Synthesis and Biological Activity of Certain Nucleoside and Nucleotide Derivatives of Pyrazofurin

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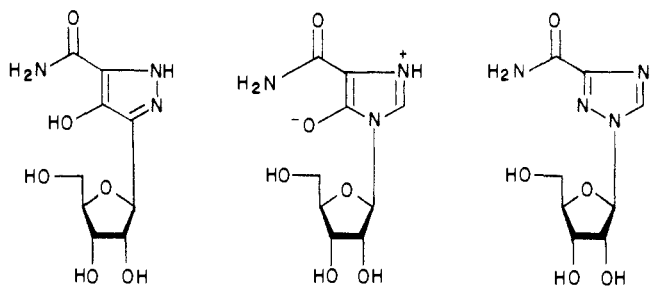
A number of nucleoside and nucleotide derivatives of 4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (pyrazofurin, 1) were prepared and tested for their antiviral and cytostatic activity in cell culture. Treatment of 1 with benzyl bromide gave 4-*O*-benzylpyrazofurin (4). Methylation of 4 with CH_2N_2 and subsequent removal of the benzyl group by catalytic hydrogenation provided 1-methylpyrazofurin (8). Direct methylation of 1 with CH_3I furnished 4-*O*-methylpyrazofurin (6). Dehydration of the pentaacetylpyrazofurin (9) with phosgene furnished 4-acetoxy-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-1-acetylpyrazole-5-carbonitrile (10). A similar dehydration of the precursor tetraacetyl derivative of 4 gave the corresponding carbonitrile, which on deprotection and subsequent treatment with hydroxylamine furnished 4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamidoxime (13). Treatment of the tetraacetyl derivative of 4 with Lawesson's reagent and subsequent deacetylation furnished a mixture of 4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-thiocarboxamide (15) and the corresponding nitrile derivative (16). Phosphorylation of unprotected 4 with $POCl_3$ and subsequent debenzylation of the intermediate 17 gave pyrazofurin 5′-phosphate (18), which provided the first chemical synthesis of 18. Similar phosphorylation of 4 with $POCl_3$ and quenching the reaction mixture with either EtOH or MeOH, followed by debenzylation, furnished the 5′-*O*-(ethyl phosphate) (**19b**) and 5′-*O*-(dimethyl phosphate) (**20b**) derivatives of pyrazofurin. DCC-mediated cyclization of 17, followed by debenzylation, gave pyrazofurin 3′,5′-(cyclic)phosphate (**21b**). The NAD analogue **23b** was also prepared by the treatment of 17 with an activated form of AMP in the presence of $AgNO_3$. The structural assignment of 7, 8, and **20a** were made by single-crystal X-ray analysis, and along with pyrazofurin, their intramolecular hydrogen bond characteristics have been studied. All of these compounds were tested in Vero cell cultures against a spectrum of viruses. Compounds 18 and **23b** were active at concentrations very similar to pyrazofurin but are less toxic to the cells than pyrazofurin. Compounds **19b**, **20b**, and the 3′,5′-(cyclic)phosphate **21b** are less active than 1. Compounds 18, **19b**, **20b**, and **23b** also exhibited significant inhibitory effects on the growth of L1210 and P388 leukemias and Lewis lung carcinoma cells in vitro, whereas B16 melanoma cells were less sensitive to growth inhibition by these compounds. Pyrazofurin derivatives modified at the 1-, 4-, or 5-position showed neither antiviral nor cytostatic activity in cell culture.

The naturally occurring azolecarboxamide nucleoside antibiotic pyrazofurin (4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide, pyrazomycin, 1) is one of three biologically active carbon-linked nucleosides isolated from the broth of a strain of *Streptomyces candidus*.[1] Pyrazofurin is structurally related to the naturally occurring imidazole nucleoside bredinin (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate, 2),[2] as well as the synthetic triazole ribonucleoside ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, 3),[3] and shows significant



1 pyrazofurin 2 bredinin 3 ribavirin

antiviral activity in cell culture against a broad spectrum of DNA and RNA viruses.[4-6] Pyrazofurin is particularly active against pox-, picorna-, toga-, myxo-, rhabdo-, and retroviruses in vitro and shows nearly as broad an antiviral spectrum as ribavirin.[6,7] Pyrazofurin is also highly effective in vitro against representative arena-, bunya-, and togavirus groups at concentrations 10–100 times less than those used with ribavirin.[8] Although pyrazofurin has a high degree of selectivity in its antiviral effects and shows a rather broad safety margin in cell culture, it exhibited a profound toxicity in mice (50% lethality at a dose of 5 mg/kg per day[5,7]). De Clercq and Torrence[6] suggest that this unexpected toxicity is probably not associated with the structural features of the molecule responsible for the antiviral potency. However, the toxicity of pyrazofurin is such that it cannot readily be separated from its antiviral efficacy in animals.[9,10] It has been presumed that the toxicity of pyrazofurin in animals may be due to its inhibition of orotidylate decarboxylase,[11,12] an enzyme of the de novo pyrimidine biosynthetic pathway responsible for the transformation of orotidine 5′-monophosphate into uridine 5′-monophosphate. Pyrazofurin is similar to ribavirin (3) in that it is converted to the 5′-triphosphate in human blood cells[13] and also converted to the 5′-

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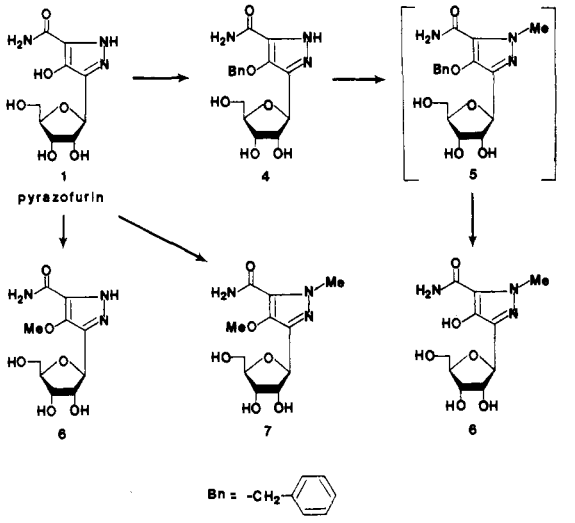
monophosphate by the cellular adenosine kinase.[14] 5'-Monophosphates of both pyrazofurin and ribavirin inhibit aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase.[15]

Pyrazofurin has been found to inhibit the growth of a number of different experimental tumor cell lines both in vitro and in vivo.[16] Pyrazofurin completely inhibited the growth of Walker carcinosarcoma 256.[17] Mammary carcinoma 755, Gardner lymphosarcoma, and X5563 plasma cell myeloma were inhibited more than 50% by pyrazofurin.[17] Pyrazofurin has been studied clinically as an anticancer agent in man.[4,12,18,19] Pyrazofurin was well tolerated by most patients at doses of 100 mg/m^2 following iv administration. Infusion of pyrazofurin to leukemic patients resulted in severe, but reversible, toxicity. In several patients, mucositis, leucopenia, and anemia were observed,[18] which limit pyrazofurin's general usage as an antitumor agent.

The second active factor produced by the strain of *Streptomyces candidus* was subsequently isolated and characterized as the α-anomer of pyrazofurin (pyrazofurin B).[20] Since the original report[1] of the isolation of pyrazofurin in 1969, the chemical synthesis of both α- and β-anomers of pyrazofurin, by widely different routes, has been documented.[21,26] The syntheses of 2'-deoxypyrazofurin,[21] the carbocyclic analogue of pyrazofurin,[27] as well as the N^1-benzyl[21] and 1,4-dimethyl[20] derivatives of pyrazofurin have been reported. We initiated a study of the structure and biological activity relationship of pyrazofurin and related nucleosides and nucleotides with the hope of finding a derivative with less toxicity. Now we report the chemical synthesis of several selected nucleoside and nucleotide derivatives of pyrazofurin. Pyrazofurin should be metabolized to its 5'-monophosphate by a cellular kinase to exhibit the potent antiviral/antitumor activity.[11,12,14] Although pyrazofurin 5'-monophosphate (**17**) was synthesized enzymatically[4,9] by the enzyme nucleoside phosphotransferase in the presence of *p*-nitrophenyl phosphate as a phosphate donor, the chemical synthesis of **17** has not been previously reported.[4]

Chemistry. Because of the electron-dense pyrazole ring system, the chemistry of pyrazofurin is quite different from that of ribavirin (**3**) or even bredinin (**2**). The apparent pK_a (in water) of pyrazofurin is 6.7. It was visualized that

Scheme I



the synthesis of the desired nucleoside and nucleotide derivatives of pyrazofurin might be realized by protecting the acidic hydroxyl group of the aglycon moiety. Benzylation of free pyrazofurin (**1**) with benzyl bromide in aqueous acetonitrile in the presence of Na_2CO_3 gave 4-*O*-benzylpyrazofurin (**4**) in excellent yield, which was found to be a useful intermediate for subsequent reactions (Scheme I). Compound **4** is indeed the 4-*O*-benzyl and not the *N*-benzyl derivative of pyrazofurin as established by comparison of the UV absorption spectrum [λ_{max} (pH 1) 245 (sh) nm; λ_{max} (pH 11) 243 nm] of **4** with that reported for 1-benzylpyrazofurin[21] [λ_{max} (pH 1) 232 and 268 nm; λ_{max} (pH 11) 239 and 312 nm]. Methylation of **4** with diazomethane and subsequent removal of the benzyl group by catalytic hydrogenation of the intermediate **5** with Pd/C in a hydrogen atmosphere gave 1-methylpyrazofurin (1-methyl-4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide, **8**). The structural assignment of **8** was confirmed by single-crystal X-ray analysis.

Direct methylation of unprotected **1** with diazomethane in dioxane gave the dimethylated derivative 1-methyl-4-methoxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (**7**) in 92% yield (Scheme I). Although compound **7** is claimed to have been synthesized, neither experimental details nor physicochemical data has been reported.[20] Since N_2-methylation cannot be ruled out, the structure of **7** was established by single-crystal X-ray analysis. Methylation of **1** with methyl iodide in dimethylformamide in the presence of K_2CO_3 readily gave 4-methoxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (**6**) as the major product (>95%), along with a trace amount of **7**. The mixture of **6** and **7** was separated on an open-bed silica gel column.

In an effort to develop a synthetic procedure that would lead to pyrazofurin derivatives modified at the 5-position, the synthesis of 4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carbonitrile (**10**) was investigated (Scheme II). Simple acetylation of **1** with acetic anhydride in the presence of 4-(dimethylamino)pyridine (DMAP) at ambient temperature gave the pentaacetyl derivative 4-acetoxy-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-1-acetylpyrazole-5-carboxamide (**9**). Compound **9** has been listed in the patent literature,[28,29] but no physicochemical data is given. Dehydration of **9** with phosgene in a mixture of pyridine and dichloromethane at room temperature gave 4-acetoxy-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-1-acetyl-

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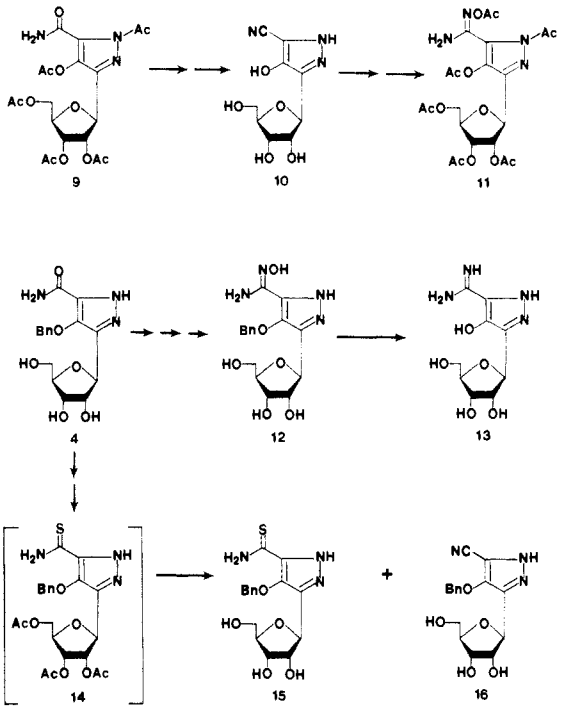
Scheme II

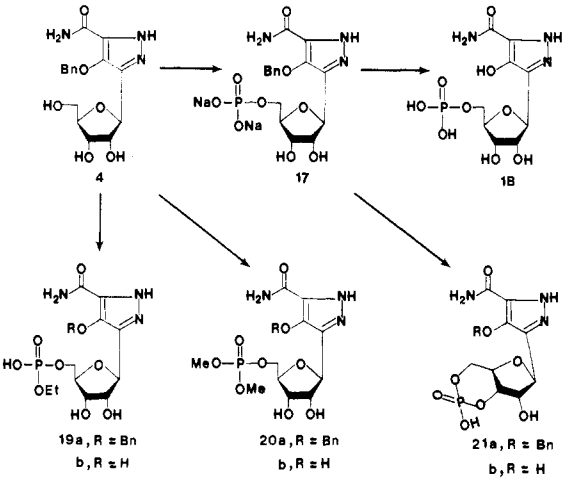


pyrazole-5-carbonitrile, which on subsequent deacetylation with methanolic sodium methoxide gave the desired **10** as a crystalline solid. Treatment of **10** with free hydroxylamine in boiling dioxane afforded a mixture of compounds from which the isolation of the deprotected carboxamidoxime derivative of **1** was rather difficult. In an attempt to isolate the carboxamidoxime derivative, the mixture was reacetylated with acetic anhydride in the presence of DMAP to obtain 4-acetoxy-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-1-acetylpyrazole-5-*N*-acetoxycarboxamidoxime (**11**). However, our attempts to deacetylate **11** with $MeOH/NH_3$ or MeOH/NaOMe under a variety of experimental conditions were unsuccessful and resulted in the formation of unidentifiable products.

A similar dehydration of 1,2′,3′,5′-tetraacetyl derivative of **4**, which in turn was obtained by the acetylation of **4** with Ac_2O/DMAP, gave the corresponding carbonitrile derivative. Further treatment of the intermediate carbonitrile with MeOH/NaOMe and subsequent reaction with hydroxylamine furnished 4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamidoxime (**12**), which was isolated and fully characterized. Catalytic hydrogenation of **12** readily provided 4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamidine (**13**). It is of particular interest that the corresponding carboxamidine derivative of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine)[30] is a competitive, reversible inhibitor of inosine phosphorolysis by human lymphoblast purine nucleoside phosphorylase but is not a substrate for this enzyme.[31] Since pyrazofurin bears structural similarity to ribavirin and has a C–C glycosyl linkage that is stable to enzymatic hydrolysis, the amidine derivative **13** might be expected to be an even more potent inhibitor of the phosphorylase enzyme.

Lawesson's reagent [2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane 2,4-disulfide] introduced in 1978, has been shown to be a mild and effective reagent to convert amides to thioamides, including sensitive amino- and hydroxy-containing compounds.[32] Treatment of the tetraacetyl derivative of **4** with Lawesson's reagent in boiling dioxane gave the unstable thioamide **14**, which without isolation was deacetylated with MeOH/NaOMe to yield a mixture of 4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-thiocarboxamide (**15**) and the corresponding nitrile derivative (**16**). Reductive cleavage of the benzyl ether **15** with either sodium in liquid ammonia or sodium naphthalene[33] in THF once again gave an intractable reaction mixture from which the desired debenzylated **15** could not be isolated. When the pentaacetyl derivative **9** was treated with Lawesson's reagent and subsequently deblocked with either $MeOH/NH_3$ or MeOH/NaOMe, a mixture of **10** and the presumed thioamide are formed. On standing the solid residue converts exclusively to **10** with the elimination of H_2S.

Scheme III



Compound **4** served as a versatile starting material for phosphorylation studies (Scheme III). Thus phosphorylation of unprotected **4** with phosphoryl chloride in trimethyl phosphate, according to the general procedure of Yoshikawa et al.,[34] provided 4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide 5′-phosphate (**17**), isolated as the disodium salt. The purity and structure of **17** was confirmed by elemental and ^1H NMR analysis. Catalytic (Pd/C) debenzylation of **17** in an atmosphere of hydrogen readily gave the desired 4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide 5′-monophosphate (pyrazofurin 5′-phosphate, **18**), which represents the first reported chemical synthesis of **18**.

Although pyrazofurin should be converted to its 5′-monophosphate (**18**) by a cellular kinase to exhibit biological activity, the direct administration of preformed **18** may not be practical because of the ionic nature of the phosphate moiety which is responsible for poor cellular penetration.[35] A practical approach may be to utilize the 5′-(*O*-alkyl phosphate) and cyclic phosphate derivatives of pyrazofurin in the hope that these lipophilic prodrug forms would enter cells by passive diffusion and thereupon be cleaved enzymatically to the free nucleotide. Consequently the chemical synthesis of such nucleotide derivatives of pyrazofurin was undertaken.

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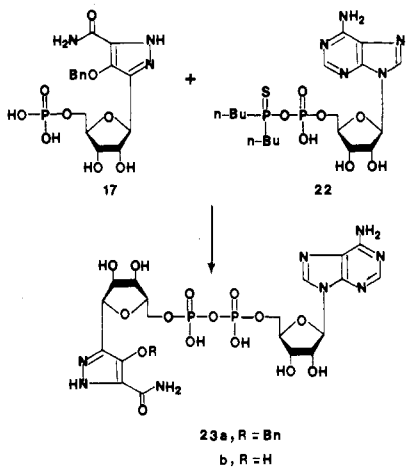
Table I. Crystal Data and Experiment Parameters

compd	1	8	7	20a
formula	$C_9H_{13}N_3O_6 \cdot H_2O$	$C_{10}H_{15}N_3O_6$	$C_{11}H_{17}N_3O_6$	$C_{18}H_{24}N_3O_9P \cdot H_2O$
$F(000)$	584	576	304	484
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_1$	$P2_1$
a, Å	6.734 (3)	7.334 (2)	7.664 (4)	8.403 (2)
b, Å	7.000 (2)	7.676 (3)	7.479 (3)	6.916 (2)
c, Å	25.283 (13)	21.111 (10)	11.404 (5)	20.311 (5)
β, deg	90	90	100.33 (4)	100.97 (2)
Z	4	4	2	2
V, Å^3	1191.8	1183.8	651.7	1158.8
d_{calcd}	1.55	1.54	1.47	1.29
$\sin \theta/\lambda$	0.65	0.65	0.70	0.70
μ, cm^{-1}	0.89	0.83	0.77	1.28
R	0.051	0.043	0.051	0.062
R_w	0.027	0.029	0.044	0.023
max peak in final difference map, e Å^{-3}	0.27	0.12	0.36	0.47
total data	1513	1572	2217	3750
unique obsd data	1091	1304	1878	2548
unobsd data	368	217	135	838

Scheme IV



Phosphorylation of **4** with phosphoryl chloride in trimethyl phosphate at low temperature, followed by quenching the reaction mixture with cold anhydrous ethanol,[36] gave a product identified as 4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide 5′-*O*-(ethyl phosphate) (**19a**). Debenzylation of **19a** with Pd/C (20%) in the presence of formic acid gave 5′-*O*-(ethyl phosphate) of pyrazofurin (**19b**). The structure of **19b** was assigned on the basis of its ^{1}H NMR (in Me_2SO-d_6, multiplet centered at δ 3.92 with POCH coupling of 7.42 Hz)[37] and by elemental analysis. However, a similar phosphorylation of **4** with $POCl_3$, followed by quenching the reaction mixture with methanol, gave 4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide 5′-(di-*O*-methyl phosphate) (**20a**). Hydrogenation of **20a** with Pd/C in the presence of formic acid afforded a good yield of pyrazofurin 5′-(di-*O*-methyl phosphate) (**20b**). The formation of the 5′-(di-*O*-methyl phosphate) derivative **20a** under these conditions was surprising. Therefore it was deemed desirable to unequivocally assign the structure of **20a** by single-crystal X-ray analysis. Finally the dicyclohexylcarbodiimide (DCC) mediated cyclization of the nucleotide **17**, according to the general procedure of Khorana and co-workers,[38] gave 4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide 3′,5′-(cyclic)phosphate (**21a**), which on subsequent catalytic hydrogenation gave pyrazofurin 3′,5′-(cyclic)phosphate (**21b**) (Scheme III). The appearance of a singlet for the anomeric proton in the ^{1}H NMR spectrum of **21b** at δ 4.89 indicated the formation of the cyclic phosphate ring.[39] Analytical HPLC also indicated compound **21b** to be free of **18**.

It has recently been observed that the synthetic oncolytic carbon-linked nucleosides tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide)[40] and selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide),[41] both synthesized and reported from our laboratories, are phosphorylated enzymatically to the corresponding 5′-phosphates,[42] which in the presence of ATP and Mg^{2+} are further converted to the NAD analogues.[43,44] These NAD analogues are powerful inhibitors of IMP dehydrogenase.[43,44] However, the NAD pyrophosphorylase in P388 lymphoblasts did not convert ribavirin to the corresponding NAD analogue,[45] which is probably why ribavirin has relatively weak antitumor activity compared to that of tiazofurin and selenazofurin. In view of these observations, the synthesis of the NAD analogue of pyrazofurin (PAD) was of particular interest.

Treatment of **17** (free acid) with the activated form of AMP, adenosine 5′-phosphoric di-*n*-butylphosphinothioic anhydride (**22**) in anhydrous pyridine in the presence of $AgNO_3$, according to the general procedure of Furusawa and co-workers,[46] gave over 90% yield of the blocked PAD analogue **23a**. Compound **23a** was purified on a DEAE-Sephadex column using a gradient of aqueous NH_4HCO_3. Debenzylation of the benzyloxy function of **23a** with hy-

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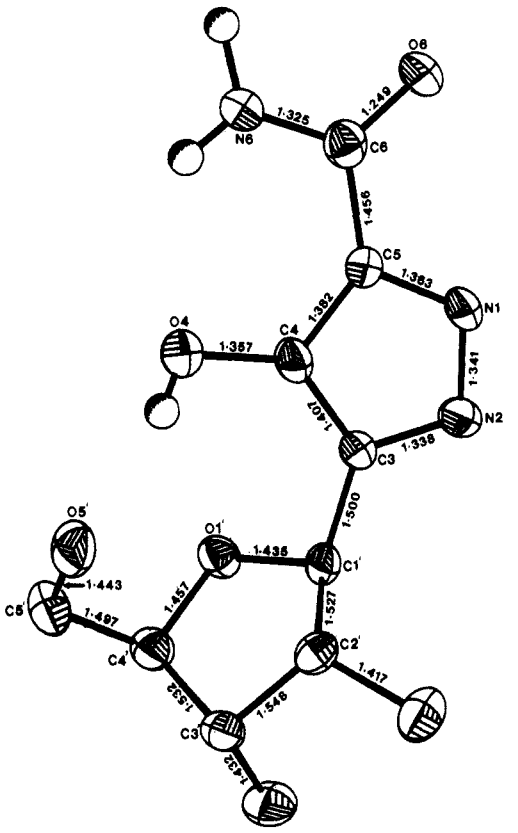

Figure 1. Compound 1.

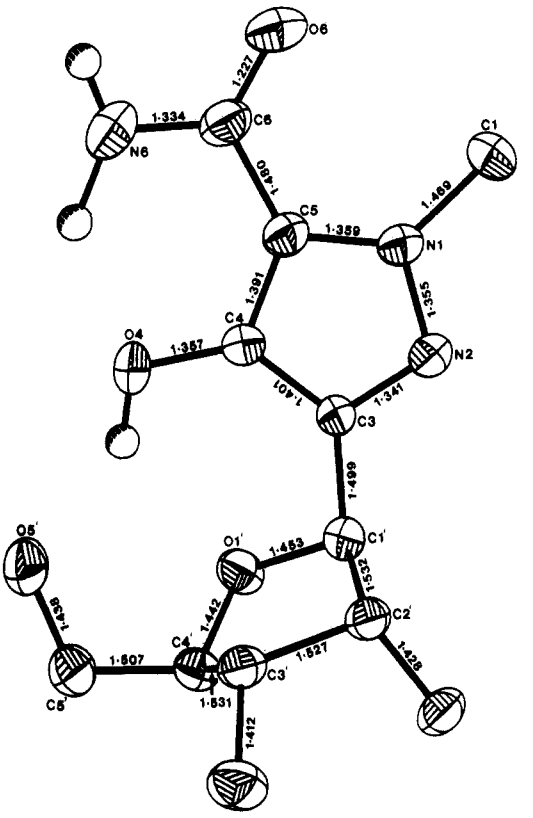

Figure 2. Compound 8.

drogenation in the presence of Pd/C gave PAD (**23b**), which was again purified on a DEAE-Sephadex column using the NH_4HCO_3 gradient. Complete removal of NH_4HCO_3 salt was achieved by repeated lyophilization and the free acid form of PAD was obtained by cation-exchange chromatography. All the chemical and spectral data are consistent with the structure of PAD as assigned.

Single-Crystal X-ray Diffraction Analyses of Pyrazofurin (1), 7, 8, and 20a. Suitable crystals of 1, 7, 8, and **20a** were chosen for single-crystal X-ray diffraction studies.[47] Data for the determination of lattice parameters and the structural study for each compound were obtained with a Nicolet P3 autodiffractometer utilizing graphite monochromated Mo Kα radiation (λ = 0.71069 Å). Lattice parameters for each crystal were obtained by using a least-squares technique of 15 centered 2θ values. Single-crystal data for each crystal was measured by using a variable-speed θ–2θ scan technique. Three standard reflections were measured every 97 reflections to check for crystal and electronic stability. In all four studies there was no indication of instability. All reflections with $I < 2\sigma(I)$ were considered to be unobserved. Experimental and crystal data are summarized in Table I.

The four structures were solved by using direct methods and refined by least-squares techniques. The difference maps of 1 and **20a** showed the presence of water of hydration in each of the crystal structures. Non-hydrogen atoms were refined anisotropically while hydrogen atoms, which were located in difference maps were refined isotropically. Exceptions to the refinement process occurred in **20a** where the phenyl group and the methyl groups were refined as rigid bodies and in 7 where the methyl group bonded to N1 was also refined as a rigid body. Also in **20a**, $O5'PO_2$ was badly disordered. The disorder was resolved by using a difference map and during further refinement the positional parameters of the partial atoms were held constant, the occupancy factors were fixed at 0.60 ($O5'PO_{2a}$) and 0.40 ($O5'PO_{2b}$), and the partial atoms were refined anisotropically. The R values for the four crystals are listed in Table I. Weights based on counting statistics

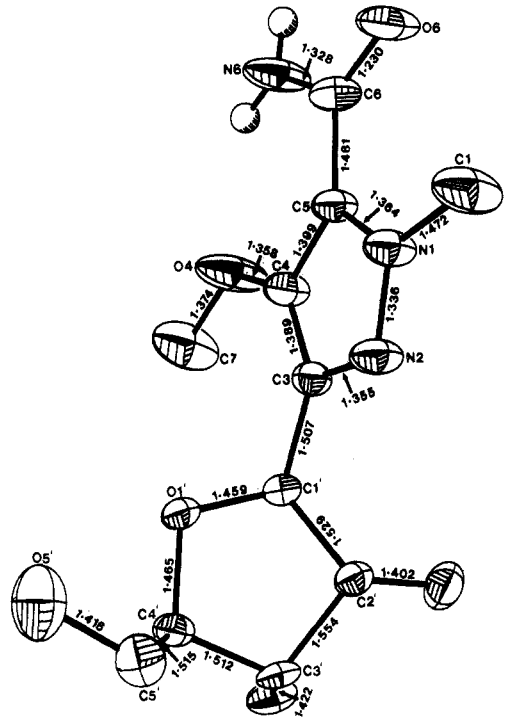

Figure 3. Compound 7.

(47) The X-ray structure of 1 was reported at a meeting (Jones, N. D.; Chaney, M. O. 9th International Congress of Crystallography, Kyoto, Japan, (1972, Abstracts, p S-48) but to our knowledge has not been published. For that reason and because of its importance to this study, the X-ray structure of 1 was determined and is reported here.

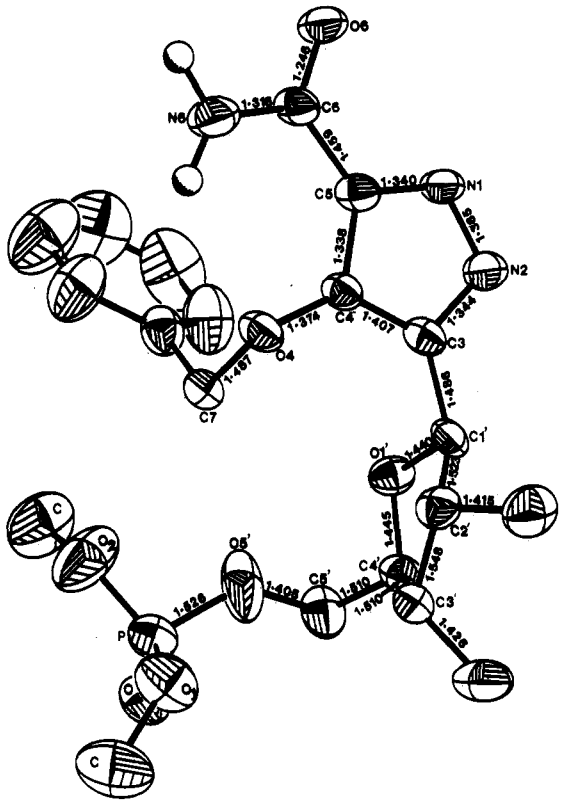

Figure 4. Compound **20a**.

were used in the refinement of the four compounds. All computer calculations performed in this study were made by using SHELX-76.[48]

Computer drawing showing structural formulas, conformation, atom labels, and interatomic bond distances are shown in Figures 1–4. The estimated standard deviations on all bond lengths are between 0.004 and 0.006 Å. The hydrogens of the carbohydrate moiety are omitted for clarity. In **20a**, O5'PO is represented by the atom in the more populated site. All drawings were made by using the SHELXTL[49] plotting program, which is part of the Nicolet R3 system. All four compounds exist in the β-anomeric configuration.

There are extensive intermolecular hydrogen bonding networks in all four structures. These generally involve all of the alcoholic hydrogens of the carbohydrate, plus in some cases one of the hydrogen atoms bonded to the carboxamide nitrogens and the hydrogen atoms of waters of hydration when present. In addition there are interesting intramolecular hydrogen bonds in all four compounds. A hydrogen bond common to all four molecules is the N6–HN1···O4 interaction. The intramolecular hydrogen bond data is summarized in Table II. Another interaction which occurs in 1 and 8 is the O4–HO4···O5' hydrogen bond. A similar hydrogen bond is not possible in 7 and **20a** as the HO4 has been replaced by a substituent in each structure. The data in Table II clearly shows that the O4–O5' hydrogen bonds are significantly shorter and therefore stronger than the N6–H^{1}N6···O4 interactions. This is likely due to the rather fixed positional relationship of N6 and O4 established by the aglycon. The O4–O5' spatial relationship is much more flexible due to the presence of the carbohydrate moiety. It is interesting to note that the O4–O2' hydrogen bond is reported[20] in the α-conformer of 1. The O–O interatomic distance of 2.551 (4) Å is shorter than those found in 1 and 8, indicating a strong hydrogen bond. The α-conformation brings O2' rather than O5' close to O4. The same authors report a O4–O5' distance of 2.61 Å in 1 in a footnote.[20] This value, although slightly shorter than the value reported in this study, supports the presence of a rather strong hydrogen bond between O4 and O5'.

Table II. Intramolecular Hydrogen Bond Data

compd	D–H···A	D–A, Å	H–A, Å	D–H–A, deg
1	N6–H^{1}N6···O4	2.895 (5)	2.28 (4)	129 (3)
7	N6–H^{1}N6···O4	2.754 (3)	2.122 (2)[a]	132.6 (2)[a]
8	N6–H^{1}N6···O4	2.786 (3)	2.00 (3)	131 (3)
20a	N6–H^{1}N6···O4	2.938 (4)	2.31 (3)	124 (3)
1	O4–HO4···O5'	2.642 (4)	1.92 (3)	166 (4)
8	O4–HO4···O5'	2.674 (3)	1.93 (3)	149 (3)

[a] The low esd values reported result from the fact that the positional parameters of H^{1}N6 were held fixed in the refinement. More reasonable uncertainties for these values would be ±0.004 Å for the H–A distance and ±4° for the D–H–A angle.

Biological Evaluations

Antiviral Activity. The nucleoside and nucleotide derivatives of pyrazofurin synthesized during this study were tested against parainfluenza type 3 (Para3), measles, vaccinia (vv), and herpes simplex type 2 (HSV2) viruses in cell culture in parallel with pyrazofurin (1) and ribavirin (3). The results of the antiviral evaluation are shown in Table III. It is evident that some of the nucleotide derivatives of 1 retain significant antiviral activity in vitro. Pyrazofurin (1) exhibited pronounced antiviral activity against all the viruses tested with ED_{50} values 10–50 times lower than that of ribavirin (3). The 5'-monophosphate (18) and the NAD analogue (**23b**) of pyrazofurin were active at concentrations very similar to that of 1. However, the 5'-(*O*-ethyl phosphate) (**19b**) had lower antiviral activity with ED_{50} concentrations 5–25 times higher than that of 18. The 5'-(di-*O*-methyl phosphate) (**20b**) was even less active with ED_{50} values approximately 10 times higher than that of **19b** and 50–250 times higher than that of 18. The 3',5'-(cyclic)phosphate (**21b**) was also considerably less active than 18 with ED_{50} values approximately 10–100 times greater than that of 18 or 1. Pyrazofurin derivatives with modifications at either the 1-, 4-, or 5-positions (4, 6–8, 10, 12, 13, 15, and 17) were inactive or exhibited drastically reduced antiviral activity as compared to that of 1.

The maximum tolerated concentration (MTC) of pyrazofurin was 1.6×10^{-3} M for the confluent monolayers of Vero cells used for the antiviral evaluations. Under identical conditions, the 5'-monophosphate (18) did not exhibit toxicity at concentrations as high as 5.0×10^{-3} M and may therefore have some therapeutic advantage over 1. The NAD analogue **23b** was also considerably less toxic than 1, with an MTC of 5.0×10^{-3} M. These observations indicate that compounds 18 and **23b** may have better antiviral potential than pyrazofurin.

Cytostatic Activity. All the newly synthesized nucleoside and nucleotide derivatives of pyrazofurin were also tested for their inhibitory effects on the growth of L1210 and P388 leukemias, B16 melanoma, and Lewis Lung carcinoma in vitro. The results of the evaluation of these cytostatic activity are summarized in Table IV. Pyrazofurin, its 5'-monophosphate (18), and the NAD analogue (**23b**), all exhibited significant inhibitory effects on the growth of L1210 and P388 leukemias and Lewis Lung

(48) Sheldrick, G. M. SHELX-76, "A Program for X-Ray Crystal Structure Determination", University of Cambridge, England, 1976.

(49) Sheldrick, G. M. SHELXTL, 4th revision, "An Integrated System for Solving, Refining and Displaying Crystal structures from Diffraction Data", University of Gottingen, Federal Republic of Germany, 1983.

Table III. Comparative in Vitro Antiviral Activity of Pyrazofurin (1), Ribavirin (3), and Certain Nucleoside and Nucleotide Derivatives of Pyrazofurin in Vero Cells

compd	MTC,[a] M	ED_{50},[b] M			
		Para3	measles	VV	HSV2
4	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	1.3×10^{-3}	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$
6	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	2.8×10^{-3}	$>5.0 \times 10^{-3}$
7	5.0×10^{-3}	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	5.0×10^{-3}	3.2×10^{-3}
8	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	1.6×10^{-3}	$>5.0 \times 10^{-3}$
10	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	5.0×10^{-3}	5.0×10^{-3}	$>5.0 \times 10^{-3}$
12	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$
13	$>5.0 \times 10^{-3}$	1.6×10^{-3}	5.0×10^{-3}	$>5.0 \times 10^{-3}$	3.1×10^{-3}
15	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	5.0×10^{-4}	2.1×10^{-3}	2.8×10^{-3}
17	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$	$>5.0 \times 10^{-3}$
18	$>5.0 \times 10^{-3}$	6.3×10^{-6}	2.0×10^{-5}	4.0×10^{-6}	2.3×10^{-5}
19b	$>5.0 \times 10^{-3}$	8.0×10^{-5}	9.7×10^{-5}	2.6×10^{-5}	5.9×10^{-4}
20b	$>5.0 \times 10^{-3}$	1.0×10^{-3}	1.4×10^{-3}	2.8×10^{-4}	$>5.0 \times 10^{-3}$
21b	$>5.0 \times 10^{-3}$	5.0×10^{-4}	$>5.0 \times 10^{-3}$	2.5×10^{-4}	1.3×10^{-4}
23b	5.0×10^{-3}	5.0×10^{-6}	1.6×10^{-5}	9.0×10^{-6}	1.0×10^{-5}
1	1.6×10^{-3}	3.0×10^{-6}	9.6×10^{-6}	2.8×10^{-6}	2.0×10^{-5}
3	$>5.0 \times 10^{-3}$	1.6×10^{-4}	9.9×10^{-5}	9.0×10^{-5}	1.9×10^{-4}

[a] The maximum tolerated concentration (MTC) is the lowest concentration of compound that produced microscopically visible cytotoxicity assessed as described in the Experimental Section. [b] The ED_{50} is the concentration of compound that produced 50% inhibition of viral CPE as compared with nondrug controls. Values represent averages of two to three separate determinations.

Table IV. In Vitro Cytostatic Activity of Pyrazofurin (1) and Certain Nucleoside and Nucleotide Derivatives of Pyrazofurin

compd	ID_{50},[a] M			
	L1210	P388	Lewis Lung	B16 melanoma
4	7.5×10^{-5}	6.2×10^{-5}	[b]	[b]
6	2.9×10^{-5}	4.0×10^{-5}	[b]	[b]
7	$>1.0 \times 10^{-4}$	$>1.0 \times 10^{-4}$	[b]	[b]
8	1.5×10^{-5}	1.7×10^{-5}	[b]	[b]
10	1.3×10^{-5}	4.5×10^{-5}	2.8×10^{-5}	7.2×10^{-5}
12	$>1.0 \times 10^{-4}$	$>1.0 \times 10^{-4}$	[b]	[b]
13	7.9×10^{-5}	$>1.0 \times 10^{-4}$	8.9×10^{-5}	$>1.0 \times 10^{-4}$
15	$>1.0 \times 10^{-4}$	$>1.0 \times 10^{-4}$	$>1.0 \times 10^{-4}$	$>1.0 \times 10^{-4}$
17	1.3×10^{-4}	9.4×10^{-5}	[b]	[b]
18	2.7×10^{-9}	1.0×10^{-9}	5.0×10^{-9}	2.4×10^{-7}
19b	3.9×10^{-9}	3.2×10^{-9}	7.0×10^{-10}	2.3×10^{-7}
20b	5.8×10^{-9}	1.2×10^{-8}	1.0×10^{-8}	2.3×10^{-6}
21b	2.5×10^{-6}	4.4×10^{-6}	1.7×10^{-6}	6.3×10^{-6}
23b	4.0×10^{-9}	8.8×10^{-9}	4.8×10^{-9}	6.0×10^{-7}
1	3.2×10^{-9}	3.3×10^{-9}	1.8×10^{-9}	6.3×10^{-8}

[a] Inhibitory dose 50 (ID_{50}) is the concentration of the compound in the culture media that produced 50% of the tumor cell growth as compared to the untreated controls. Values represent averages of two to three separate determinations. [b] Not determined.

carcinoma cells in vitro, with similar ID_{50} values in the nanomolar concentration range. However, B16 melanoma cells were less sensitive to growth inhibition by these compounds. The ID_{50} value of pyrazofurin was approximately 20–35 times higher against B16 melanoma than against other cell lines. Compounds **18** and **23b** were less effective than **1** in inhibiting B16 melanoma with ID_{50} values 4–10 times higher than that of pyrazofurin and 50–250 times higher than their ID_{50} values against the other cell lines tested.

The 5′-(*O*-ethyl phosphate) (**19b**) had approximately the same cytostatic activity as the 5′-monophosphate (**18**), while the 5′-(di-*O*-methyl phosphate) (**20b**) had an ID_{50} value 2–12 times higher than that of **18**. Modification at the 1-, 4-, or 5-position of **1** (compounds **4**, **6**, **8**, **10**, **13**, and **17**) considerably reduced the cytostatic activity in cell culture, and compounds **7**, **12**, and **15** were inactive against L1210 and P388 leukemias.

Experimental Section

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance (^1H NMR) spectra were determined at 89.6 MHz with a JEOL FX 90Q spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard. The presence of water as indicated by elemental analysis was verified by ^1H NMR. Infrared spectra (IR) were obtained on a Beckman Acculab 2 spectrophotometer and ultraviolet spectra (UV; sh = shoulder) were recorded on a Cary Model 15 spectrophotometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and Robertson Labs, Florham Park, NJ. Thin-layer chromatography (TLC) was run on silica gel 60 F-254 plates (EM Reagents). E. Merck silica gel (230–400 mesh) was used for flash column chromatography. preparative liquid chromatography (LC) was run utilizing the Waters Prep 500 LC system. All solvents used were reagent grade and were dried over molecular sieve (4A). Detection of components on TLC was by UV light and with 10% H_2SO_4 in MeOH spray followed by heating. Evaporations were carried out under reduced pressure with the bath temperature below 30 °C.

4-(Benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide (4). To a solution of pyrazofurin (1; 10.36 g, 40 mmol) in water (200 mL) containing Na_2CO_3 (4.24 g, 40 mmol) was added benzyl bromide (7.6 g, 60 mmol) in CH_3CN (200 mL), all at once with stirring. After stirring for 18 h at room temperature, the solution was evaporated to dryness. The residue was dissolved in a minimum amount of hot water and allowed to stand at room temperature overnight. The white crystals that deposited were collected by filtration to yield 8.4 g. The mother liquor was chromatographed on a reverse-phase silica gel column (Prep 500) using 5% MeOH in water as the eluent. The fractions containing the desired product were pooled and evaporated to dryness. The residue was triturated with ether (50 mL) and filtered to give 4.1 g of white crystals. Recrystallization of the combined solids from aqueous acetonitrile afforded 12.2 g (87.4%) of pure 4; mp 163–165 °C; IR (KBr) ν 1050 (=COC), 1650 (C=O), 2900–3430 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 245 (sh) nm (ϵ 3200); UV λ_{max} (pH 7) 245

(sh) nm (ε 4200); UV λ_{max} (pH 11) 243 nm (ε 6300); ^{1}H NMR (Me_2SO-d_6) δ 4.72 (d, 1, J = 6.20 Hz, C_1'H), 5.17 (s, 2, CH_2), 7.40 (m, 7, $CH_2C_6H_5$ and $CONH_2$), and other sugar protons. Anal. ($C_{16}H_{19}N_3O_6$) C, H, N.

1-Methyl-4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (N^1-Methylpyrazofurin, 8). A solution of 4 (2.0 g, 7.7 mmol) in dioxane (100 mL) was saturated with diazomethane and the mixture was allowed to stand at room temperature for 18 h. Acetic acid was added to destroy excess diazomethane and the solution was evaporated to dryness. The residue containing 1-methyl-4-(benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide (5) was dissolved in MeOH (75 mL) and hydrogenated on a Parr hydrogenator at 45 psi for 5 h in the presence of Pd/C (10%, 100 mg). The mixture was filtered through a Celite pad and washed with hot MeOH (3 × 25 mL), and the combined filtrates were evaporated to a syrup. The syrup was dissolved in CH_3CN (10 mL) and allowed to stand at room temperature for several days. The title compound crystallized out, 0.30 g. Additional amount of 8 was obtained after chromatographing the mother liquor on a silica gel column (2 × 25 cm) using CH_2Cl_2/MeOH (9:1, v/v) as the eluent. Total yield of 8 was 0.70 g (52.5%, from aqueous acetonitrile); mp >195 °C dec; IR (KBr) ν 1650 (C=O), 2930 (NCH_3), 3360 and 3470 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 230 nm (ε 8200), 267 (5600); UV λ_{max} (pH 7) 232 nm (ε 6500), 308 (5200); UV λ_{max} (pH 11) 237 nm (ε 6000), 309 (8200); ^{1}H NMR (Me_2SO-d_6) δ 3.92 (s, 3, CH_3), 4.71 (d, 1, J = 6.10 Hz, C_1'H), 7.22 (br s, 3, $CONH_2$, C_4-OH), and other sugar protons. Anal. ($C_{10}H_{15}N_3O_6$) C, H, N.

4-Methoxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (4-O-Methylpyrazofurin, 6). Pyrazofurin (1.0 g, 3.8 mmol) in dimethylformamide (25 mL) containing methyl iodide (0.59 g, 4.18 mmol) and K_2CO_3 (0.57 g, 4.18 mmol) was stirred at room temperature for 24 h. The reaction mixture was diluted with 0.5 N acetic acid (25 mL) and evaporated to dryness. The residue was purified on a silica gel column (2.5 × 25 cm) using CH_2Cl_2/MeOH (9:1, v/v) as the eluent to yield 1.0 g (95.2%) of the title compound after crystallization from water; mp >210 °C dec; IR (KBr) ν 1015 (=COC), 1670 (C=O), 3340–3440 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1 and 7) 224 nm (ε 14 500); UV λ_{max} (pH 11) 224 nm (ε 15 000); ^{1}H NMR (Me_2SO-d_6) δ 3.78 (s, 3, CH_3), 4.67 (d, 1, J = 6.45 Hz, C_1'H), 7.32, 7.16 (2 br s, 2, $CONH_2$), 12.80 (br s, 1, NH), and other sugar protons. Anal. ($C_{10}H_{15}N_3O_6 \cdot H_2O$) C, H, N.

1-Methyl-4-methoxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (1,4-Dimethylpyrazofurin, 7). To a hot solution of pyrazofurin (1.0 g, 3.8 mmol) in dioxane (free from peroxides) was added an ice-cold solution of diazomethane in dioxane. The reaction was monitored by TLC (silica gel, CH_2Cl_2/MeOH, 9:1, v/v), with additional amounts of diazomethane in dioxane being added. When the reaction had gone ~75%, enough diazomethane was added to impart a dark yellow color and the reaction mixture was allowed to stand in the refrigerator overnight. TLC indicated that the reaction had gone almost to completion with only small amounts of mono- and higher methylated products. The mixture was acidified with acetic acid to destroy excess diazomethane and then evaporated to dryness. The residual syrup was triturated with CH_3CN (10 mL) to give crystalline 7 (0.35 g). Additional material was obtained by chromatographing the mother liquor (silica gel using CH_2Cl_2/MeOH, 9:1, v/v, as solvent). Recrystallization of the combined solids from water gave 1.02 g (92%) of the title compound; mp 179–180 °C; IR (KBr) ν 1010 (=COC), 1640 (C=O), 2920 (NCH_3), 3310–3410 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1 and 7) 228 nm (ε 6200); UV λ_{max} (pH 11) 228 nm (ε 6000); ^{1}H NMR (Me_2SO-d_6) δ 3.82 (s, 3, C_4-OCH_3), 3.96 (s, 3, N_1-CH_3), 4.65 (d, 1, J = 6.45 Hz, C_1'H), 7.18, 7.70 (2 s, 2, $CONH_2$), and other sugar protons. Anal. ($C_{11}H_{17}N_3O_6$) C, H, N.

4-Acetoxy-3-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1-acetylpyrazole-5-carboxamide (O^4,N^1,2′,3′,5′-Pentaacetylpyrazofurin, 9). A mixture of pyrazofurin (10.36 g, 40 mmol) and 4-(dimethylamino)pyridine (DMAP, 25 mg) in acetic anhydride (100 mL) was stirred at ambient temperature for 18 h. The solution was evaporated to dryness and the residue was coevaporated several times with toluene. TLC (silica gel, $CHCl_3$/acetone, 8:2, v/v) indicated a mixture of two products. The mixture was purified on a silica gel column by preparative LC techniques with acetone/hexane (4:6, v/v) as the solvent. Two bands were eluted, which were evaporated to dryness. The first band gave the title compound as amorphous foam, 9.8 g (52.2%); IR (KBr) ν 1685 (C=O of amide), 1785 (C=O of acetyl) cm^{-1}; UV λ_{max} (MeOH) 252 nm (ε 29 100); ^{1}H NMR ($CDCl_3$) δ 2.08–2.12 (3 s, 9, 3 OAc), 2.36 (s, 3, C_4-acetoxy), 2.70 (s, 3, NAc), 5.64 (d, 1, J = 3.10 Hz, C_1'H), 6.20, 6.79 (2 br s, 2, $CONH_2$), and other sugar protons. Anal. ($C_{19}H_{23}N_3O_{11} \cdot H_2O$) C, H, N. The second product was found to be tetraacetylpyrazofurin.

4-Hydroxy-3-β-D-ribofuranosylpyrazole-5-carbonitrile (10). To a solution of 9 (9.4 g, 2 mmol) (or a mixture of 9 and tetraacetylpyrazofurin) in CH_2Cl_2 (100 mL) containing pyridine (5 mL) was added phosgene in toluene (12.5%, 50 mL) dropwise at a rate that maintained a constant temperature. The resulting dark red solution was poured onto crushed ice (100 g) and extracted with water (3 × 50 mL). The combined water extracts were then extracted once with CH_2Cl_2 (100 mL). The organic phases were combined and dried over anhydrous Na_2SO_4. The solution was filtered and evaporated to dryness. The residue was subjected to silica gel flash chromatography (4.1 × 15 cm column, acetone/hexane, 1:4, v/v) to give pure 4-acetoxy-3-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1-acetylpyrazole-5-carbonitrile as a gum (8.0 g, 88.6%).

The above gum (5.0 g, 11 mmol) was dissolved in MeOH (100 mL) and 1 N NaOMe was added to pH 9. The mixture was stirred for 16 h at room temperature and neutralized with Doxex-50 H^+ resin and the solvent evaporated. The residual syrup was triturated with anhydrous ether (50 mL). The solid was collected by filtration and crystallized from aqueous ethanol to yield 2.3 g (86.2%) of the title compound; mp 138–139 °C; IR (KBr) ν 2230 (C≡N), 2950–3430 (OH, NH) cm^{-1}; UV λ_{max} (pH 1) 257 nm (ε 5100); UV λ_{max} (pH 7) 296 nm (ε 5500); UV λ_{max} (pH 11) 296 nm (ε 8000); ^{1}H NMR (Me_2SO-d_6) δ 4.73 (d, 1, J = 6.44 Hz, C_1'H), and other sugar protons. Anal. ($C_9H_{11}N_3O_5$) C, H, N.

4-Acetoxy-3-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1-acetylpyrazole-5-N-acetoxycarboxamidoxime (11). To a solution of 10 (2.41 g, 10 mmol) in anhydrous dioxane was added free hydroxylamine (0.5 g, 15 mmol) and the mixture was heated under reflux for 1 h, with the exclusion of moisture. The reaction mixture was evaporated to dryness and held at high vacuum overnight. The dry residue was treated with acetic anhydride (25 mL) and DMAP (100 mg). The mixture was stirred at room temperature for 5 h and evaporated to dryness. Coevaporation with absolute ethanol (3 × 25 mL) gave dry residue, which was purified on a silica gel column (2.4 × 45 cm) using toluene/ethyl acetate (7:3, v/v) as the solvent. The appropriate homogenous fractions were pooled and evaporated to dryness and the residue was triturated with CCl_4 (25 mL) to give crystalline solid, 2.5 g (47.5%); mp 238–240 °C; IR (KBr) ν 1760 and 1780 (C=O of acetyl), 3370, 3480 (NH_2) cm^{-1}; UV λ_{max} (pH 1) 230 nm (ε 9200); UV λ_{max} (pH 7) 230 nm (ε 8700); UV λ_{max} (pH 11) 300 nm (ε 6000); ^{1}H NMR (Me_2SO-d_6) δ 2.00, 2.01, 2.04 (3 s, 9, 3 OAc), 2.12 (s, 3, NAc), 2.30 (s, 3, C_4-OAc), 2.72 (s, 3, NOAc), 5.54 (s, 1, C_1'H), 6.79 (br s, 2, NH_2), and other sugar protons. Anal. ($C_{21}H_{26}N_4O_{12}$) C, H, N.

4-(Benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamidoxime (12). A solution of 4 (2.0 g, 5.7 mmol) in anhydrous pyridine (50 mL) containing acetic anhydride (10 mL) and DMAP (25 mg) was stirred at room temperature under anhydrous conditions for 18 h before it was poured onto ice–water (100 mL). After stirring overnight, the mixture was extracted with CH_2Cl_2 (3 × 75 mL). The combined, dried (Na_2SO_4) organic layer was evaporated to yield 2.85 g (96.6%) of the tetraacetyl derivative of 4 as homogeneous gum.

To a solution of the above tetraacetyl derivative (2.5 g) in CH_2Cl_2 (75 mL) containing pyridine (3.5 mL) was added dropwise a solution of phosgene in benzene (1.2 equiv). After the addition was complete, the reaction mixture was allowed to warm to room temperature for 2 h and then poured onto crushed ice (75 g). The aqueous solution was extracted with CH_2Cl_2 (3 × 50 mL). The combined, dried (Na_2SO_4) organic layer was evaporated to dryness and the residue dissolved in MeOH (50 mL). NaOMe (1 N) was added until pH 10 was maintained. After standing overnight, the solution was neutralized with Dowex-50 H^+ resin and filtered and the filtrate evaporated to dryness. The residue was purified on a silica gel column (2.3 × 25 cm) using $CHCl_3$/MeOH (6:1, v/v) as the solvent to yield the deblocked nitrile, 1.90 g. The nitrile

was dissolved in absolute ethanol to which was added 5 equiv of free NH_2OH. The solution was heated under reflux for 10 h and allowed to stand at room temperature overnight. The solution was evaporated to dryness and the residue was chromatographed on a silica gel column (2.5 × 25 cm) using $CHCl_3$/MeOH (5:1, v/v) as the solvent to yield 1.50 g (85.2%) of the title compound after crystallization from aqueous MeOH; mp 125–127 °C; IR (KBr) ν 1075 (=COC), 2940–3300 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 235 nm (ϵ 7300); UV λ_{max} (pH 7) 235 nm (ϵ 6400); UV λ_{max} (pH 11) 235 nm (ϵ 6900); ^1H NMR (Me_2SO-d_6) δ 4.68 (d, 1, J = 6.45 Hz, C_1'H), 4.98 (s, 2, CH_2), 5.46 (s, 2, NH_2), 7.38 (m, 5, C_6H_5), 9.55 (s, 1, NOH), 12.61 (s, 1, NH), and other sugar protons. Anal. ($C_{16}H_{20}N_4O_6$) C, H, N.

4-Hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamidine (13). To a solution of **12** (50 mg, 0.13 mmol) in 95% EtOH (10 mL) was added 20% Pd/C (20 mg) and the mixture was shaken on a Parr hydrogenator for 48 h at 50 psi. The mixture was filtered on a Celite pad and the filtrate evaporated to dryness. The residue was purified on a preparative TLC silica gel plate and developed with EtOAc/EtOH/H_2O (2:1:1, v/v). The appropriate band was scraped from the plate and eluted with MeOH. The solution was evaporated to dryness. The residue was dissolved in hot EtOH (95%) and filtered. On standing fluffy white needles separated, yield 24 mg (62.7%); mp 188 °C dec; IR (KBr) ν 1670 (C=NH), 3200–3400 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 227 (sh) nm (ϵ 8800), 276 (4400); UV λ_{max} (pH 7) 326 nm (ϵ 4400); UV λ_{max} (pH 11) 325 nm (ϵ 7000); isosbestic point λ_{max} 295 nm (ϵ 3000); ^1H NMR (Me_2SO-d_6) δ 4.83 (d, 1, J = 5.10 Hz, C_1'H), 7.50 (2 br s, 4, C_4-OH, C=NH, NH_2), 12.50 (br s, 1, NH), and other sugar protons. Anal. ($C_9H_{14}N_4O_5 \cdot 2H_2O$) C, H, N.

4-(Benzyloxy)-3-β-D-ribofuranosylpyrazole-5-thiocarboxamide (15) and 4-(Benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carbonitrile (16). A portion of the tetraacetyl derivative of 4 from reaction **12** (3.0 g, 5.8 mmol) was dissolved in anhydrous dioxane (50 mL) containing 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane 2,4-disulfide (Lawesson's reagent, 1.20 g). The solution was heated under reflux for 2 h before it was poured into ice–water (50 mL). After stirring for 30 min, the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was purified on a silica gel column (2.4 × 40 cm) using acetone/hexane (1:4, v/v) as the solvent. Fractions containing the desired product were pooled and evaporated to dryness to yield 2.5 g of the blocked thioamide (**14**) as a yellow foam. The solution of **14** (2.5 g) in MeOH (65 mL) was adjusted to pH 10 with NaOMe. After stirring at room temperature for 2 h, the mixture was neutralized with Dowex-50 H^+ resin and then evaporated to dryness. The residue was triturated with $CHCl_3$ (50 mL) and the solid that separated was collected and applied to a flash silica gel column. The column was eluted with $CHCl_3$/MeOH (6:1, v/v). The first nucleoside product to elute was found to be **16**, which was isolated as a syrup to yield 0.15 g (70%); IR (KBr) ν 2240 (C≡N) cm^{-1}.

The second product to elute was identified as **15**, which was crystallized from aqueous ethanol to yield 1.32 g (62.3% overall yield); mp 146–148 °C; IR (KBr) ν 1080 (=COC), 1200, 1500 (C(S)N), 3300–3360 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 253 nm (ϵ 6600), 297 (9500); UV λ_{max} (pH 7) 253 nm (ϵ 7500), 297 (11 000); UV λ_{max} (pH 11) 265 nm (ϵ 8800), 305 (12 100); ^1H NMR (Me_2SO-d_6) δ 4.71 (d, 1, J = 5.90 Hz, C_1'H), 5.07 (s, 2, CH_2), 7.38 (m, 5, C_6H_5), 8.82, 9.60 (2 s, 2, $C(S)NH_2$), 12.96 (br s, 1, NH), and other sugar protons. Anal. ($C_{16}H_{19}N_3O_5S$) C, H, N, S.

4-(Benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide 5'-Monophosphate Disodium Salt (17). To an ice-cold solution of **4** (2.0 g, 5.7 mmol) in freshly distilled trimethyl phosphate (50 mL) was added $POCl_3$ (2 mL). The reaction mixture was stirred at ice-bath temperature and monitored frequently by TLC. When all the starting material had disappeared (1–2 h), the mixture was poured into ice–water (200 mL). The aqueous solution was extracted with ether (3 × 50 mL) and then adjusted to pH 2 with 2 N NaOH. The solution was applied to a column of activated charcoal (50 g, acid-washed Au-4), and the column was washed thoroughly with water until the eluate was salt free. The column was eluted with a solution of EtOH/H_2O/NH_4OH (10:10:1, v/v). The fractions containing pure (TLC, silica gel, CH_3CN/0.1 N NH_4Cl, 7:3, v/v) nucleotide were pooled and evaporated to dryness. The residue was dissolved in a small amount of water and passed through a column of Dowex-50 H^+ resin (20–50 mesh, 25 mL). The column was washed with water, and the fractions containing the nucleotide were collected. The solution was concentrated to a small volume (~20 mL) and passed through a column of Dowex 50W-X8 (20–50 mesh, Na^+ form, 25 mL). The column was washed with water, and the fraction containing the nucleotide sodium salt was evaporated to dryness. The solid residue was triturated with absolute EtOH (20 mL) before the white solid was collected by filtration and dried (P_2O_5) to provide 1.50 g (55.6%) of the title compound; mp >145 °C dec; IR (KBr) ν 1050 (=COC), 1665 (C=O), 2940–3440 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1 and 7) 240 (sh) nm (ϵ 4000); UV λ_{max} (pH 11) 240 (sh) nm (ϵ 5400); ^1H NMR (Me_2SO-d_6) δ 5.00 (s, 2, CH_2), 5.09 (d, 1, J = 7.46 Hz, C_1'H), 7.07, 7.21 (2 s, 2, $CONH_2$), 7.32 (m, 5, C_6H_5), and other sugar protons. Anal. ($C_{16}H_{18}N_3O_9Na_2P \cdot H_2O$) C, H, N, P.

4-Hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide 5'-Monophosphate (Pyrazofurin 5'-Monophosphate, 18). Compound **17** (2.0 g, 4.2 mmol), formic acid (5 mL), and 10% Pd/C (0.10 g) in MeOH (100 mL) were shaken under hydrogen atmosphere (50 psi) for 24 h. The catalyst was removed by filtration on a Celite pad and the filtrate evaporated to dryness. The residual solid was dissolved in water (50 mL) and passed through a column of Dowex-50 H^+ resin (25 mL). The eluate was lyophilized to obtain off-white powder, 1.10 g (77.2%); mp 90–95 °C; IR (KBr) ν 1675 (C=O), 3200–3380 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 257 nm (ϵ 4300); UV λ_{max} (pH 7) 257 nm (ϵ 3700); UV λ_{max} (pH 11) 303 nm (ϵ 5400); ^1H NMR (Me_2SO-d_6) δ 4.73 (d, 1, J = 6.32 Hz, C_1'H), 7.40 (br s, 3, C_4-OH and $CONH_2$), and other sugar protons. Anal. ($C_9H_{14}N_3O_9P \cdot 1^1/_4H_2O$) C, H, N, P.

4-(Benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide 5'-(*O*-Ethyl phosphate) (19a). $POCl_3$ (2 mL) was added slowly to an ice-cold solution of **4** (2.0 g, 5.7 mmol) in freshly distilled trimethyl phosphate (25 mL). The solution was stirred for 2 h at 0–5 °C before cold anhydrous EtOH (25 mL) was added. The mixture was kept at 0 °C for 12 h. Water (100 mL) was added and the pH of the aqueous solution was adjusted to 7 with 2 N NaOH. The solution was extracted with ether (3 × 50 mL), then concentrated to ~50 mL, and chromatographed on a Prep-500 reverse-phase column using 10% MeOH in 0.2 N AcOH. The fractions containing the desired nucleotide product were pooled and evaporated to dryness. The residual syrup was triturated with ether (3 × 50 mL), which crystallized on standing for 2 weeks, to yield 1.50 g (58%) of the title compound; mp 113–115 °C; IR (KBr) ν 1020 (COC), 1100, 1160, 1210 (EtOPO), 1660 (C=O), 3150–3410 (NH_2, OH) cm^{-1}; UV λ_{max} (pH 1 and pH 7) 246 nm (ϵ 6400); UV λ_{max} (pH 11) 243 nm (ϵ 7800); ^1H NMR (Me_2SO-d_6) δ 1.14 (t, 3, J = 7.42 Hz, $POCH_2CH_3$), 3.87 (m, 2, $J_{H,P}$ = 7.42 Hz and $J_{H,H}$ = 7.42 Hz, $POCH_2CH_3$), 4.70 (d, 1, J = 6.45 Hz, C_1'H), 5.02 (s, 2, $CH_2C_6H_5$), 7.08, 7.40 (br s and m, 7, $CONH_2$, $CH_2C_6H_5$), and other sugar protons. Anal. ($C_{18}H_{24}N_3O_9P \cdot H_2O$) C, H, N, P.

4-Hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide 5'-(*O*-Ethyl phosphate) (19b). In the same manner as for **18**, hydrogenation of **19a** (1.1 g) in EtOH (100 mL) containing formic acid (5 mL) in the presence of 20% Pd/C (100 mg) gave the title compound, 0.75 g (85%); mp 68–71 °C; IR (KBr) ν 1030 (POEt), 1190 (POH), 1660 (C=O), 3200–3400 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 218 nm (ϵ 6600), 263 (5500); UV λ_{max} (pH 7) 263 nm (ϵ 5000), 307 (sh) (1600); UV λ_{max} (pH 11) 234 (sh) nm (ϵ 5300), 307 (8600); ^1H NMR (Me_2SO-d_6) δ 1.16 (t, 3, J = 7.42 Hz, CH_3), 3.92 (m, 2, $J_{H,P}$ = 7.42 Hz, CH_2), 4.74 (d, 1, J = 6.09 Hz, C_1'H), 7.39 (br s, 3, C_4-OH and $CONH_2$), and other sugar protons. Anal. ($C_{11}H_{18}N_3O_9P$) C, H, N, P.

4-(Benzyloxy)-3-β-D-ribofuranosylpyrazole-5-carboxamide 5'-(Di-*O*-methyl phosphate) (20a). Compound **4** (2.0 g, 5.7 mmol) was suspended in trimethyl phosphate (25 mL) and cooled to 0 °C. $POCl_3$ (2 mL) was added dropwise with stirring. After 3 h, the reaction was quenched by the addition of MeOH (50 mL) and the solution was stored at 0 °C overnight. The mixture was poured into toluene (100 mL). The layers were separated, and the lower layer was again poured into toluene (100 mL). The toluene was decanted from an oily residue, which was dissolved in EtOAc (50 mL) and adsorbed onto silica gel (~15 g). The silica gel was placed on top of a silica gel column (2.5 × 40 cm) and eluted with $CHCl_3$/MeOH (6:1, v/v). The fractions containing

the desired product were pooled and evaporated to dryness. The residue was crystallized from water to yield 1.70 g (62.9%) of the title compound; mp 100–102 °C; IR (KBr) ν 1035 (COC), 1185 (P), 1665 (C=O), 3290–3450 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 246 nm (ϵ 6300); UV λ_{max} (pH 7) 246 nm (ϵ 7400); UV λ_{max} (pH 11) 241 nm (ϵ 15 500); ^1H NMR (Me_2SO-d_6) δ 3.63 (d, 6, $J_{H,P}$ = 10.85, 2 CH_3), 4.76 (d, 1, J = 5.43, $C_{1'}H$), 5.05 (s, 2, CH_2), 6.80, 7.18 (2 s, 2, $CONH_2$), 7.40 (m, 5, $CH_2C_6H_5$), 13.12 (d, 1, $J_{NH,P}$ = 11.01 Hz), and other sugar protons. Anal. ($C_{18}H_{24}N_3O_9P\cdot H_2O$) C, H, N, P.

4-Hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide 5′-(Di-O-methyl phosphate) (20b). In the same manner as for **18**, hydrogenation of **20a** (2.0 g) in MeOH (100 mL) containing formic acid (5 mL) in the presence of 20% Pd/C (150 mg) gave the title compound, which was purified on a silica gel column (2 × 40 cm) using $CHCl_3$/MeOH (6:1, v/v) as the eluent. Crystallization from aqueous ethanol gave an analytical sample, 1.25 g (75%); mp 178–180 °C; IR (KBr) ν 1040 (POC), 1680 (C=O), 2940–3250 (OH, NH_2) cm^{-1}; UV λ_{max} (pH 1) 262.5 nm (ϵ 6500); UV λ_{max} (pH 7) 265.5 nm (ϵ 4700), 305 (2900); UV λ_{max} (pH 11) 307 nm (ϵ 8900); ^1H NMR (Me_2SO-d_6) δ 3.64 (d, 6, $J_{H,P}$ = 11.81, 2 OCH_3), 4.78 (d, 1, J = 5.08 Hz, $C_{1'}H$), 7.44 (br s, 2, $CONH_2$), 8.83 (br s, 1, C_4-OH), 12.92 (br s, 1, NH), and other sugar protons. Anal. ($C_{11}H_{18}N_3O_9P\cdot{}^1/_2H_2O$) C, H, N, P.

4-Hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide 3′,5′-(Cyclic)phosphate (Pyrazofurin 3′,5′-(Cyclic)phosphate, 21b). Compound **17** (0.50 g, 1.05 mmol) was dried by repeated evaporation with anhydrous pyridine. The residual syrup was dissolved in dry pyridine (15 mL) and added dropwise to a refluxing dioxane (200 mL) solution of dicyclohexylcarbodiimide (DCC; 1.24 g, 6 mmol). After all the pyridine was added, the solution was refluxed an additional 6 h. The reaction mixture was evaporated to dryness, and the residue was dissolved in water (20 mL) and extracted with ether (3 × 50 mL). The aqueous solution was adjusted to pH 7 with NH_4HCO_3 and applied to a DEAE-Sephadex column (25 × 45 cm) equilibrated with 0.05 M NH_4HCO_3. The column was washed with 0.05 M NH_4HCO_3 (500 mL) at a flow rate of 3 mL/min. Fractions containing the cyclic phosphate were pooled and lyophilized until free of NH_4HCO_3. The residue was then dissolved in water (15 mL) and passed through a column of Dowex-50 H^+ resin (50–100 mesh, 2 × 15 cm). The eluate was lyophilized to a white powder (**21a**, 0.35 g), which was used for debenzylation studies without further purification.

A solution of **21a** (0.10 g) in 95% EtOH (25 mL) containing 20% $Pd(OH)_2$/C (25 mg) and cyclohexene (8 mL) was heated under reflux for 2 h. The catalyst was removed by filtration on a Celite pad and the filtrate evaporated to dryness. The residue was dissolved in water (25 mL), adjusted to pH 7 with NH_4HCO_3, and applied to a DEAE-Sephadex column (1 × 15 cm) equilibrated with 0.05 M NH_4HCO_3. The column was washed with 0.05 M NH_4HCO_3 (200 mL) and then eluted with a gradient of 0.05 M NH_4HCO_3 (250 mL) to 1.0 M NH_4HCO_3 (250 mL) at a flow rate of 3 mL/min. Fractions containing the desired product were pooled and lyophilized until free of NH_4HCO_3. The residue was dissolved in water (20 mL) and passed through a Dowex-50 H^+ resin column (1 × 15 cm). The eluate was lyophilized to give a light tan colored powder, which was triturated with anhydrous MeOH, filtered, and dried to yield 78 mg of pure **21b**; mp 133 °C dec; IR (KBr) ν 1060 (POC), 1220 (P=O), 1650 (C=O), 3000–3440 (NH_2, OH) cm^{-1}; UV λ_{max} (pH 1) 263 nm (ϵ 3700); UV λ_{max} (pH 7) 268 nm (ϵ 2800); UV λ_{max} (pH 11) 235 nm (ϵ 2900), 305 (5700); ^1H NMR (Me_2SO-d_6) δ 4.89 (s, 1, $C_{1'}H$), 7.43 (br s, 3, C_4-OH, $CONH_2$), and other sugar protons. Anal. ($C_9H_{12}N_3O_8P\cdot 3H_2O$) C, H, N, P.

4-(Benzyloxy)-5-carbamoyl-3-β-D-ribofuranosylpyrazolyl(5→5′)adenosine Pyrophosphate (23a). A mixture of **17** (0.30 g, 0.63 mmol) and adenosine 5′-phosphoric di-*n*-butylphosphinothioic anhydride[46] (**22**; 0.62 g, 1.26 mmol) was rendered anhydrous by repeated evaporation with dry pyridine. The residue was dissolved in dry pyridine (50 mL) to which was added $AgNO_3$ (0.68 g, 4 mmol) and the mixture was stirred overnight with the exclusion of moisture. H_2S was bubbled into the reaction mixture for 15 min, and the black precipitate that formed was removed by filtration. The filtrate was evaporated to dryness. The aqueous solution of the residue was adjusted to pH 7 with NH_4HCO_3 and applied to a DEAE-Sephadex column (2.5 × 40 cm) equilibrated with 0.5 M NH_4HCO_3. The column was washed with 0.05 M NH_4HCO_3 (1 L) and then eluted with a gradient of 0.05 M NH_4HCO_3 (1 L) to 6 N NH_4HCO_3 (1 L) at a flow rate of 3 mL/min. Fractions containing the desired product were pooled and lyophilized until free of NH_4HCO_3. The residue was dissolved in water (25 mL) and passed through a column of Dowex-50 H^+ resin (50–100 mesh, 2 × 20 cm). The eluate was lyophilized to an amorphous powder to give 0.50 g (90.6%) of **23a**; mp 160 °C dec; IR (KBr) ν 1075 (COC), 1230 (P=O), 1660 (C=O), 3170–3420 (NH_2, OH) cm^{-1}; UV λ_{max} (pH 1) 257 nm (ϵ 15 900); UV λ_{max} (pH 7 and 11) 257 nm (ϵ 15 700); ^1H NMR (Me_2SO-d_6) δ 4.70 (d, 1, J = 6.72 Hz, $C_{1'}H$ of **17**), 5.03 (s, 2, CH_2), 5.93 (d, 1, J = 5.19 Hz, $C_{1'}H$ of Ado), 7.40 (m, 9, C_6H_5, $CONH_2$, and NH_2), 8.16, 8.48 (2 s, 2, C_2H and C_8H of Ado), and other sugar protons. Anal. ($C_{26}H_{32}N_8O_{15}P_2\cdot 2H_2O$) C, H, N, P.

5-Carbamoyl-3-β-D-ribofuranosylpyrazolyl(5→5′)-adenosine Pyrophosphate (PAD, 23b). To a solution of **23a** (100 mg) in 95% ethanol (25 mL) was added 20% $Pd(OH)_2$/C (25 mg) and cyclohexene (8 mL), and the mixture was heated under reflux for 2 days. The catalyst was removed by filtration on a Celite pad and the filtrate evaporated to dryness. The residue was dissolved in water (15 mL), adjusted to pH 7 with NH_4HCO_3, and applied to a DEAE-Sephadex column (2.5 × 45 cm) equilibrated with 0.05 M NH_4HCO_3. The column was washed with 0.05 M $NaHCO_3$ (150 mL) and eluted with a gradient of 0.05 M NH_4HCO_3 (200 mL) to 5 N NH_4HCO_3 (200 mL) at a flow rate of 3 mL/min. Fractions containing the desired product were pooled and lyophilized until free from NH_4HCO_3. The aqueous solution (25 mL) of the residue was passed through a Dowex-50 H^+ resin column (2 × 25 cm) and the eluate was lyophilized repeatedly to yield PAD, 85 mg (93%); mp 175 °C dec; IR (KBr) ν 1060 (POC), 1230 (P=O), 1640 (C=O), 3000–3400 (NH_2, OH) cm^{-1}; UV λ_{max} (pH 1) 259 nm (ϵ 17 000); UV λ_{max} (pH 7) 259 nm (ϵ 18 000); UV λ_{max} (pH 11) 259 nm (ϵ 15 400), 306 (8700); ^1H NMR (Me_2SO-d_6) δ 4.72 (d, 1, J = 6.10 Hz, $C_{1'}H$ of **17**), 5.92 (d, 1, J = 4.06 Hz, $C_{1'}H$ of Ado), 7.28, 7.41 (2 s, 4, $CONH_2$ and NH_2), 8.15, 8.49 (2 s, 2, C_8H and C_2H of Ado), and other sugar protons. Anal. ($C_{19}H_{26}N_8O_{15}P_2\cdot 3H_2O$) C, H, N, P.

Antiviral Evaluation. Test compounds were evaluated for their ability to inhibit virus-induced cytopathic effect (CPE) produced by herpes simplex virus type 2 (HSV-2, 333), vaccinia virus (VV), measles, and parainfluenza virus type 3 (para-3) in African green monkey kidney (Vero) cells (American Type Culture Collection, Rockville, MD). Vero cells were maintained in antibiotic-free Eagle minimum essential medium (EMEM) with Earle's salts supplemented with 10% heat-inactivated newborn bovine serum (Grand Island Biological Co., Grand Island, NY). For antiviral experiments, cells were inoculated into 96-well tissue culture plates (Corning Glassworks, Corning, NY) at a concentration of 4×10^4 cells/0.2 mL per well and cultured for 24 h at 37 °C in 5% CO_2 to confluency.

Monolayers were inoculated with a predetermined number of $TCID_{50}$ (50% tissue culture infective dose) units of virus that will produce complete destruction of the cell monolayer in 72 h. The number of $TCID_{50}$ units in 0.1 mL/well were as follows: HSV-2, 100; VV, 200; measles, 100; para-3, 60. After 30-min adsorption at 37 °C, test compounds were added (0.1 mL/well) in seven 0.5 log concentrations ranging from 1×10^{-5} to 1×10^{-2} M resulting in final well concentrations of 5×10^{-6} to 5×10^{-3} M. Active compounds were tested at concentrations of 5×10^{-7} to 5×10^{-4} M. At each concentration, duplicate wells were used for evaluation of antiviral activity and single uninfected wells for cytotoxicity evaluation.

After 72-h incubation at 37 °C in 5% CO_2/air, the concentration of compound that produced 50% inhibition of viral CPE was determined (ED_{50}). A given concentration of compound was considered cytotoxic if it produced any microscopically visible changes in cellular morphology or in the density of the cell monolayer due to lysis, rounding up, or detachment of cells. The lowest concentration where cytotoxicity to uninfected, confluent Vero cell monolayers was observed was considered to be the maximum tolerated concentration (MTC).

Cytostatic Activity Evaluation. A. L1210 and P388 Leukemias. Compounds were evaluated for their ability to inhibit growth of murine leukemia L1210 and lymphoid neoplasm P388

(American Type Culture Collection, Rockville, MD) maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY) and 20 mM Hepes buffer. For growth experiments, cells were adjusted to 1×10^5 cells/mL and distributed to 24-well tissue culture plates (0.5 mL/well). Test compounds were dissolved in growth medium, sterilized by passage through an 0.22-μm membrane filter, serially diluted, and added to wells (0.5 mL/well). Compounds were tested in duplicate at log concentrations ranging from 1×10^{-10} to 1×10^{-4} M. Following 48-h incubation at 37 °C, cell counts were determined with a Coulter Model ZF cell counter. Cell growth in the presence of test compounds was expressed as a percentage of growth in untreated control wells and the concentration of compound producing 50% inhibition of cell growth was determined (ID_{50}).

B. B16 Melanoma and Lewis Lung Carcinoma. Lewis Lung carcinoma was maintained as a monolayer in RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum. B16 melanoma was maintained as a monolayer in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated fetal calf serum, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, and MEM vitamin solution. For determination of cell growth inhibition using either cell line, cells were seeded at 2.5×10^4 cells per well in 24 tissue culture plates. Cells were grown 24 h at 37 °C in 5% CO_2, and then growth medium was replaced with medium containing the compound of interest at log concentrations ranging from 1×10^{-10} to 1×10^{-4} M. After an additional 72-h incubation, cells were washed twice with phosphate buffered saline, trypsized to single cell suspensions, and counted with a Coulter Model ZF cell counter. Cell growth at each dose level was expressed as a percentage of growth in control wells and the dose resulting 50% inhibition of growth was determined.

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Registry No. 1, 30868-30-5; 4, 99298-13-2; 4 (tetraacetyl derivative), 99298-21-2; 4 (5-carbonitrile), 99298-22-3; **5**, 99298-15-4; 6, 99298-16-5; **7**, 41329-11-7; **8**, 99298-14-3; **9**, 59252-13-0; **9** (5-carbonitrile), 99298-17-6; **10**, 99298-18-7; **11**, 99298-19-8; **12**, 99298-20-1; **13**, 99298-23-4; 14, 99298-24-5; **15**, 99298-25-6; 16, 99298-22-3; **17**, 99298-26-7; **18**, 65446-02-8; **19a**, 99298-27-8; **19b**, 99298-28-9; **20a**, 99298-29-0; **20b**, 99298-30-3; **21a**, 99298-31-4; **21b**, 99298-32-5; **22**, 57816-25-8; **23a**, 99298-33-6; **23b**, 99298-34-7.

Supplementary Material Available: Tables of positional parameters (Tables V–VIII), anisotropic thermal parameters of the non-hydrogen atoms (Tables IX–XII), and positional and isotropic thermal parameters of the hydrogen atoms (Tables XIII–XVI) for compounds **1**, **8**, **7**, and **20a**, respectively (12 pages). Ordering information is given on any current masthead page.

Mapping the Turkey Erythrocyte β Receptor: A Distance Geometry Approach

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Extensions and refinements of the receptor mapping method as originally developed by Crippen are presented. In a set of newly developed algorithms measures are taken to reduce the number of required energy parameters to a statistically acceptable degree. The most important measure is the incorporation of lipophilicity as a hydrophobic bonding parameter to describe the binding of parts of the ligands to lipophilic areas on the receptor. In order to test the applicability of our set of programs, we mapped the turkey erythrocyte β receptor using a data set of Bilezikian. It was found that the experimentally determined free energies of binding can be reasonably described using a nine-point geometrical representation of the receptor site and only six energy parameters. The deduced model predicts that the phenyl rings of phenylethanolamines and phenoxypropanolamines occupy different parts of the receptor site.

Conformational flexibility of ligands poses a major problem in pharmacophoric pattern search and receptor mapping techniques. It has become clear that it is unrealistic to assume that ligands always bind to the receptor in their conformations of minimal energy as the free energy of association generally outweighs the energy of a conformational change of the ligand. It is therefore necessary to take into account all conformations having energies up to a few kilocalories/mole above the global minimum.

Conformational flexibility can be conveniently condensed in a distance matrix. Considering the total set of possible conformations, the entries in the upper triangle of such a matrix usually are the maximum distances between any pair of preselected atoms or dummy points in the molecule, whereas the entries in the lower triangle are the corresponding minimum distances.

The methodology of distance geometry is a powerful tool in handling the information contained in such matrices. During the last few years especially Crippen[1] has applied the distance geometry approach as a receptor mapping technique.

By comparing the distance matrices of the ligands in the data set, Crippen was able to deduce the common structural features of a set of ligands. Substituent points were subsequently positioned relative to this common base group. Complementary receptor "site points" were then proposed to account for the binding of the structural features of the ligand ("ligand points"). Furthermore, Crippen supposed that the total binding energy of a ligand to its receptor is equal to the sum of the individual interactions between ligand points and site points. Given the experimentally determined free energy of binding, the method enables one to calculate the individual binding energy contributions of any ligand point–site point interaction.

Thus, next to the incorporation of conformational flexibility in the calculations, the strong point of the method is that it enables the researcher to propose new, stronger binding ligands on basis of the geometry and energy parameters of the deduced receptor site model.

A major problem with respect to the general applica-

(1) Crippen, G. *J. Med. Chem.* 1980, *23*, 599.

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