temperature above 50° during the evaporations. The remaining residue was assumed to be pure bis-(tri-n-butylammonium)pyrophosphate and was dissolved in pyridine (40 ml) and stored under refrigeration (5°) until needed.

Triethylammonium bicarbonate. A 1 M solution of triethylammonium bicarbonate (pH 7.5) was prepared as described by Khorana and Conners[141] and stored under refrigeration until needed and then diluted as required.

DEAE cellulose (bicarbonate). DE-52 (Whatman #24522) (300 g) was stirred with 0.05 M triethylammonium bicarbonate (pH 7.5) (2 L.) for 10-15 min. Carbon dioxide was added to readjust the pH to 7.5. The conductivity was recorded and the mixture allowed to stand for 15 min. without stirring. The supernatant liquid with the DE-52 fines was decanted. Treatment with 0.05 M triethylammonium bicarbonate (pH 7.5) was repeated until a constant pH and conductivity were obtained.

Benzoylisothiocyanate was purchased from Trans-World Chemicals, redistilled and stored under refrigeration until needed.

Formycin was purchased from Meiji Seika Kasha Co., Tokyo.

Other chemicals used in these investigations were high grade, commercially available materials and were not further purified unless otherwise specified.

Other Methods and Techniques

Evaporations were conducted on a rotary evaporator using a hot

water bath as a heat source and under aspirator vacuum for low boiling (bp $\leq$ ethanol) solvents or high vacuum for higher boiling solvents, unless otherwise specified.

Spectroscopic Analysis of the Product Ratio from the Methylation of Formycin. Formycin (1 g) was methylated as described in the Syntheses section. After a removal of the solvent and the addition of 1 N ammonium hydroxide, the mixture was evaporated to dryness in vacuo. The resulting residue was coevaporated with ethanol (3 x 25 ml), triturated with chloroform (2 x 50 ml), and then dissolved in aqueous methanol. An aliquot was applied to a tlc plate, and the chromatogram developed with solvent B. The band containing a mixture of 11 and 171 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;[100]

$$Am_{\lambda_1} = (a_{11\lambda_1} C_{11} + a_{171\lambda_1} C_{171})b$$

$$Am_{\lambda_2} = (a_{11\lambda_2} C_{11} + a_{171\lambda_2} C_{171})b$$

where Am is the observed absorption of the mixture of 11 and 171 at the chosen wavelengths λ_1 and λ_2, a_{11} and a_{171} are the absorptivities of 11 and 171 at the chosen wavelengths, and b is the cell path length in cm. 314 nm where a_{11} is at a maximum and $a_{11} > a_{171}$ and 288 nm where a_{171} is at a maximum and $a_{171} > a_{11}$ were chosen as λ_1 and λ_2 respectively.[100]

Syntheses

4-Methylformycin (11) and 6-Methylformycin (171): Formycin (1) (2 g) was dissolved in dry dimethylformamide (60 ml), methyl iodide (2 ml) was added, and the mixture then stirred at room temperature for 25.5 hr. 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 x 50 ml), the diethyl ether was discarded, and the residue dissolved in water (30 ml). The pH of the resulting solution was adjusted to approximately 9.5 by the addition of 1 N ammonium hydroxide. A white solid separated after stirring at room temperature for 0.5 hr, and this was followed by refrigeration for 1.5 hr. The solid was collected by filtration, washed with cold water (10 ml), and air dried to yield 1.2 g (50%) of 171; mp 224-227° dec. Pure 171 was obtained by two recrystallizations from water; 650 mg; mp 231-232° dec. Anal. Calc'd. for $C_{11}H_{15}N_5O_4 \cdot 2H_2O$ (317.3): C, 41.64; H, 6.04; N, 22.07. Found: C, 41.53; H, 5.86; N, 21.96. The hydration of 171 was verified by pmr. Uv: see Table 1. Pmr: see Table 2. Cmr: see Table 3. Ms: see Table 4. Tlc: see Table 6.

The aqueous solution (filtrate and washings), after removal of 171, was evaporated to dryness in vacuo on a steam bath. The residue was redissolved in water (25 ml), and Dowex 1 X 8 ($^-$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 hr to give **11** (250 mg, 12%). An additional quantity (120 mg) of **11** was obtained by extraction of the Dowex 1 X 8 ($^-$OH) resin with a water-concentrated ammonium hydroxide mixture (4:1, v/v) (4 x 50 ml) followed by evaporation of the extracts and stirring the resulting residue with ethanol (50 ml). The total yield of **11** was 370 mg (17.5%). Pure **11** was obtained by recrystallization from aqueous ethanol and drying *in vacuo* at 110°; 200 mg; mp 268-269° dec. *Anal.* Calc'd. for $C_{11}H_{15}N_5O_4$ (281.3): C, 46.97; H, 5.38; N, 24.90. Found: C, 46.71; H, 5.31; N, 25.06. *Uv*: see Table 1. *Pmr*: see Table 2. *Cmr*: see Table 3. *Ms*: see Table 4. *Tlc*: see Table 6.

The reaction mixture was analyzed by uv spectroscopy as outlined in the preceding section. The concentrations (C) of **11** and **171** were calculated and C_{171}/C_{11} was found to be 2.24.

N^7-Methylformycin (**150**), *Method A*: 3-(β-D-Ribofuranosyl)-7-chloropyrazolo[4,3-d]pyrimidine (**48**)[44] (500 mg) was mixed with methylamine·HCl (130 mg), anhydrous sodium acetate (500 mg), and ethanol (50 ml). The mixture was stirred and heated at reflux for 2 hr. The reaction mixture was evaporated to dryness *in vacuo*, the residue was then coevaporated with water (2 x 10 ml) and then *iso*-propanol (3 x 20 ml). The residue was extracted with hot *iso*-propanol (2 x 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 #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" x 12"), and the column eluted with solvent C 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 150; mp foams above 145°. The solid was dried *in vacuo* at 110°. *Anal.* Calc'd. for $C_{11}H_{15}N_5O_4 \cdot H_2O$ (299.3): C, 44.14; H, 5.73; N, 23.4. Found: C, 44.4; H, 5.96; N, 23.74. The hydration of 150 was verified by pmr. *Uv*: see Table 1. *Pmr*: see Table 2. *Cmr*: see Table 3. *Ms*: see Table 4. *Tlc*: see Table 6.

Method B: 7-Chloro-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-pyrazolo[4,3-d]pyrimidine (47)[44] (5 g) was mixed with methylamine hydrochloride (5 g), anhydrous sodium acetate (5 g) and ethanol (400 ml). The mixture was stirred and heated at reflux for 4 hr and then evaporated to dryness *in vacuo*. The residue was extracted with hot *iso*-propanol (2 x 150 ml). Silica gel (J. T. Baker #5-3405) (30 g) was added to the combined extracts and the mixture evaporated to dryness *in vacuo*. The dry powder was placed on top of a dry column (2" x 12") of silica gel and the column eluted with solvent C. The first 100 ml of eluate was discarded. The next 500 ml of eluate contained two uv absorbing products, each of which gave a correct uv spectrum for 150. The solution containing these products was evaporated to dryness *in vacuo*, and the residue dissolved in 20% aqueous methylamine (40 ml). After standing at room temperature

for 15 hr, the solution was evaporated to dryness *in vacuo* and the residue coevaporated with water (3 x 50 ml) and then ethanol (3 x 50 ml). The residue was dissolved in water (25 ml) and the pH of the solution was then adjusted to approximately 5 with glac. acetic acid. The resulting solution was evaporated to dryness *in vacuo* and the residue coevaporated with ethanol (3 x 25 ml). The residue was dissolved in *iso*-propanol (50 ml), silica gel (J. T. Baker #5-3405) (10 g) was added and the mixture was then evaporated to dryness *in vacuo*. The dry powder was placed on a silica gel dry column (1.5" x 15") and the column eluted with solvent C. The fractions containing uv absorbing material were combined and evaporated to dryness *in vacuo*. The residue was recrystallized from an ethanol-ethyl acetate mixture to obtain 1.5 g (41%) of a white crystalline solid which was identical spectroscopically (uv, pmr) and chromatographically to *150* obtained by method A.

Water Hydrolysis of 171: An aqueous solution of *171* (100 mg/10 ml) was heated at reflux for 24 hr. The course of the reaction was monitored by tlc using solvent D as the developing solvent. At the end of 6 hr of reflux only a trace of unreacted *171* remained, and three new products had been formed as determined by tlc. No further change was noted at the end of 24 hr of 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 x 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: $\lambda_{max}^{pH\ 1}$ = 272 nm; $\lambda_{max}^{pH\ 11}$ = 280 nm; ms: M^+ = m/e 282) was identified as 6-methylformycin B (172) by tlc comparison in three solvent systems to (172) prepared by the nitrosyl chloride reaction with 171. The second product eluted from the column was identified as N^7-methylformycin (150) by its uv spectra and tlc comparison to authentic 150. The third product was identified as formycin B (6) by its uv spectra, ms (M^+ = m/e 268), and tlc comparison to authentic formycin B.

3-Methyl-4-nitropyrazole-5-(N-methyl)carboxamide (174): Ethyl 3-methyl-4-nitropyrazole-5-carboxylate[106] (21 g) was added to 40% aqueous methylamine (300 ml) and the mixture was heated on a steam bath, under a reflux condenser, for 1.5 hr. The condenser was removed and heating was continued for an additional 0.5 hr. The resulting solution was then evaporated to dryness in vacuo. The residue was dissolved in water (70 ml) and the pH was adjusted to approx. 6 by the addition, with stirring, of dilute hydrochloric acid. The mixture was heated to boiling and water was slowly added to dissolve all the solid (final volume approx. 500 ml). The boiling solution was treated with charcoal, filtered, and the filter cake washed with boiling water (75 ml). The combined filtrate and wash was refrigerated for 16 hr. The crystalline solid

was collected by filtration, washed with water and air dried to yield 14.22 g (73%) of 174; mp 216-220°. A second crop (1.6 g, mp 210-218°) was obtained by concentration of the filtrate to approx. 100 ml and refrigeration of the concentrate. A pure sample (mp 218-220°) was obtained by recrystallization from water (80% recovery) and drying at 110° in vacuo. Anal. Calc'd. for $C_6H_8N_4O_3$ (184.2): C, 39.14; H, 4.38; N, 30.42. Found: C, 39.21; H, 4.10; N, 30.61. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 277 (7.92); pH 11, 317 (10.5); methanol, 271 (7.0).

4-Amino-3-methylpyrazole-5-(N-methyl)carboxamide hemihydrate (175): 3-Methyl-4-nitropyrazole-5-(N-methyl)carboxamide (174) (11 g) was dissolved, by heating, in a mixture of ethanol (400 ml) and water (50 ml). The solution was then allowed to cool to room temperature. A slurry consisting of 10% Pd/C (1 g) in water (50 ml) was added to the mixture which was then shaken in a hydrogen atmosphere (2.8 Kg/cm^2, initial pressure) for 2 hr. The catalyst was removed by filtration thru a celite bed, washed with a boiling ethanol-water (3:2, v/v) mixture (200 ml) and the combined filtrate and wash evaporated to dryness in vacuo. The residue was coevaporated with ethanol (2 x 50 ml) to obtain 9 g (92%) of 175 as a light purple solid; mp 179-181°. A pure sample of 175 (mp 181-182°) was obtained by three recrystallizations from water (60% recovery) followed by drying in vacuo at 80°. Anal. Calc'd. for $C_6H_{10}N_4O \cdot 0.5 H_2O$ (163.2): C, 44.16; H, 6.79; N, 34.34. Found: C, 44.21; H, 7.26; N, 34.17. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 11, 232 (6.69), 280 (4.98);

methanol 280 (4.57). Tlc: solvent A, R_{174} = 0.73; solvent C, R_{174} = 0.6.

3,6-Dimethylpyrazolo[4,3-d]pyrimidin-7-one (176): Method A: 4-Amino-3-methylpyrazole-5-(N-methyl)carboxamide hemihydrate (175) (1 g) was mixed with 99% formamide (6 ml) and then placed in a preheated oil bath at 180°. The temperature of the oil bath was slowly raised to 200° over a period of 1 hr and then maintained at 200-205° for an additional 1.75 hr. After cooling (< 100°), H_2O (20 ml) was added to the dark colored solution and the mixture refrigerated for 16 hr. The dark brown solid was collected by filtration, recrystallized twice, with charcoal treatment, from water to yield 410 mg (41%) of 176, which was dried in vacuo at 110°; mp 229-231°. Anal. Calc'd. for $C_7H_8N_4O$ (164.2): C, 51.21; H, 4.91; N, 34.12. Found: C, 51.22; H, 4.99; N, 34.20. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 281.5 (7.14); pH 11, 281.5 (6.4), 233 (18.6); ethanol, 271.5 (6.81). Tlc: solvent A, R_{175} = 0.79; solvent E, R_{175} = 0.87; solvent D, R_{175} = 1.11.

Method B: 4-Amino-3-methylpyrazole-5-(N-methyl)carboxamide hemihydrate (2 g) was mixed with diethoxymethyl acetate[107] (20 ml) and the mixture then stirred and heated at reflux for 5 hr. The resulting solution was evaproated in vacuo on a hot water bath, ethanol (25 ml) was added and the solution was again evaporated in vacuo. Recrystallization of the residue from water, with charcoal treatment, yielded 1.31 g (65%) of a solid which was identical (uv, mp) with the product obtained by method A.

6-Methylformycin B (172): 6-Methylformycin dihydrate (171) (150 mg) was mixed with pyridine (5 ml) and chloroform (5 ml). The mixture was stirred and heated in an oil bath at 60° (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° (bath temperature) for 2 hr. The reaction mixture was evaporated in vacuo, and the resulting oily residue was coevaporated with ethanol (3 x 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 ammonium hydroxide. The basic solution was applied to a column Dowex 1 X 8 (formate) (50 ml), and the column washed with water (400 ml). The column was then eluted with 0.1 N formic acid (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 x 20 ml) and then recrystallized from ethanol to obtain 60 mg (45%) of 172; mp 201-203°. The compound was dried at 110° in vacuo. Anal. Calc'd. for $C_{11}H_{14}O_5N_4$ (282.3): C, 46.81; H, 5.00; N, 19.85. Found: C, 46.76; H, 4.84; N, 19.92. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 275 (9.0); pH 11, 281.5 (6.2); 230 (23.6). Pmr: δ 3.52 (s, NCH_3), δ 4.93 (d, $H_{1'}$), δ 8.16 (s, H_5). Tlc: see Table 6.

4-Methylformycin B (177): 4-Methylformycin (11) (150 mg) was mixed with pyridine (5 ml) and chloroform (5 ml). The mixture was stirred and heated in an oil bath at 60°. A solution of nitrosyl

chloride in chloroform (5 ml, 1.4 mmole/ml) was added over an interval of 5 minutes. The mixture was stirred and heated at 65-70° (bath temperature) for 2 hr. The mixture was then evaporated *in vacuo*, and the oily residue was coevaporated with ethanol (3 x 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 ammonium hydroxide. The solution was then applied to a column of Dowex 1 X 8 (formate) (50 ml). The column was washed first with water (400 ml) and then eluted with 0.1 *N* formic acid 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 x 25 ml) and then recrystallized from ethanol to obtain 56 mg (37%) of 177; mp 239-240°. The compound was dried *in vacuo* at 110° for analysis. *Anal.* Calc'd. for $C_{11}H_{14}O_5N_4$ (282.3): C, 46.81; H, 5.00; N, 19.85. Found: C, 46.63; H, 4.96; N, 19.86. *Uv*, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 277.5 (10.3); pH 11, 302 (7.9), 263.5 sh (7.2), 237.5 (11.4). *Pmr*: δ 3.93 (s, NCH_3), δ 5.03 (d, $H_{1'}$), δ 8.13 (s, H_5). *Tlc*: see Table 6.

1-Methylformycin B (178): 1-Methylformycin (7) (300 mg) was mixed with chloroform (10 ml) and pyridine (10 ml). The mixture was stirred and heated in an oil bath at 60° (bath temperature). A solution of nitrosyl chloride in chloroform (10 ml, 1.4 mmole/ml) was added over a period of 10 min. The resulting mixture was stirred and heated at 65° (bath temperature) for 2 hr. The reaction mixture was evaporated *in vacuo* and the residue coevaporated with

Table 6

Comparative Tlc Data for the N-Methyl Derivatives of 1 and 6

Compound	Solvent A	Solvent F
7	R_1=0.91	R_1=0.00
8	R_1=0.89	R_1=1.36
11	R_1=0.73	R_1=0.17
171	R_1=0.80	R_1=0.33
150	R_1=0.81	R_1=1.47
172	R_6=0.82	R_6=1.20
177	R_6=0.85	R_6=0.42
178	R_6=0.94	R_6=1.38

ethanol (3 x 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 ammonium hydroxide. The solution turned a deep wine red in color once basic. The solution was applied to a column of Dowex 1 X 8 (formate) (50 ml). The column was washed with water (400 ml) and then eluted with 0.1 N formic acid 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 nm 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 x 50 ml). Tlc (solvent F) showed one major component with trace impurities indicating 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 178. The solid was dried at 110° in vacuo; mp 189-192° dec. Anal. Calc'd. for $C_{11}H_{14}O_5N_4$ (282.3): C, 46.81; H, 5.00; N, 19.85. Found: C, 46.81; H, 4.91; N, 19.86. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 280 broad (4.8), 226 (9.7); pH 11, 308 sh(3.0), 290 broad (5.7), 230 (2.1). Pmr: δ 4.14 (s, NCH_3), δ 4.87 (d, $H_{1'}$), δ 7.83 (s, H_5). Tlc: see Table 6.

Formycin-5'-phosphate (57): Formycin (2.3 g) was suspended in trimethyl phosphate (60 ml). The stirred suspension was cooled to

0° and phosphoryl chloride (2.4 ml) was then added dropwise (~30 min) maintaining a temperature of 0° ± 2°. Water (150 μL) was added and the mixture stirred for 30 min more at 0° ± 2°. The solid had all dissolved by this time and the mixture was refrigerated at 0-5° for 16 hr. The solution was then poured onto crushed ice (200 ml) with stirring and the pH adjusted to 2 with cold (5°) 4 N sodium hydroxide, maintaining a temperature of <10°. The aqueous solution was then stirred with enough activated charcoal (~40 g) to absorb all uv absorbing material. The charcoal was filtered and washed with water until the wash was no longer acidic (~2 L). The charcoal was then washed with an ethanol:conc. ammonium hydroxide:water (25:1:24, v/v/v) solution until little or no uv absorbing material was eluting (~2 L). The eluate was filtered through a celite bed and then evaporated *in vacuo* (temp. <40°) to a volume of approx. 50 ml. The remaining solution was then applied to a column of Dowex 1 X 8 (formate) (50 ml). The column was washed with water (~2 L) until uv absorbing material was no longer eluting. The column was then washed with 0.1 N formic acid until little or no uv absorbing material was eluting (~2 L). The formic acid solution was evaporated *in vacuo* (temp. <40°) and the residue coevaporated with ethanol until a white solid free of formic acid was obtained. Yield; 2.3 g of 57, which was chromatographically homogeneous. Tlc: solvent G, R_1 = 0.55; solvent H, R_1 = 0.17.

Formycin-5'-triphosphate, sodium salt (64): Morpholine (0.34 ml) was added to a solution of 57 in water (10 ml) and t-butanol (10 ml).

The solution was heated to reflux and, with continuous stirring, a solution of dicyclohexylcarbodiimide (0.83 g) in t-butanol (15 ml) was added slowly over a period of approx. 4 hr. The mixture was stirred and refluxed for 3 hr more and then allowed to stand at room temperature for 16 hr. The mixture was then filtered and the solid washed with t-butanol (10 ml). The combined filtrate and wash was extracted with diethyl ether (3 x 25 ml). The aqueous solution was then evaporated in vacuo (temp. <40°). The residue was dissolved in methanol (3 ml) and then diethyl ether (35 ml) was added to precipitate a sticky solid. The supernatant liquid was decanted from the precipitate and the precipitate was then triturated with diethyl ether (35 ml). The resulting solid was coevaporated in vacuo (temp. <40°) with pyridine (3 x 30 ml) and then a solution of bis-(tri-n-butylammonium)pyrophosphate in pyridine (50 ml, 0.1 mmole/ml) was added. After evaporation of the mixture in vacuo (temp. <40°) and redissolving of the residue in pyridine (50 ml), the mixture was allowed to stand at room temperature for 3 hr. The mixture was then evaporated in vacuo (temp. <40°) and the residue dissolved in 0.05 M triethylammonium bicarbonate (pH 7.5) (20 ml). The solution was evaporated in vacuo (temp. <40°), the residue redissolved in 0.05 M triethylammonium bicarbonate (pH 7.5) (10 ml) and the resulting solution applied to a column (3.5 x 40 cm) of DEAE-cellulose (bicarbonate). The column was washed with 0.05 M triethylammonium bicarbonate (pH 7.5) (100 ml) and then eluted with a linear gradient of triethylammonium bicarbonate (pH 7.5) using 0.05 M buffer (2 L)

in the mixing chamber and 0.5 M buffer (2 L) in the reservoir. Fractions of 15-20 ml were collected at 6 min intervals. An elution profile was obtained by plotting concentration (approximated by uv measurements) vs. fraction number. Homogeneous fractions in the triphosphate peak of the elution profile were determined by tlc (solvent I) analysis and were pooled. The resulting solution was evaporated in vacuo (temp. <30°). The residue was repeatedly co-evaporated with ethanol (50 ml) to remove triethylammonium bicarbonate. The residue was then dissolved in methanol (10 ml) and a solution of sodium perchlorate monohydrate (1.05 g) in acetone (50 ml) was added with stirring. The mixture was centrifuged and the supernatant liquid decanted. The solid was washed with acetone (2 x 50 ml) and then collected by filtration and dried in vacuo at ambient temperature. The FTP (64) thus obtained (220 mg) was homogeneous on tlc (solvent I), paper chromatography (solvent I) and high voltage electrophoresis (solvent J) with a migration comparable to the migration of ATP.

Formycin-N^6-oxide (162): A mixture of 1 (5 g), m-chloroperbenzoic acid (5 g) and methanol (250 ml) was stirred and refluxed for 2.5 hr. An additional portion of m-chloroperbenzoic acid (5 g) was added and the stirring and refluxing continued for 2.5 hr more. The hot reaction solution was then poured into ethyl acetate (1.5 L) with rapid stirring. After standing at room temperature for 14 hr, the mixture was filtered. The yellow solid was washed with ethyl acetate (100 ml) and air dried to give 4 g of impure 162. The

solid was suspended in boiling ethanol (700 ml). The boiling solution was treated with charcoal, filtered, and the filter cake washed with boiling ethanol (100 ml). The ethanol solutions were combined and evaporated to dryness *in vacuo*. The solid residue was washed with ethyl acetate (50 ml) and air dried; 3.55 g (67%) of *162* was obtained. The product was homogeneous on tlc (solvent H) and gave uv spectra identical to the spectra reported for *162* by Long.[21a]

4-Amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamidoxime (*186*): Formycin-N^6-oxide (*162*) (670 mg) was dissolved in water (30 ml). Dowex 50 X 4 (H^+) (8 g) was added and the mixture stirred at room temperature for 15 hr. The Dowex was removed by filtration and washed with water (50 ml). The filtrate and wash were discarded. The resin was then stirred with a water:conc. ammonium hydroxide (2:1, v/v) mixture for 0.5 hr. The resin was removed by filtration, washed with water (30 ml) and again treated with dilute ammonia as previously described. The ammonia solutions and water washes were combined and evaporated to dryness *in vacuo*. The residue was co-evaporated with water (5 x 20 ml) and then dissolved in boiling water (40 ml). The boiling solution was treated with charcoal, then filtered and again evaporated to dryness *in vacuo*. The residue was coevaporated with methanol (3 x 10 ml) and then stirred with ethanol (20 ml) to produce a white solid which was collected by filtration and air dried; 270 mg (42%); mp 180-185° dec. Pure *186* was obtained by recrystallization from methanol and drying at 110° *in vacuo*; mp 190-192° dec. *Anal*. Calc'd. for $C_9H_{15}N_5O_5$ (273.3):

C, 39.56; H, 5.53; N, 25.53. Found: C, 39.59; H, 5.56; N, 25.77. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 11, 270 (6.15), 237 (6.29). Tlc: solvent F, R_1 = 0.71; solvent H, R_1 = 1.17.

4-Amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide (ΨAPCA-riboside) (17), Method A: Formycin-N^6-oxide (162) (2 g) was dissolved in water (100 ml). Dowex 50 X 4 (H^+) (20 g) was added and the mixture stirred at room temperature for 18 hr. The resin was removed by filtration and washed with water (200 ml). The resin was then stirred with a mixture of water:conc. ammonium hydroxide (1:1, v/v) (120 ml) for 1 hr. The mixture was then filtered and the resin washed with water (100 ml), combining the wash with the ammonia filtrate. The solution was then evaporated to dryness in vacuo to give a brown foam, which was dissolved in 0.2 N sodium hydroxide (80 ml). Raney nickel (3 g) was added and the mixture shaken under hydrogen (2.8 Kg/cm^2) for 4 days. The reaction mixture was filtered and the nickel washed with boiling water (200 ml), adding the wash to the filtrate. The basic solution was then stirred with Amberlite IRC-50 (80 g) until neutral (approx. 20 min). The resin was then removed by filtration and washed with water (3 x 100 ml). The washes were combined with the filtrate and the solution evaporated to dryness in vacuo. The residue was dissolved in boiling water (50 ml). The boiling solution was treated with charcoal and then filtered and freeze-dried to give a light yellow solid; 880 mg. The solid was dissolved in water (50 ml). Silica gel (J. T. Baker #5-3405) (2 g) was added and the mixture evaporated to dryness in vacuo. The dry

mixture was placed on top of a silica gel dry column (2" x 8"). The column was eluted with solvent B collecting the first 50 ml of eluate. The methanolic solution was filtered and evaporated to dryness *in vacuo*. The residue was dissolved in water (20 ml) and the solution freeze-dried to obtain a white solid; 300 mg. Reverse phase HPLC analysis showed that this material was approximately ninety percent pure. Samples (3 x 60 mg) of this material were chromatogramed on a Porasil B-C_{18} column (8 x 1800 mm) using solvent A as the mobile phase. The major product (K' = 0.52) from each sample was collected and then the three solutions were combined, concentrated *in vacuo* to 20 ml and freeze-dried to give 90 mg of pure **17**; mp: wide range >85°. *Anal*. Calc'd. for $C_9H_{14}N_4O_5$ (258.2): C, 41.86; H, 5.46; N, 21.70. Found: C, 41.64; H, 5.52; N, 21.63. $[\alpha]_D^{24.5}$ = -56.5 (C = 1, H_2O). *Uv*, λ_{max} (nm), (ε x 10^{-3}): H_2O, 282 (4.8), 232.5 sh (4.5); pH 11, 282 (4.9), 232.5 sh (4.9). *Ms*: M^+, m/e 258. *Pmr*: δ 4.73 (d, $H_{1'}$), J_{1-2} 6.5 Hz. *Tlc*: solvent A, R_1 = 1.11; solvent F, R_1 = 0.11; solvent H, R_1 = 1.07, R_{186} = 0.91.

Method B: 4-Amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamidoxime (**186**) (5.1 g) was dissolved in 0.2 *N* sodium hydroxide (500 ml). Water wet Raney nickel (15 g) was added and the mixture shaken under hydrogen (2.8 Kg/cm^2) for 4.33 days. The nickel was removed by filtration and washed with boiling water (500 ml). The wash and filtrate were combined and stirred with Amberlite IRC-50 (70 g) until neutral (approx. 30 min). The resin was removed by filtration and then washed with water (2 x 200 ml). The washes were combined with

the filtrate and the resulting solution was evaporated to dryness *in vacuo*. The brown residue was stirred with boiling methanol (200 ml). After standing at room temperature for 12 hr a brown insoluble solid was removed from the mixture by filtration and discarded. The methanol filtrate was evaporated to dryness *in vacuo* and the residue redissolved in boiling methanol (100 ml). The boiling solution was treated with charcoal and then filtered. The charcoal was washed with boiling methanol (40 ml) and the two alcoholic solutions combined and evaporated to dryness *in vacuo*. The residue was stirred with hot methanol (50 ml) and the mixture then evaporated to dryness *in vacuo*. This coevaporation with methanol was repeated two more times, then the residue was extracted with boiling methanol (100 ml) and the mixture filtered to remove insoluble material. After standing at room temperature for 12 hr, the crystalline solid, which had deposited from the methanol filtrate, was collected and air dried; 1.5 g (31%) of 17 which was sufficiently pure for subsequent syntheses was obtained.

4-(N'-Benzoylthiocarbamoyl)aminopyrazole-3-carboxamide (199): 4-Aminopyrazole-3-carboxamide (15)[19] (7.6 g) was dissolved, by warming, in water (200 ml). A mixture of benzoylisothiocyanate (10.4 g) and ethanol (20 ml) was added slowly (approx. 2 min) to the rapidly stirred solution of 15. The mixture was stirred at room temperature for 1 hr, during which time the sides of the reaction flask were frequently scraped clean of adhering solid. The solid was then collected, washed with hot ethanol (250 ml) and air dried;

11.95 g. The solid was recrystallized from a methanol-acetone mixture to obtain 8.7 g (50%) of 199. Pure 199 was obtained by recrystallization of a sample (1 g) from a methanol-acetone mixture and drying in vacuo at 110°; 550 mg; mp: 254-257°, melts and then resolidifies, then decomposes at 338-340°. Anal. Calc'd. for $C_{12}H_{11}N_5O_2S$ (289.3): C, 49.82; H, 3.83; N, 24.21. Found: C, 49.89; H, 3.62; N, 24.42. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 302 (11.7), 268 (24.0), 236 sh (14.5); pH 11, 307 (9.26), 262 sh (14.8), 236.5 (17.8); methanol, 308 (12.0), 265 (23.0), 238.5 sh (12.6). Pmr: δ 7.4-8.1 (m, 7H, C_6H_5 and amide NH_2), δ 9.14 (s, 1H, H_3), δ 11.3 (s, 1H, NH), δ 13.35 (s, 1H, NH), δ 13.72 (s, 1H, NH). Tlc: solvent B, R_f = 0.83; solvent K, R_f = 0.68; solvent C, R_f = 0.86.

4-(N'-Benzoyl-S-methylisothiocarbamoyl)aminopyrazole-3-carboxamide (201): 4-(N'Benzoylthiocarbamoyl)aminopyrazole-3-boxamide (199) (500 mg) was dissolved in 0.1 N sodium hydroxide (75 ml). Methyl iodide (0.15 ml) was added and the mixture stirred at room temperature for 1 hr, then acidified (approx. pH 6) with glac. acetic acid. The solid was collected, then washed with water (20 ml) and air dried; 500 mg (95%). Pure 201 was obtained by recrystallization, with charcoal treatment, from methanol and drying at 110° in vacuo; 260 mg; mp 196-197°. Anal. Cald'd. for $C_{13}H_{13}N_5O_2S$ (303.3): C, 51.48; H, 4.32; N, 23.09. Found: C, 51.54; H, 4.23; N, 23.25. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 241 (17.1); pH 11, 305 sh (9.25), 232 (16.1); methanol, 305 (17.0), 283 (16.2), 241 (13.3). Pmr: δ 2.67 (s, 3H, SCH_3), δ 7.4-8.3 (m, 7H, C_6H_5 and amide NH_2), δ 8.44

(s, 1H, H_3), δ 10.95 (s, 1H, NH), δ 13.42 (s, 1H, NH). Tlc: solvent C, R_f = 0.86; solvent K, R_f = 0.59, R_{199} = 0.86.

5-Aminopyrazolo[4,3-d]pyrimidin-7-one (203): A mixture of 201 (5.56 g) and N,N-dimethylformamide, which had been saturated with ammonia at 0°C (150 ml) was placed in a sealed reaction vessel and heated, at 125° for 2 hr. After cooling, the reaction mixture was evaporated on a steam bath, in the hood. The solid residue was washed with water (25 ml) and air dried; 3.42 g; mp > 300°. Tlc in solvent D showed that this product was composed of two major components. By tlc comparison with the product from a previous small scale reaction and by uv analysis of the mixture, it was determined that one of the components was the desired 203 and it was assumed that the other product was the intermediate 202. The solid mixture (3.42 g) was stirred and refluxed with 1 N sodium hydroxide (70 ml) for 3.5 hr. The solution was acidified (approx. pH 6) with conc. hydrochloric acid and the mixture refrigerated (5°) for 14 hr. The solid which had separated was collected, washed with water (25 ml) then acetone (25 ml) and air dried. The dry solid was powdered and then extracted with boiling ethanol (3 x 50 ml). The ethanol insoluble solid was recrystallized twice from water to obtain 1.14 g (41%) of impure 203. Pure 203 was obtained by reprecipitation (at approx. pH 6) from hot sodium hydroxide with dilute hydrochloric acid, then recrystallization from water and then reprecipitation (at approx. pH 6) from hot dilute sodium hydroxide with glac. acetic acid; 380 mg; mp > 300° dec. Tlc: solvent A, C and F all showed single elongated spots. The

solid was dried *in vacuo* at 110°. *Anal.* Calc'd. for $C_5H_5N_5O$ (151.1); C, 39.74; H, 3.33; N, 46.34. Found: C, 38.46, 38.24; H, 3,52, 3.44; N, 45.57, 45.47. The elemental analyses indicated that *203* was hydrating. *Anal.* Calc'd. for $C_5H_5N_5O \cdot 0.25\ H_2O$ (155.6): C, 38.59; H, 3.56; N, 45.00. A sample of *203* was exposed to the atmosphere for several days and then again analyzed. Found: C, 38.05; H, 3.44; N, 45.15. *Uv*, λ_{max} (nm), ($\varepsilon\ 10^{-3}$): pH 1, 279 (4.36); pH 11, 299 (5.06); methanol, 300 (4.67), 243.5 sh (5.68). *Pmr*: δ 6.24 (bs, 2H, NH_2), δ 7.67 (s, 1H, H_3).

4-(N'-Benzoylthiocarbamoyl)amino-3-(β-D-ribofuranosyl)pyrazole-5-carboxamide monohydrate (*200*): 4-Amino-3-(β-D-ribofuranosyl)-pyrazole-5-carboxamide (*17*) (1.3 g) was mixed with benzoylisothiocyanate (830 mg) and N,N-dimethylformamide (25 ml). The mixture was stirred at room temperature for 2 hr and then evaporated to a heavy oil *in vacuo*. The oil was triturated with hot toluene (2 x 25 ml) and then dissolved in methanol (20 ml). The methanolic solution was added slowly to chloroform (200 ml) with rapid stirring. The volume of the resulting mixture was reduced to approximately 100 ml by boiling on a steam bath. After standing at room temperature for 14 hr, the white solid, which had separated, was collected by filtration, then washed with chloroform and air dried; 1.93 g (91%); mp: wide range > 120°. Tlc in solvent D showed that the product contained trace impurities. Pure *200* was obtained by recrystallization of a small sample from a methanol:ethyl acetate mixture. *Anal.* Calc'd. for $C_{17}H_{19}N_5O_6S \cdot H_2O$ (439.5): C, 46.45; H, 4.82; N, 15.94.

Found: C, 46.40; H, 4.91; N, 15.51. The hydration of 200 was confirmed by the pmr spectrum. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 283.5 sh (12.7), 245 (19.8); pH 11, 236 (20.0); methanol, 282 sh (9.01), 242 (16.0). Pmr: δ 4.92 (d, 1H, $H_{1'}$), δ 7.0-8.3 (m, C_6H_5 and amide NH_2), δ 11.5 (s, 1H, NH), δ 11.94 (s, 1H, NH), δ 12.99 (s, 1H, NH). Tlc: solvent A, R_1 = 0.86, R_{17} = 0.78; solvent F, R_1 = 1.17, R_{17} = 2.40.

5-Amino-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidin-7-one (5-aminoformycin B) (19): Compound 200 (1.63 g) was dissolved in 0.1 N sodium hydroxide (40 ml). Methyl iodide (600 mg) was added and the mixture was stirred at room temperature for 2.5 hr. A few drops of glac. acetic acid were added to acidify (approx. pH 6) the mixture, which was then evaporated to dryness in vacuo. The residue was coevaporated with methanol (2 x 20 ml) to obtain a yellow foam. The foam was dissolved in N,N-dimethylformamide, which had been saturated with ammonia at 0°, (20 ml). The resulting solution was placed in a sealed reaction vessel and heated, in an oil bath, at 130° ± 5° (bath temperature) for 3 hr. The reaction mixture was then evaporated on a steam bath, in the hood, to give a brown oil. The oil was coevaporated, in vacuo, with water (2 x 20 ml), then with ethanol (20 ml). The residue was recrystallized from water (10 ml) to obtain a brown solid; 480 mg. The water filtrate was saved for later processing. Uv analysis of the solid confirmed that 19 had been obtained. Tlc analysis (solvent F) showed that the solid consisted of one major product with R_f = 0.38 and several minor

products. The solid (450 mg) was dissolved in a methanol:water (3:1, v/v) mixture (40 ml). Silica gel (J. T. Baker #5-3405) (10 g) was added and the mixture evaporated to dryness in vacuo. The dry mixture was coevaporated with methanol (3 x 30 ml) and then placed on top of a shallow bed (64 x 20 mm) of silica gel, which had been prewashed with methanol. The bed was washed with methanol (4 x 100 ml) and the combined washes was filtered and then evaporated to dryness in vacuo. The solid residue was stirred with hot ethanol (25 ml) and, after cooling to room temperature, the mixture was filtered and the solid washed with ethanol (20 ml); 310 mg. The ethanol extraction was repeated to obtain 220 mg (20% yield) of 19 which showed only trace impurities on tlc (solvent F) analysis. Pure 19 was obtained by recrystallization of a sample from a water:acetonitrile mixture; mp wide range > 250° with decomposition. A sample of 19 was dried in vacuo at 110° just prior to elemental analysis. Anal. Calc'd for $C_{10}H_{13}N_5O_5$ (283.2): C, 42.41; H, 4.63; N, 24.73. Found: C, 42.24; H, 4.56; N, 24.42. A sample of 19 which was not dried just prior to elemental analysis gave C, H and N which corresponded to the hemihydrate. Uv, λ_{max} (nm), ($\varepsilon \times 10^{-3}$): pH 1, 282 (7.02); pH 11, 301 (6.28), 249 sh (8.62); methanol, 300 (4.97), 249.5 sh (8.62). Pmr: δ 4.80 (d, 1H, H_1', J_{1-2} = 6 Hz), δ 5.97 (s, 2H, NH_2), δ 10.8 (bs, 1H, ring NH), δ 13.33 (bs, 1H, ring NH). Tlc: solvent F, R_1 = 0.91, R_{200} = 0.53; solvent H, R_1 = 1.09.

The water filtrate from the first recrystallization of 19 was evaporated to dryness in vacuo. The residue was dissolved in 0.5 N

sodium hydroxide (20 ml) and the solution was refluxed for 1 hr. The solution was acidified (approx. pH 6) with formic acid and then evaporated to dryness *in vacuo*. The residue was stirred with water (5 ml) and the pH adjusted (pH > 10) with conc. ammonium hydroxide. The basic solution was applied to a column of Dowex 1 X 8 (formate) (100 ml). The column was washed with water (1.2 L) and then eluted with a linear formic acid gradient, using water (1 L) in the mixing chamber and 0.5 *N* formic acid in the reservoir. Fractions of approximately 18 ml were collected at 6 ml intervals and analyzed by uv and tlc (solvent F). Fractions which contained only *19* (#46-55) were pooled and evaporated, *in vacuo*, to dryness. The residue was coevaporated with ethanol (3 x 25 ml) and then recrystallized from a methanol-ethanol mixture to obtain additional *19*; 19 mg. The fractions from the formate column which were predominately *19*, but contained also small amounts of other products, were pooled and evaporated to dryness *in vacuo*. After coevaporation with ethanol (3 x 25 ml), 70 mg of slightly impure *19* was obtained.

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VITA

Name:	Arthur Frank Lewis
Birthdate:	August 29, 1939
Birthplace:	Cooledge, Arizona
High School:	North Phoenix High School
University:	University of Redlands, Redlands, California (1957-1959)
	Arizona State University Tempe, Arizona (1959-1962)
	University of Utah Salt Lake City, Utah (1970-1979)
Degree:	B.S. in Chemistry Arizona State University Tempe, Arizona
Professional Organizations:	American Chemical Society
	Division of Medicinal Chemistry, American Chemical Society
	International Society of Heterocyclic Chemistry
Professional Positions:	Chemist, Riker Labs. Inc., Northridge, California (1962-1964)
	Head, Organic Preparations Laboratory, University of Utah, Salt Lake City, Utah (1965-1970)
	Research Associate, Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah (1970-1979)

Publications:

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