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DIMENSIONAL PROBES OF BINDING AND ACTIVITY

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INTRODUCTION

One of the important fundamental aspects of studying enzymatic reactions is the discernment of the interaction between enzymes and their requisite substrates or cofactors, i.e., the definition, in detail, of the active binding sites on or within the enzymes. One strategy that is employed in the analysis of enzyme characteristics is the synthesis and testing of compounds that are designed to mimic the natural substrate(s) of an enzyme. The possible consequences of this substitution include various types of inhibition or induction or the formation of a product analogue which itself may confer a direct or indirect physiological effect. In this regard and in the area of nucleic acid chemistry, there has been considerable interest in the synthesis of analogues of naturally occurring nucleic acid bases and their corresponding nucleosides, nucleotides, and coenzymes.[1] Compounds have been designed to provide information regarding the electronic, spatial, steric, and conformational requirements for an analogue to function specificially as an inhibitor, substrate, or cofactor for an enzyme.[2] If we take the naturally occurring purines and their derivatives as examples, these have been subject to three major types of modification. First, the periphery of the five- and six-membered fused rings has been altered by substitution or else by replacement, addition, or relocation of nitrogens. Second, the addition of derivatizing agents to intact purines has led to sterically blocked, fluorescent, or photoaffinity relatives of the originals. Third, the attached ribofuranosyl or deoxyribofuranosyl group has been replaced with other sugar or pseudo sugar moieties.

We have introduced a different approach, that of synthesizing <u>de novo</u> analogues of the purines, and especially of adenine, that differ from the natural bases, ribonucleosides, or ribonucleotides by defined dimensional changes. With these <u>dimensional probes</u>, it is possible to assess the size of the space available or required for the purine (e.g., adenine) moiety in enzyme-coenzyme binding sites. In the best cases, the dimensional probes also serve as fluorescent probes. When the concept was initiated toward the possible provision of answers in dimensionally quantitative terms, with the use of specific sets of enzymes and cofactors, encouragement came from the demonstrated[3] conformational fluctuation and flexibility of proteins.

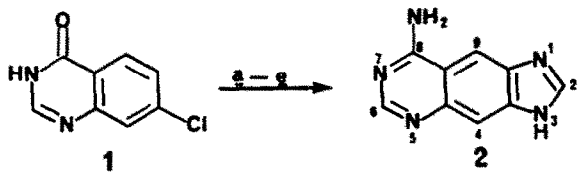
There are essentially five levels of experimentation with dimensional probes,[4-8] and the most valuable results are to be obtained when the final two stages can be reached: (1) synthesis and characterization of the requisite heterocyclic nuclei; (2) <u>N</u>-ribosidation at the proper location on the heterocycle; (3) mono-, di-, and triphosphorylation at the 5'-hydroxyl of the ribosyl unit; (4) determination of enzyme binding and activity relative to the natural substrate or cofactor; and (5) NMR spectroscopy for conformational analysis and fluorescence spectroscopy for metal ion and enzyme binding. In the subsequent sections, we shall examine, but not necessarily in chronological order, the extent to which these levels have been reached in this laboratory and in other laboratories where the concept is being utilized. We shall not discuss the synthetic methodology in detail where it follows standard procedures but only where it departs from the predictable or represents the application of new techniques. Enzymic and/or biological activity will be discussed, whenever such information has been provided, when a particular compound is described, whether at the heterocyclic base, nucleoside, or nucleotide level.

LINEAR HETEROCYCLIC BASES

Benzene Ring Spacer

6,6,5-Ring Systems. Our first experiments were designed to answer the question as to what properties might be associated with compounds in which the pyrimidine ring and the imidazole ring of the purine system are separated by a benzene ring to form an extended or "stretched-out" purine model.[7] The initial synthetic target was 8-aminoimidazo[4,5-g]quinazoline (2), to which we gave the trivial name lin-benzoadenine. The prefix refers to the linear disposition of the three rings in compound 2, and the numbering is as shown. The formal insertion of a spacer into the center of the adenine ring system, in this case a benzene ring (actually four additional carbon atoms), stretches the natural adenine linearly by 2.4 A, the width of the benzene ring known accurately from many X-ray structure determinations. The stretched-out analogue (2) has the advantage of retaining unobstructed the normal binding sites, e.g., N1, N^6, and

Scheme I



a, HNO_3, H_2SO_4; b, NH_3, C_4H_9OH, 175 °C; c, Pd/C, H_2, HCOOH; d, P_2S_5, py; e, NH_3, C_4H_9OH, 200 °C

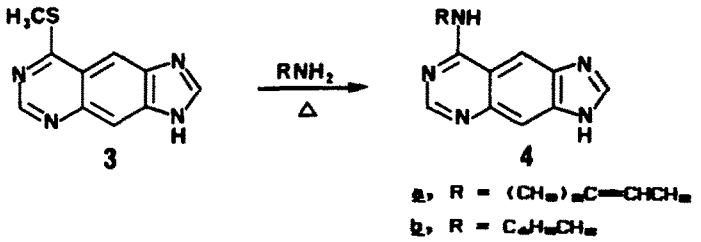
N7, of adenine. In general, if a compound of this type or one with the basic nucleus were found to possess interesting activity in vitro, it might be hoped that transport in vivo would be similar for analogue and natural substance while it would be expected that the metabolic pathway for analogue and natural substance would be very different.

The synthesis of lin-benzoadenine (2) was achieved by several converging routes,[7] and a convenient one (from 1) is abbreviated in Scheme I. Separation of isomers was necessary after the nitration stage, but otherwise the synthesis was straightforward. This synthesis provided imidazo[4,5-g]quinazolin-8(7H)-one (lin-benzohypoxanthine) after the catalytic hydrogenation step (c) and 8-mercaptoimidazo[4,5-g]quinazoline, the lin-benzo derivative of the antileukemia drug 6-mercaptopurine,[10] in the penultimate step. The latter intermediate exhibits antiviral activity in vitro and has generated some interest since it inhibits focus formation in mouse sarcoma virus. At separate stages in the synthesis of 2, it was found possible to replace the 4-, 9-, and 2-hydrogens selectively by deuterium. This facilitated the ^1H NMR chemical shift assignments and subsequently permitted the analysis of stacking interactions of various lin-benzoadenine nucleotides in aqueous solution.[11] Since the pK_a values of lin-benzoadenine were determined to be 5.6 and 11.7 in 66% DMF solution,[12] i.e., compound 2 is a stronger base than adenine, it was early recognized that any comparison of analogue and natural substrates would best be carried out with enzyme systems at slightly alkaline pH. The completed synthesis of lin-benzoadenine offered an immediate reward in terms of observed enzymatic activity. With adenosine deaminase from calf intestinal mucosa, lin-benzoadenine is hydrolyzed to lin-benzohypoxanthine at about 85% the rate that the normal substrate adenosine is hydrolyzed to inosine.[13] The contrast in

the behavior of 2 with that of adenine is especially noteworthy since adenine is not converted to hypoxanthine by this enzyme. Xanthine oxidase from buttermilk, a complex enzyme that catalyzes the air-oxidation of hypoxanthine to uric acid and has broad substrate specificity, catalyzes the oxidation of lin-benzohypoxanthine to lin-benzoxanthine then to lin-benzouric acid at a conversion rate apparently faster than that for hypoxanthine.[15] These findings indicate that both adenosine deaminase and xanthine oxidase can accommodate the 2.4 Å-laterally extended substrates lin-benzoadenine (2) and lin-benzohypoxanthine, respectively, and act on them efficiently.

Because of the biological activity demonstrated by lin-benzoadenine and its synthetic precursors, we were prompted to prepare cytokinin analogues of the lin-benzo variety. The natural cytokinins are plant hormones which bring about cell division and cell differentiation.[16] Since there were no previously known natural or synthetic cytokinins containing a central heterocyclic nucleus larger than bicyclic, the possibility of activity for the tricyclic benzoadenines 4a and 4b should help define the limits of the spatial parameters for the central moiety in cytokinins. 8-(3-Methyl-2-butenylamino)imidazo[4,5-g]quinazoline (4a) and 8-benzylaminoimidazo[4,5-g]quinazoline (4b) were prepared by heating an ethanolic solution of 8-methylmercaptoimidazo[4,5-g]quinazoline (3) with 3-methyl-2-butenylamine and benzylamine, respectively, at 200 °C (Scheme II).[17] The substituted linear-benzoadenines do possess cytokinin activity. They are

Scheme II



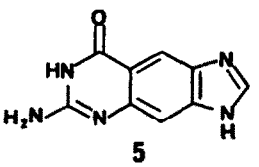
3 4

a, R = $(CH_3)_2C{=}CHCH_2$

b, R = $C_6H_5CH_2$

less active than their respective adenine models, i⁶Ade and bzl⁶Ade; however, they permit the same maximum yields to be obtained as do their adenine models. Moreover, they are fluorescent, emitting in the 380 nm range when excited above 300 nm.[15,17] The results suggest that the cytokinin activity may be related to the area defined by a cross section of the central nucleus. The rectangle enclosing the lin-benzoadenine nucleus is 1.7 times larger in area than the corresponding area for adenine. Analogues with an even larger central nucleus have lower activity or no activity.[17]

lin-Benzoguanine, 6-aminoimidazo[4,5-g]quinazolin-8(7H)-one (5), has been synthesized by two routes, each one starting with a substituted central benzene ring and proceeding via elaboration of the terminal rings in either sequence.[18]



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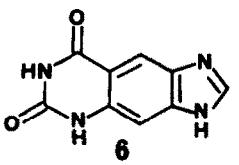

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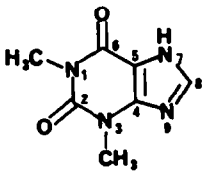
The sequences from ethyl 4-nitroanthranilate or ethyl 4-chloroanthranilate follow standard procedures, and the sequence utilizing the former starting material appears to be more reliable. A variant of the procedure starting with N-(carboethoxy)-4-nitroanthranilic acid provided lin-benzoxanthine (6), also through the use of standard reactions.[16] lin-Benzoguanine is fluorescent and emits at maximum wavelength (456 nm) in neutral aqueous buffer upon excitation at 332 nm.[16]

The biological activity of theophylline (7) and caffeine (8) made it of interest to synthesize the laterally extended analogues (9, 10) of these



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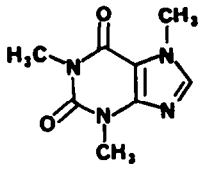

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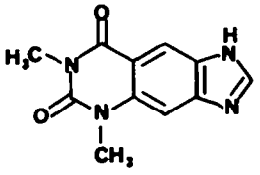

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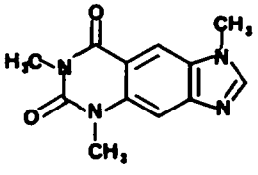

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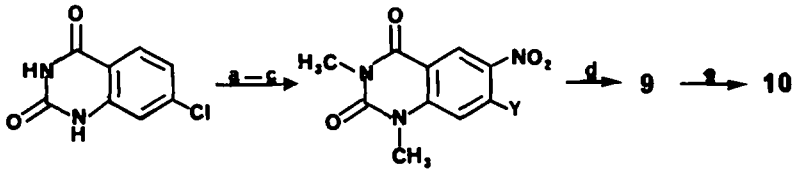
purine derivatives. lin-Benzotheophylline (7) was made by Schneller and Christ[17] by the method shown in Scheme III, starting with 7-chloroquinazoline-2,4(1H,3H)-dione (11). Either the amino or hydrazino

Scheme III

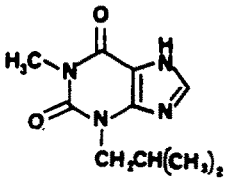


11 12

a, HNO_3, H_2SO_4; b, $(CH_3)_2SO_4$, OH^-; c, NH_2 or N_2H_4; d, Pd/C, H_2, HCOOH; e, $(CH_3)_2SO_4$, OH^-

intermediate 12 (Y = NH_2 or $NHNH_2$) yielded lin-benzotheophylline on catalytic hydrogenation in formic acid. lin-Benzocaffeine (10) was obtained on methylation of 9 along with the 3,5,7-trimethyl isomer.[17] The structure of the latter, and indirectly of lin-benzocaffeine, was established by direct synthesis from 12, Y = CH_2NH. A slight variation in the procedure produced lin-benzo-1-methylxanthine.[16]

lin-Benzotheophylline was also synthesized by the same route (Scheme III) in this laboratory[17] and was tested as a potential phosphodiesterase inhibitor. Theophylline is active[20] and 3-isobutyl-1-methylxanthine (IBMX) (13) is maximally active[21-23] as inhibitors of cyclic nucleotide phosphodiesterases in many tissues, including coronary arteries. They produce relaxation of smooth muscle but increase the levels of both cAMP and cGMP in these tissues. Since several diseases are associated with abnormal metabolism of cyclic nucleotides, the



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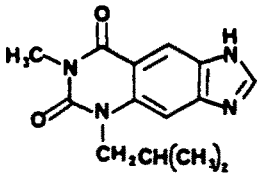


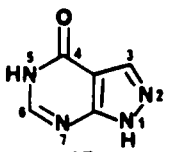
14

possibility exists of altering the course of the diseases with specific phosphodiesterase inhibitors. lin-Benzotheophylline was found to have sufficient activity, of the same order as that of theophylline,[86] to encourage us to synthesize "lin-benzo-IBMX" (14).[87] The synthesis proceeded by elaboration of ethyl 2-amino-4-chloro-5-nitrobenzoate with methyl isocyanate to 7-chloro-3-methyl-6-nitroquinazoline-2,4(1H,3H)-dione, followed by treatment with sodium hydride and isobutyl iodide in dimethylformamide. The resulting 1-isobutyl compound related to 12 with Y = Cl was converted to the desired product, 7-methyl-5-(2-methylpropyl)imidazo[4,5-g]quinazoline-6,8(5H,7H)-dione (lin-benzo-IBMX) (14), which was catalytically hydrogenated in formic acid.[88] In terms of potency of inhibition with three different phosphodiesterase preparations (red blood cells, cat heart, and bovine brain), the lin-benzo-IBMX was more active than theophylline but less active than IBMX (13). In terms of additional desirable behavior, it did not affect cAMP-stimulated protein kinase activity even at a concentration of 10^{-4}M of 14.[89] In an article in press,[90] Schneller, Ibay, Martinson, and Wells report the examination of a series of compounds related to 14 as inhibitors of the separate peak I and peak II forms of cyclic nucleotide phosphodiesterase from pig coronary artery, including the linear benzo-separated analogues of 7-benzyl-3-isobutyl-1-methylxanthine, 3-isobutyl-1,8-dimethylxanthine, 3-isobutyl-8-t-butyl-1-methylxanthine, 3-isobutyl-8-methoxymethyl-1-methylxanthine, and 1-isoamyl-3-isobutylxanthine. While the active sites of both enzyme forms tolerated the stretched-out xanthines, most of the lin-benzo analogues were less effective inhibitors of these phosphodiesterases than IBMX. In one case, however, the lin-benzo analogue of 7-benzyl-IBMX was a more potent inhibitor of peak I activity than IBMX.

lin-Benzoxanthine with 2,3-dimethyl substitution (see 2 for general numbering system) resulted from nitration of methyl 1,2-dimethylbenzimidazole-5-carboxylate followed by reduction and ring closure with urea.[91] The linear, rather than angular, nature of the product was indicated by the lack of o-coupling exhibited by the NMR signals of the benzenoid protons.

In a search for anthelmintic agents effective in ridding the body of parasitic worms, Kumar, Kansal, and Bhaduri[94] expanded the scope of earlier work[97] to include the synthesis of lin-benzohypoxanthine substituted with a carbethoxyamino group on the 2-position, with or without n-butyl on the 7-nitrogen. The methodology was similar to that previously described (Scheme I), but S-methylisothiourea was used for the imidazole ring closure and ethyl chloroformate was used to complete the carbethoxyamino substitution. The anthelmintic activity of these compounds against Hymenolepis nana in mice and Ancylostoma ceylanicum in hamsters has been reported but without obvious comparison with the activity of known anthelmintics.

Closely related in structure to hypoxanthine, the 8-aza-7-deaza analogue, pyrazolo[3,4-d]pyrimidin-4(5H)-one (allopurinol) (15),[98,99] has xanthine oxidase inhibitory properties[100,101] that have led to its use as a major medicinal product for controlling gout and related metabolic disorders.[98]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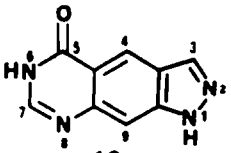
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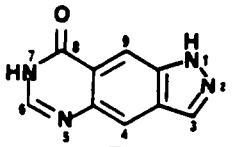
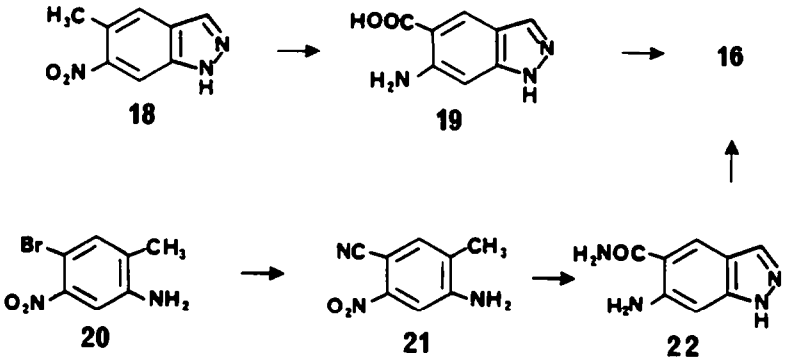

17

The obvious target molecules for synthesis of lin-benzo analogues are lin-benzoallopurinol (16) and lin-benzoisoallopurinol (17) for determination of their substrate or inhibitor activity with xanthine oxidase. The synthesis of lin-benzoallopurinol, pyrazolo[4,3-g]quinazolin-5(6H)-one (16), has been reported independently from two laboratories (Scheme IV).[33-34] The Cuny-Lichtenthaler synthesis[33,35,36] started with 5-methyl-6-nitroindazole (18) and proceeded through appropriate stages of protection, oxidation, esterification, reduction, and hydrolysis, or through variations of this sequence, to 6-aminoindazole-5-carboxylic acid (19), which was then cyclized

Scheme IV



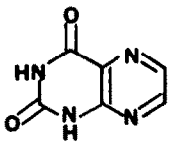
to lin-benzopurinol (16) by heating with formamide. The synthesis from our laboratory[34] started with displacement of the bromine in 4-bromo-2-methyl-5-nitroaniline (20) with cuprous cyanide to 21, followed by ring closure with nitrous acid, reduction, and partial hydrolysis to 6-aminoindazole-5-carboxamide (22), which was then cyclized to 16 by heating in formic acid. Intermediate 19 was also converted to the 7-methoxy, 7-ethoxy, and 7(8H)-keto derivatives of 16. Amination of the latter dione was reported to yield the 7-amino derivative of 16,[37] and this compound could be obtained directly from the intermediate 19 by thermal ring closure with guanidine.[34]

The synthesis of lin-benzoisoallopurinol, pyrazolo[3,4-g]quinazolin-8(7H)-one (17), was effected by starting with the position isomer of 18, namely, 6-methyl-5-nitroindazole, and following a similar sequence to that described for 16 (Scheme IV).[33] Compound 17 is also recognizable as the benzologue of the C-aglycone of formycin B. The synthetic sequence was applied to the construction of the 6(5H)-keto derivative of 17 as well.

lin-Benzoallopurinol (16) was found to be fluorescent, as were the lin-benzoadenine derivatives.[4-8] In ethanol solution, the fluorescence quantum yield for 16 was 0.19, the fluorescence lifetime was 4 ns, and the fluorescence emission maximum was 380 nm upon excitation at 315 nm.[34]

With regard to the activity of lin-benzoallopurinol (16) with xanthine oxidase, with oxygen as the final electron acceptor, we compared the rate of uric acid formation (as an indication of the rate of consumption of hypoxanthine) with the rate of disappearance of lin-benzoallopurinol and found them to be of the same order.[34] The apparent Michaelis constants for the two substrates, similarly measured, were also found to be nearly identical, K_m = 1.6 x 10^{-4} M, and in the range of values reported for hypoxanthine. lin-Benzoallopurinol was readily oxidized to the 7(8H)-keto derivative.[37,38] Our conclusion was that lin-benzoallopurinol served as a substrate for xanthine oxidase essentially indistinguishable from hypoxanthine,[34] while the conclusion from Lichtenthaler's laboratory was that it behaved as a true inhibitor.[35,36] In a carefully controlled experiment, samples of xanthine oxidase were preincubated[39] with allopurinol (15) or lin-benzoallopurinol (16) to compare their behavior (or the behavior of their oxygenated derivatives) as inhibitors with an unincubated sample.[34] The allopurinol, as previously known, reduced the rate of uric acid formation from hypoxanthine - in our experiment by a factor of 500 - whereas the lin-benzoallopurinol did not reduce the rate of uric acid formation significantly. Thus, while allopurinol and its 6(7H)-keto derivative are true inhibitors, lin-benzoallopurinol, as a substrate, can be called more properly a competitive alternative inhibitor.[40] Cuny, Lichtenthaler, and Jahn[36] have reported that the 7-amino derivative of lin-benzoallopurinol has double the inhibitor activity against xanthine oxidase as does allopurinol, making this derivative of interest for further study of its biological activity. Finally, a compound with the lin-benzoallopurinol nucleus and substitution at the 3(CH_3), 4 (piperonyl), and 7 (SH) positions has been described that originated with piperonylidene thiobarbituric acid.[41]

6,6,6-Ring Systems. Folic acid is a hematopoietic vitamin in humans and a dietary requirement in poultry. Schneller's laboratory has undertaken the synthesis of benzo-separated folate compounds for the purpose of defining more completely the active sites of enzymes involved in the metabolism of folic acid.[42] A recalcitrant synthetic problem lies in directing the synthesis so that the unsymmetrical substitution on the terminal ring is properly oriented. More immediate success has been achieved in extending a simpler pteridine ring system, namely, that of lumazine (23). Thus, both lin-benzolumazine (24) and compound 26 were synthesized.[43] The latter may be viewed as an "inside-out" lumichrome (25), the irradiation product of riboflavin. Both 24 and 26



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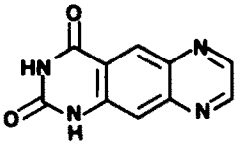

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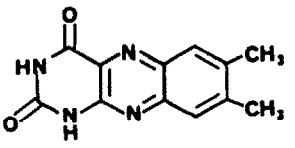

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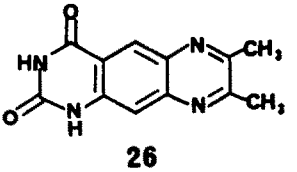

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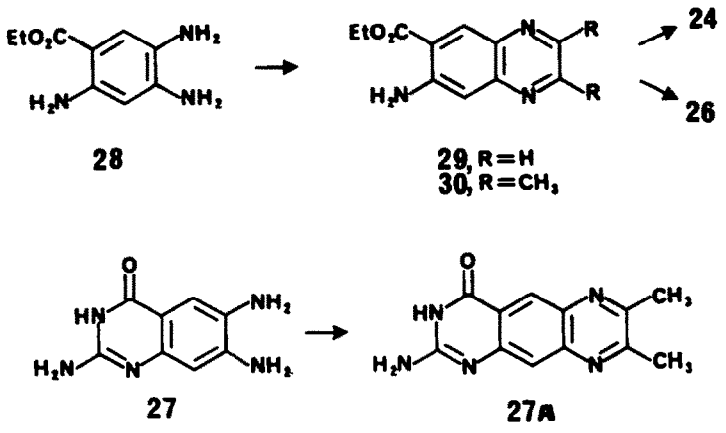
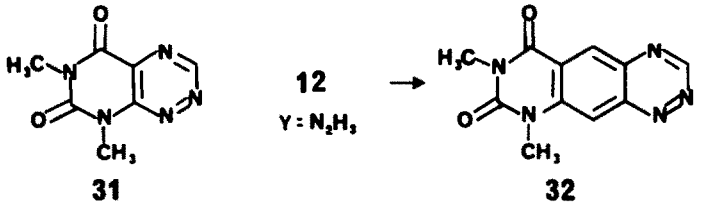
Scheme V



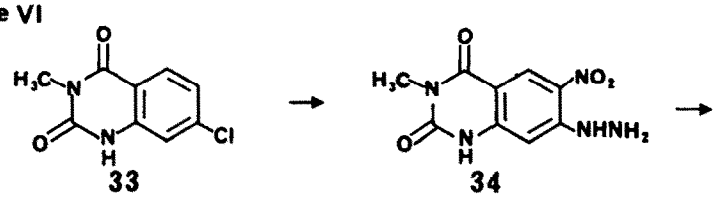
could be obtained by annelation of ethyl 2,4,5-triaminobenzoate (28) hydrochloride by glyoxal and 2,3-butanedione, respectively, followed by reaction of 29 and 30 with urea (Scheme V). Catalytic hydrogenation of 30 yielded a tetrahydroquinoxaline derivative which on reaction with chloroformamidine and oxidation with basic hydrogen peroxide produced the tricyclic system (27a). The structure of 27a was confirmed by independent synthesis from 2,6,7-triaminoquinazoline-4(3H)-one (27) and 2,3-butanedione. The same intermediate (28) had served, without isolation, in our preferred route to lin-benzoguanine.[15] Compound 26 is described as exhibiting yellow-green fluorescence;[20] a neutral aqueous solution of lumazine (23) has a bluish-green fluorescence, and an aqueous solution of lumichrome (25) is blue. A more quantitative, direct comparison of the fluorescence of all four compounds (23-26) would be desirable.

The preferred synthetic route to lin-benzofervenulin (32), the stretched-out analogue of the antibiotic fervenulin (31), involved the conversion of the

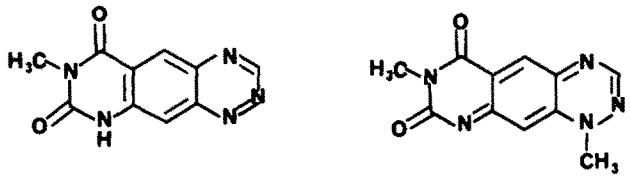


intermediate 12 (Y = $NHNH_2$) to the ethoxymethylene derivative with triethyl orthoformate followed by catalytic hydrogenation with palladium on charcoal.[17] There has been no report of the presence or absence of antibiotic activity for the orange lin-benzofervenulin. The closely related lin-benzoreumycin (35) was made similarly, starting with the reaction of methyl 2-amino-4-chlorobenzoate with methyl isocyanate to give 7-chloro-3-methylquinazoline-2,4(1H,3H)-dione (33, related to 11), followed by nitration and hydrazine displacement to give

Scheme VI



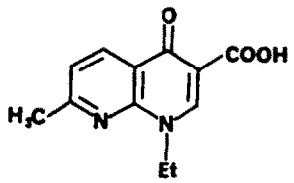
33 34



35 36

7-hydrazino-3-methyl-6-nitroquinazolin-2,4(1H,3H)-dione (34, related to 12), as in Scheme VI.[18] The final conversion to lin-benzoreumycin (35) was effected through the ethoxymethylene derivative as in the case of lin-benzofervenulin (32). An isomer of compound 36, which could be called lin-benzotoxoflavin, thus far has resisted synthesis.[18]

Nalidixic acid (37), which is bactericidal for most of the common gram-negative bacteria that cause urinary tract infections,[19] is the progenitor of thousands of synthetic analogues, some of which have enhanced bactericidal activity, appear insusceptible to plasmid-mediated resistance, and show disproportionately superior activity against chromosomal mutants exhibiting biochemical resistance.[19] These compounds act as inhibitors of bacterial DNA gyrase. lin-Benzo derivatives of nalidixic acid (38) and its congeners have been



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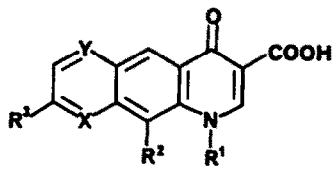


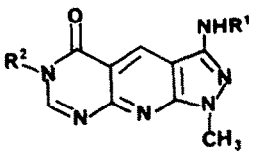
38

R^1	R^2	R^3	X	Y
H	Me	H	N	CH
Et	Me	H	N	CH
$-(CH_2)_3-$		H	N	CH
$-(CH_2)_3-$		H	CH	N
$-MeCHCH_2CH_2-$		H	N	CH
$-MeCHCH_2CH_2-$		Me	N	CH

made by Jordis, Cai, and Sauter,[20] who found that their various synthetic lin-benzo analogues (38) possessed useful fluorescence properties and antibacterial activity at concentrations equal to or greater than the therapeutic level for nalidixic acid. Prior to this work, Tanaka and Nagata[21] had reported a related compound in the lin-benzo series: 38, with R^1 = Et, R^2 = H, R^3 = H, X = CH, Y = N. Details of the syntheses of compounds of type 38 are not yet available, but one major strategy involved blocking the position between the two nitrogen groups with a methyl, trimethylene, or substituted trimethylene bridge to prevent the formation of angular annelation products. The right-hand side quinolinone and the left-hand side quinoline rings were closed by standard methodology.

Heterocyclic Ring Spacer

6,6,5-Ring System. Substituted pyrazolo[3',4':6,5]pyrido[2,3-d]pyrimidines (39) were made about twenty years ago and were discussed on the basis of their relationship to tetrasubstituted pyridines.[46] Substituted purines containing a pyridine spacer (40) have been synthesized more recently.[47] It should be said, however, that if one is seeking to use stretched-out analogues as dimensional



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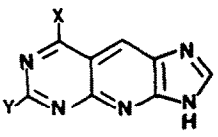
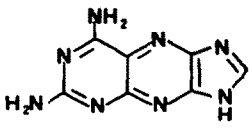


40

probes of binding sites, it is advisable to alter only one parameter at a time, i.e., the dimensions of the substrate or cofactor. Inclusion of the extra nitrogen in the central ring, at the same time as the peripheral rings are being separated by about 2.4 A, introduces an extraneous feature into the binding possibilities available for the extended molecule. Any activity function that is preserved (or reversed) will of course be of interest, but a comparison of relative activities of substrate and pyrido-extended substrate cannot offer as distinct an interpretation as with the lin-benzo analogue. Most of the examples presented in this section are intended to illustrate the synthetic availability of triheterocyclic ring systems that bear a relationship to simpler ring systems exhibiting biological activity of some kind. They cannot be regarded strictly, according to the description provided in the Introduction, as dimensional probes since they represent alteration in electronic and hydrogen-bonding properties as well. Unfortunately, scant information has been provided as to activity of any kind. Correlations, then, are still to be made.

A pyrazino-separated analogue (41) related to 2,6-diaminopurine was obtained by Taylor and Sherman[80] over twenty-five years ago in the course of work on pteridines. More recently, Taylor and Inbaskaren[81] synthesized 1,3-dimethyl-5,7-diaminoimidazo[4,5-b]pyrimido[5,4-e]pyrazin-2(1H)-one (42) and 1,3,6,8-tetramethylimidazo[4,5-b]pyrimido[5,4-e]pyrazin-2,5,7(1H,6H,8H)-trione (43) from a common precursor (44) in glacial acetic acid (Scheme VII). The synthetic methodology is noteworthy on two counts. The first is that the precursor 4,5-diimino-1,3-dimethylimidazolidin-2(1H)-one (44) was obtained by a facile ring contraction, reminiscent of a benzylic acid-type rearrangement, of 1,3-dimethyl-6-imino-5-phenoxyiminouracil (45) on heating in ethanol containing a trace of morpholine or on stirring at room temperature in 5% aqueous sodium hydroxide. The second point is that the diimino compound was highly reactive



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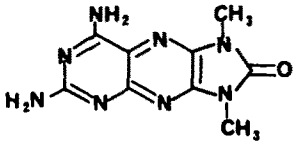

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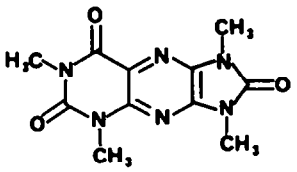

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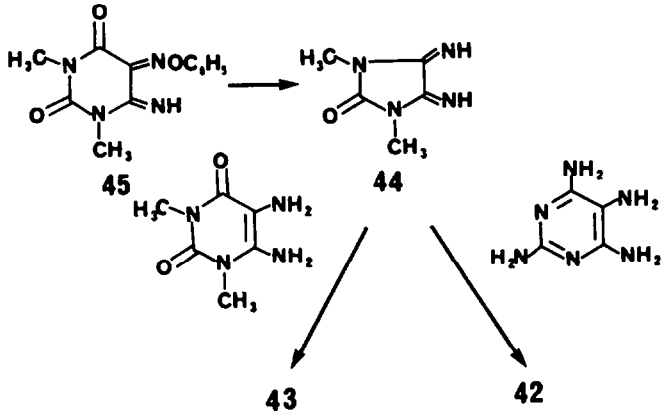
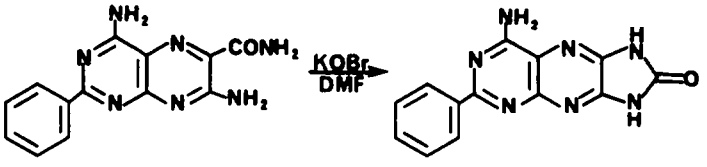
Scheme VII



45 44 43 42

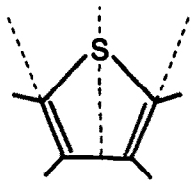
with the representative o-phenylenediamine whereas the corresponding dicarbonyl compound, 1,3-dimethylparabanic acid, failed to react. The authors point out that the comparison between imine and carbonyl reactivity has interesting consequences in other areas of heterocyclic synthesis. The tricyclic compounds 42 and 43 are described as yellow. Compound 43, at least, despite its relative insolubility, might be a reasonable candidate for testing as an inhibitor of cyclic nucleotide phosphodiesterase. In a final example of a purine ring laterally extended by the insertion of a pyrazino ring, the Hofmann rearrangement route to compound 47 from 46 is of interest.[88] Various pteridine



46 47

compounds were being synthesized as diuretics. As we shall see below, 4H-imidazo[4,5-g]pteridines have been found in nature as their D-ribitol derivatives.

6,5,6-Ring System. The thiophene ring is considered to be bioisosteric with the benzene ring, but when it is used as an internal spacer, the peripheral rings are not only stretched out but tilted outwards in a direction dependent upon the location of the sulfur, e.g. 48. Triheterocyclic compounds of interest



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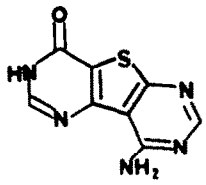

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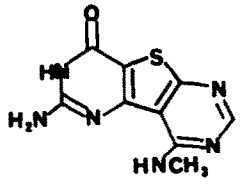
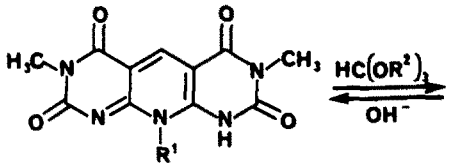


50

here that contain a central thiophene ring are among those made by Clark and Hitiris[53] for potential central nervous system activity and low toxicity. Structures of which 49 and 50 are examples represent a certain duality and have terminal pyrimidine rings like those of the purine nucleic acid bases. They would be good candidates for an analysis of deaminase and oxidase activities inter alia.

6,6,6-Ring System. Triheterocyclic ring systems containing a central pyridine and two terminal pyrimidine rings (51) have been made by the condensation of 6-chloro-5-formyl-3-methyluracil with companion 6-alkylamino-substituted pyrimidines.[54] Compounds of this type are also intermediates in the reaction of triethyl or trimethyl orthoformate with 6-alkylamino-3-methyluracils in dimethylformamide to give the corresponding 8-O-ethyl or methyl products 52.[55] The final conversion of 51 to 52 (R^2 = C_2H_5 or CH_3), which occurs also



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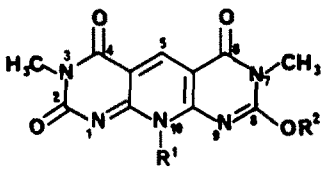

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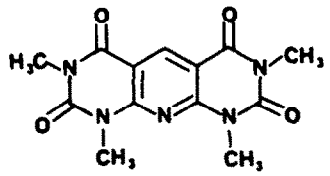

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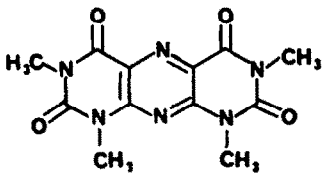
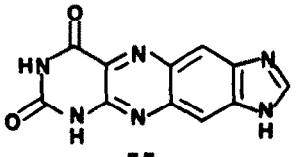


54

from 51 isolated separately, is stated to be the first example of the O-alkylation of a pyrimidine moiety with a trialkyl orthoformate. The structures of this family of compounds were established by X-ray crystallographic analysis of the product of 8-ethoxy displacement by benzylamine in the 10-n-propyl member (52, R^1 = n-Pr, R^2 = Et). Compounds of type 52 are reported to have strong oxidizing ability and a remarkable autorecycling in the oxidation of alcohols.[55,56] Compound 53, with no substituent on N-10,[57] has been made most recently by heating a mixture of 6-amino-1,3-dimethyluracil and 6-amino-1,3-dimethyl-5-formyluracil, product of a Vilsmeier reaction, in tetralin.[58]

The analogue of 53, but with a pyrazine spacer (54), was formed by the thermolysis of 6-azido-1,3-dimethyluracil in formamide,[57] and intermediates along the pathway were tested for ultimate conversion to 54. The isomer having the terminal rings facing in the opposite direction was the product of irradiation of 6-azido-1,3-dimethyluracil in methanol.[60] No direct comparison of any biological activity of these two isomers has been reported.

6,6,6,5- and 6,6,6,6,6-Ring Systems. The product of condensation of 1,2,4,5-benzenetetramine with alloxan (molar ratio 2:1) in 2N HCl was heated in formic acid and concentrated HCl to yield the extended ring system 55.[41] The imidazo[4,5-]alloxazine has a reduction potential of -0.082 V in comparison



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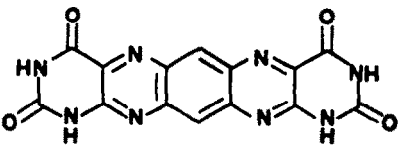
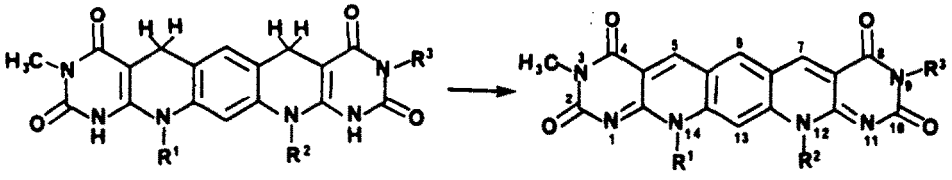


56

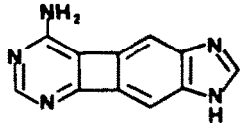
with alloxazine at -0.252 V relative to the SCE.[42] UV absorption maxima were observed in absolute ethanol-dimethylformamide at 270 and 375 nm. Compound 55 may also be regarded as a xanthine in which the rings are separated by a quinoxaline unit, similar in width to the naphthalene unit discussed below. The fluorescent product formed, when the molar ratio of 1,2,4,5-benzenetetramine to alloxan was 1:3 in the initial condensation, was presumed to be 56, a kind of benzene-separated double-pteridine system, with UV maxima at 285, 390, and 420 nm.[41] Similar pentacyclic molecules more closely related to 5-deazaflavins (5-deazaisoalloxazines) at each end have been prepared by a route through the partially reduced ring system 57, which could be oxidized by diethyl azodicarboxylate to 1,3,9,11,12,14-hexaazapentacene-2,4,8,10(1H,3H,9H,11H)-tetraones (58).[43] The NMR chemical shifts for the 5- and 7-protons for the 58 series of compounds were 10 ppm or greater. Their redox potentials at 25 °C in dimethylformamide with $LiClO_4$ were ca. -0.590 V vs. the Ag/AgCl electrode for the first one-electron transfer compared with the values of ca. -1.13 V for the monomeric 5-deazaflavins. The double-headed 5-deazaflavin compounds were effective for oxidizing secondary alcohols at raised temperature in the absence of a base and at low temperature (10-15 °C) in the presence of sunlight. Autorecycling oxidation occurs under these conditions, with 50-180 mols of the representative alcohol, cyclopentanol, being oxidized in 24 hours per mol of one of the compounds of type 58, according to the findings of Yoneda, Kuroda, Koga, and Ibuka.[43]



57 58

Benzocyclobutadiene Ring Spacer

We have been attempting to synthesize the linear benzocyclobutadieno-extended adenine analogue 59, lin-bcb-adenine, because the spacer is intermediate in width (3.9 A) between the benzene in lin-benzoadenine (2) (2.4 A)



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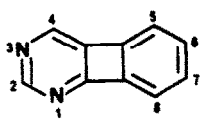

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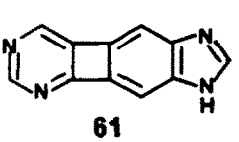
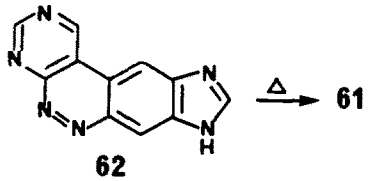


61

and the naphthalene in lin-naphthoadenine (4.8 Å) discussed below. The three different widths of spacers between the constant terminal rings will hopefully permit fine-tuning of the dimensional restrictions of certain enzyme-coenzyme interactions.[4-7] When we learned that the primary structural feature of 59, namely, the 1,3-diazabiphenylene ring (60), was unknown, we developed two independent syntheses, the first involving thermal extrusion of nitrogen from a condensed pyridazine precursor and the second utilizing a diethynylpyrimidine in a cobalt-catalyzed co-oligomerization reaction.[44,45] However, the pilot 4-chloro-1,3-diazabiphenylene underwent such facile transformations to isoquinolines upon treatment with either acid or base that we were diverted to an examination of the mechanistic pathways for the unusual rearrangements. We have postulated the intermediacy of 1,3-benzodiazocines between a pair of electrocyclic reactions, followed by small-molecule extrusion.[44] We have also examined the behavior of two monoazabiphenylenes, 4-methoxy-1-azabiphenylene and 1-methoxy-2-azabiphenylene, with methanolic sodium methoxide and we found that their equally unusual rearrangement products could be rationalized by consideration of the locus of methanol addition and sequential electrocyclic reactions.[44] Nevertheless, the mechanistic precursor-product study remained a diversion while the synthesis of a lin-bcb-purine constituted the challenge. Two distinct syntheses of the pyrimido[6,5-i]imidazo[4,5-g]cinnoline ring system (62) have been accomplished.[47] The first of these began with 2-acetamido-4-chloro-5-nitroacetophenone, which was elaborated by fusion, in sequence, of the



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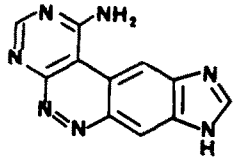
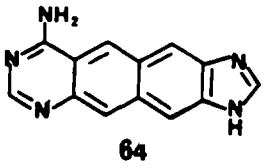


63

imidazole, pyridazine, and pyrimidine rings to provide the tetracyclic system 62 lacking peripheral substitution. The second synthesis, which utilized a palladium-catalyzed cross-coupling reaction of 4,6-dimethoxypyrimidin-5-yl zinc chloride and 3,4-dinitrobromobenzene, followed by closure of the imidazole and pyridazine rings, resulted in 10-aminopyrimido[6,5-i]imidazo[4,5-g]cinnoline (63). This compound, by a stretch of the ring system and of the imagination, is an angular analogue of adenine with a cinnoline spacer. Pyrolysis of 63 at 800 °C appeared to lead to the extrusion of HCN rather than N_2. By contrast, flash vacuum pyrolysis (810-860 °C and 10^{-3} torr) of compound 62 resulted in the extrusion of nitrogen to provide imidazo[4,5-h]-1,3-diazabiphenylene (61), the first representative of a new class of linearly extended purine analogues.

Naphthalene Ring Spacer

The synthesis of lin-naphthoadenine (9-amino-3H-benzimidazo[5,6-g]-quinazoline) (64),[48] lin-naphthoxanthine (65),[37] and lin-naphthohypoxanthine (66)[37] required the construction of the requisite tetra-ß-substituted naphthalene intermediates. We developed methodologies that insured the linear array of the four contiguous rings. Along one route, this included cobalt-catalyzed cyclotrimerization of acetylenic components and generation of Diels-Alder o-xylylene intermediates thermally.[37] Along the other route,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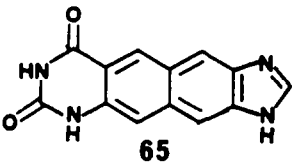
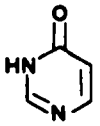

65

Diels–Alder *o*-xylylene intermediates were generated under Finkelstein reaction conditions.[46] In both routes, stepwise conversions turned a terminal anhydride, N-hydroxyimide, or isatoic anhydride into a suitably substituted pyrimidine ring. Whereas *lin*-benzoadenine (2) was deaminated with adenosine deaminase, *lin*-naphthoadenine (64) was not a substrate. The conclusion was reached that a lateral extension of 4.8 A is too great for a satisfactory fit at the enzyme site, whereas a 2.4-A extension is well tolerated, while adenine itself is not a substrate.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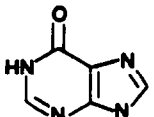
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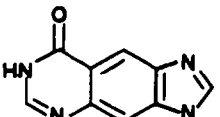
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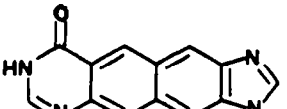

66

In the case of substrates for xanthine oxidase, it is now possible to compare an entire series of dimensionally related analogues of hypoxanthine (66–69). It was shown earlier by Krenitsky, Neil, Elion, and Hitchings,[47] with xanthine oxidase and with ferricyanide as the ultimate electron acceptor, that 4-hydroxypyrimidine (shown as keto form, 67) is oxidized just as rapidly as hypoxanthine (68). In a direct comparison of rates of oxidation of hypoxanthine, *lin*-benzohypoxanthine (69),[18] and *lin*-naphthohypoxanthine (66)[37] with xanthine oxidase and air, we found that the V_{max} values increase in the order 68 < 69 < 66. The actual values observed were 4.15×10^{-4}, 1.78×10^{-3}, and 5.21×10^{-4} mol min^{-1} (mg of protein)$^{-1}$, respectively. The K_m values for *lin*-benzohypoxanthine and *lin*-naphthohypoxanthine were similar to each other (1.57×10^{-5} and 1.01×10^{-5} mol L^{-1}) and about an order higher in concentration than the K_m value for hypoxanthine. Two differences in behavior appear at the *lin*-naphthohypoxanthine level of extension. It functions as a competitive inhibitor of the oxidation of hypoxanthine with xanthine oxidase (K_i 3.04×10^{-4} mol L^{-1}) and it is oxidized only at the pyrimidine carbon. Hypoxanthine and *lin*-benzohypoxanthine are oxidized first at the pyrimidine carbon and then at the imidazole carbon.[18] We can conclude that the ability of buttermilk xanthine oxidase to produce *lin*-benzouric acid (oxidation at each end of the molecule) and not *lin*-naphthouric acid requires that the binding pocket at an oxidation site on or within the enzyme be unable to accommodate the larger lateral extension of the naphthalene derivatives necessary for oxidation in the imidazole ring.[37]

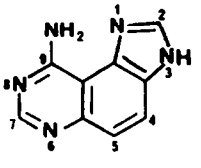
lin-Naphthoadenine (64), *lin*-naphthohypoxanthine (66), and *lin*-naphthoxanthine (65) are brilliantly fluorescent, exhibiting high fluorescence quantum yields and long lifetimes in purged ethanol: 64, Φ 0.57; τ 20.5 nsec; 66, Φ

0.70, τ 24.8 nsec; 65, Φ 0.88, τ 33.1 nsec. In cases where the lin-naphtho analogues exhibit activity or inhibition in specific enzyme systems, their uses as fluorescent probes will complement those of the lin-benzo analogues.[5,67] Larger spacer units such as biphenylene (6.3 Å) and anthracene (7.2 Å) can be envisaged between the pyrimidine and imidazole rings of the purines, but predictable water insolubility and probable lack of activity with the usual enzymes are deterrents to the synthesis of such over-extended analogues.

ANGULAR HETEROCYCLIC BASES

Benzene Ring Spacer

6,6,5-Ring Systems. Similar to lin-benzoadenine (2) in containing the binding sites analogous to the 1,N⁶ binding sites found in adenine and related nucleosides and nucleotides are prox-benzoadenine (70) and dist-benzoadenine (71). In the abbreviated nomenclature, prox for proximal and dist for distal refer to the spatial relationship of the amino groups in the respective compounds to the imidazole ring. The rings are angulated within a common plane. The term benzo presents no ambiguity since only when the ring is central is it devoid of nitrogen and accordingly "benzo". The structural differences among 2, 70, and 71 reside in the spatial relationships of the pyrimidine and imidazole



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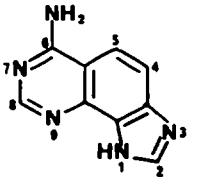
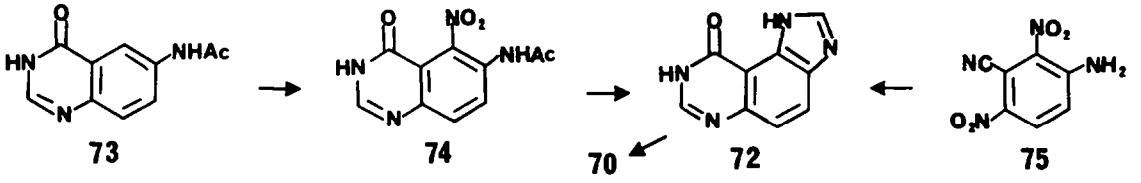


71

Scheme VIII



73 74 70 72 75

rings with respect to the central benzene ring and in the nature of the internal hydrogen bonding likely to occur in isomers 70 and 71. They are not actually dimensional probes according to the description in the Introduction since they also involve orientational changes. Our original synthesis of prox-benzoadenine (Scheme VIII) was approached through imidazo[4,5-f]quinazolin-9(8H)-one, prox-benzohypoxanthine (72). Nitration of 6-acetamido-quinazolin-4(3H)-one (73) gave 6-acetamido-5-nitroquinazolin-4(3H)-one (74), acid hydrolysis removed the acetyl group, and catalytic hydrogenation over palladium on carbon in formic acid closed the imidazole ring.[70] A more recent synthesis of prox-benzohypo- xanthine from Schneller's laboratory,[71] which starts with a tetrasubstituted benzene, 3-amino-2,6-dinitrobenzonitrile (75), compresses the steps of terminal ring cylizations to catalytic hydrogenation over palladium on carbon in formic acid, reflux in formic acid, reflux in formic acid-toluene, and treatment with ammonia in methanol under pressure.

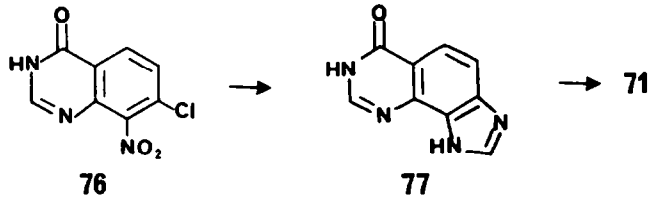
prox-Benzohypoxanthine was oxidized with buttermilk xanthine oxidase and air in the pyrimidine ring and in the imidazole ring, but two orders of magnitude more slowly than hypoxanthine and lin-benzohypoxanthine were oxidized under the

same conditions.[18] The relatively slow oxidation of prox-benzohypoxanthine is a probable consequence of the steric shielding of the carbonyl by the adjacent imidazole ring or by hydrogen bonding through the N-1 hydrogen. prox-Benzohypoxanthine was not converted to its ribotide when incubated with intact human erythrocytes or with 5-phosphoribosyl-1-pyrophosphate and hypoxanthine-guanine phosphoribosyl transferase (HGPRT), nor did it block the synthesis of GMP or IMP from guanine or hypoxanthine in the enzyme preparation.[71] prox-Benzohypoxanthines with 8-n-butyl substitution and with or without 2-methyl substitution have been made as part of a broad search for anthelmintic agents.[84]

Thiation of prox-benzohypoxanthine with phosphorus pentasulfide in pyridine yielded imidazo[4,5-f]quinazolin-9(8H)-thione.[70] This compound was found to inhibit plaques of Herpes virus but led to no prolongation of life in Herpes-infected animals. The thione was convertible to prox-benzoadenine (70) either by heating with ammonia in butanol or by treatment with hydrazine followed by hydrogenolysis. prox-Benzoadenine did not react with adenosine deaminase under conditions that converted lin-benzoadenine to lin-benzohypoxanthine.[18]

When 7-chloroquinazolin-4(3H)-one (1) was nitrated,[9] the minor mononitro product, 7-chloro-8-nitroquinazolin-4(3H)-one (76) was carried through a sequence (Scheme IX) similar to that abbreviated in Scheme I for the 6-nitro isomer. This sequence proceeded through dist-benzohypoxanthine (77) to dist-benzoadenine (71).[70] dist-Benzohypoxanthine was oxidized with xanthine

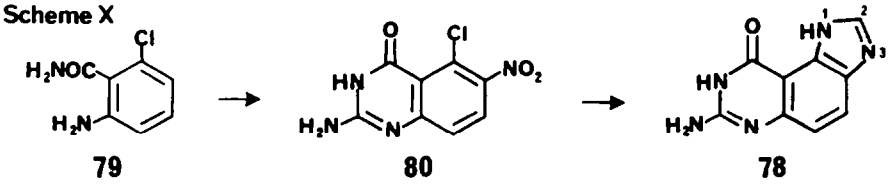
Scheme IX



oxidase as rapidly as hypoxanthine and lin-benzohypoxanthine, but only to the monooxidized stage.[9] The structure of the single oxidized product was established by NMR spectroscopy, including deuterium-exchange identification of protons, as dist-benzoxanthine, with the imidazole ring intact.[18] dist-Benzoadenine (71), like prox-benzoadenine, did not react with adenosine deaminase.

For the synthesis of prox-benzoguanine (78), Schneller et al. utilized chloroformamidine hydrochloride in annelation of the pyrimidine ring with the correct substitution pattern.[71] After ring closure of 2-amino-6-chlorobenzamide (79) with chloroformamidine hydrochloride, nitration gave 2-amino-5-chloro-6-nitroquinazolin-4(3H)-one (80) (Scheme X). Amination of compound 80 produced 2,5-diamino-6-nitroquinazolin-4(3H)-one which could be converted to 7-amino-

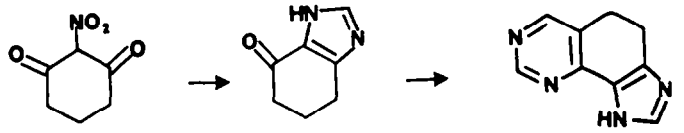
Scheme X



imidazo[4,5-f]quinazolin-9(8H)-one, prox-benzoguanine (78), in the usual

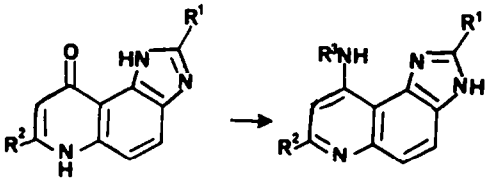
sequence for imidazole ring formation. The locus of direct methylation of prox-benzoguanine was determined as N3 by independent synthesis of the separate 1- and 3-methyl isomers of 78. prox-Benzoguanine (78), like prox-benzohypoxanthine (72), was neither a substrate nor an inhibitor of the purine salvage enzyme HGPRT.[71]

A novel approach to the distal system involved reduction of 2-nitro-1,3-cyclohexanedione (81) in formic-acetic anhydride followed by prolonged reflux in a mixture of formic acid and formamide (→ 82 → 83). Dihydro-dist-benzopurine (83) was isolated as a minor component of a complex mixture.[72]



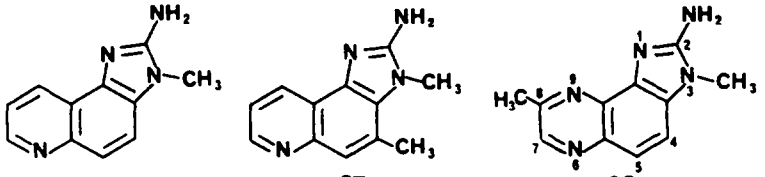
81 82 83

Closely related to the prox-benzoadenine system 70 is the 9-aminoimidazo-[4,5-f]quinoline angular tricyclic system with a benzene spacer. Substituted compounds of type 84, prepared by cyclization of the corresponding benzimidazolylaminocrotonates, were treated with phosphorus oxychloride followed by an amine to yield substituted compounds of type 85 that were classed as anthelmintics.[73-74]



84 85

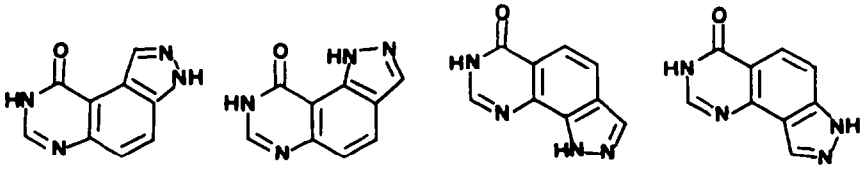
Compounds containing the same ring skeleton include the mutagen 2-amino-3-methylimidazo[4,5-f]quinoline (86), which has been found to be present in broiled fish,[75-76] heated beef extract,[77] and fried beef.[80] The 4-methyl-



86 87 88

substituted compound (87) related to 86 has also been isolated from broiled fish, and the closely related 2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline (88) is another potent mutagen isolated from fried beef.[81] The structures of these compounds were established by straightforward unequivocal syntheses.

Of the four possible angular benzologs of allopurinol, prox-benzoallopurinol (89), prox-benzoisoallopurinol (90), dist-benzoallopurinol (91) and dist-benzoisoallopurinol (92), three have been synthesized (89, 90, 92) and one has

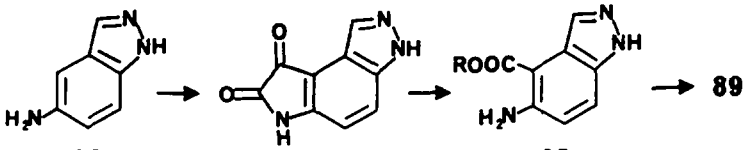


89 90 91 92

been listed in a patent claim (91).[33] The two compounds that deserve through structural analogy to be related by name to allopurinol (15) are 89 and 91. The juxtaposition of the pyrazole nitrogens to the pyrimidinone carbonyl in 90 has led us to name this prox-benzoisoallopurinol[88] instead of "prox-benzoallopurinol" which has appeared in the literature[33,35,38] without regard to the lack of exact structural analogy to allopurinol. Anyone who refers to the original literature may be confused since the abbreviated names of the compounds do not conform to our precise designations, but the systematic chemical names will serve as satisfactory guides.[34,89]

prox-Benzoallopurinol (89) was synthesized by Cuny, Lichtenthaler, and Jahn[34] in a five-step sequence starting with 5-aminoindazole (93) (Scheme XI).

Scheme XI



93 94 95

The isatin intermediate 94 was obtained by using the Sandmeyer procedure,[84] that is, chloral hydrate and hydroxylamine to give the 5-(isonitrosoacetamido)-indazole followed by acid ring closure. Oxidation of 94 with hydrogen peroxide followed by treatment with diazomethane furnished 95, which was fused with formamide in the Niementowski reaction[85] to yield pyrazolo[4,3-f]quinazolin-9(8H)-one, or prox-benzoallopurinol (89).

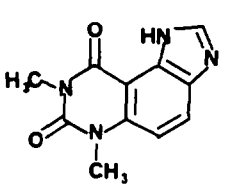
dist-Benzoallopurinol (91) has been mentioned[33,34] but not described. The compounds which we call prox-benzoisoallopurinol (90) and dist-benzo-isoallopurinol (92) are also recognizable as angular benzologs of the C-aglycone of formycin B. Pyrazolo[3,4-f]quinazolin-9(8H)-one, or prox-benzoisoallopurinol (90), was synthesized in Lichtenthaler's laboratory[33,35,38] and our laboratory[88] by two essentially identical routes. One route started with 6-aminoindazole to close the pyrimidinone ring as in the synthesis of 89 from 5-aminoindazole. The direct ring closure of the intermediate 6-aminoindazole-7-carboxylic acid by heating with formamidine acetate in ethanol[84] has recently been found to avoid the decarboxylation that accompanied its fusion with formamide.[88] The other route to 90 started with 6-methylquinazolin-4(3H)-one and involved familiar steps of nitration, reduction, diazotization, and internal azo coupling. The convergence of these routes from a known indazole in one case and a known quin-azoline in the other provided unequivocal proof of structure.

The synthesis described for dist-benzoisoallopurinol, pyrazolo[3,4-h]quin-azolin-6(7H)-one (92), was similar in sequence to those in Schemes IV and XI.[88]

The product of oxidation of prox-benzoisoallopurinol (90) with xanthine oxidase was identified by independent synthesis as pyrazolo[3,4-f]quinazolin-7,9(6H,8H)-dione.[88] Like lin-benzoallopurinol (16), this substrate was found to be a competitive alternative inhibitor[87] of xanthine oxidase.[87,88] Preincuba-tion of xanthine oxidase with prox-benzoisoallopurinol permitted the oxidation of hypoxanthine at the same rate as the untreated enzyme; similar preincubation of xanthine oxidase with allopurinol (15) caused the enzyme to lose nearly all its catalytic ability. Thus, prox-benzoisoallopurinol (90) and lin-benzo-allopurinol (16) do not inhibit xanthine oxidase by the powerful secondary

mechanism of allopurinol and are inhibitors only in concentrations comparable to their K_m's. While they are relatively unpromising as xanthine oxidase inhibitors, it is clear that angular and linear isomers can be accommodated satisfactorily at the active site of xanthine oxidase and hence are informative spatial probes.[88]

Other Ring Systems. Employing chemistry similar to that used in the lin-benzo series, Schneller and Christ[17,88] have used starting isomers that resulted in angular products.[89] The compounds synthesized include prox-benzoxanthine



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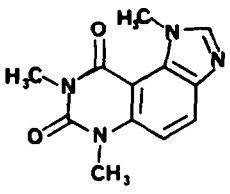

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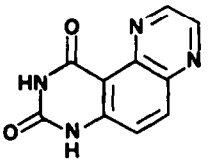

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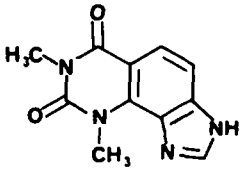

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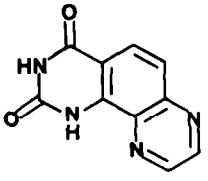
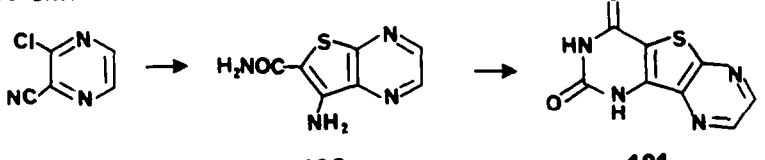


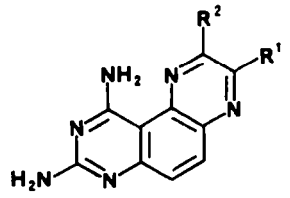
100

(see earlier), prox-benzotheophylline (96), prox-benzocaffeine (97), prox-benzolumazine (98) (6,6,6-ring system), dist-benzotheophylline (99) and dist-benzolumazine (100) (6,6,6-ring system). Thiophene analogues of the dist-benzolumazine type (6,5,6-ring system) have also been made by Schneller and Christ,[88] of which 101 is representative. Treatment of 3-chloropyrazine-2-carbonitrile (102) with 2-mercaptoacetamide led to 103, which was ring-closed with ethyl chloroformate to furnish the dist-thieno analogue 101 (Scheme XII). Compounds related to 100, of the class represented by 104, can be regarded as substituted prox-benzopteridines. In a research program investigating possible folate antagonists, Johnson, Elslager, and Werbel[90] synthesized compounds of this type, two of which ($R^1 = R^2 = CH_3$, and R^1 or $R^2 = CH_3$ or C_6H_5) were active in vitro against Streptococcus faecalis at concentrations below 2.5 μg/ml. The key intermediate in the synthesis was 2,4,5,6-tetraminoquinazoline, which was condensed with representative 1,2-diketones to elaborate the pyrazine ring.

Scheme XII



102 103 101



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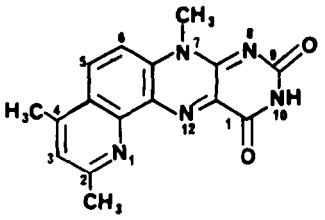

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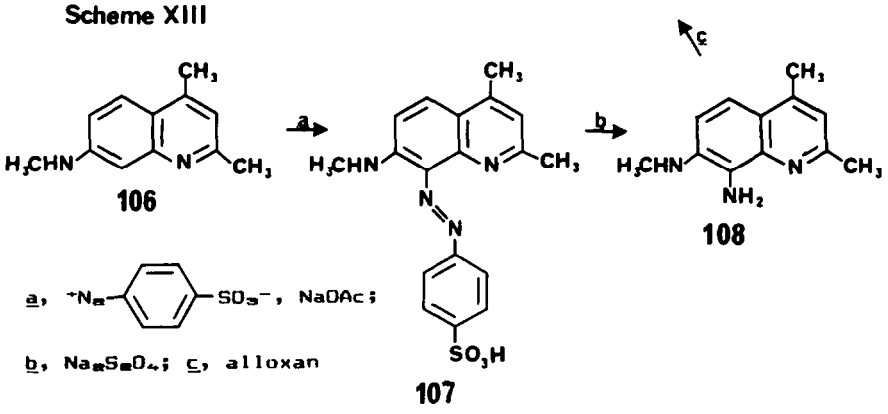
Scheme XIII



106 107 108

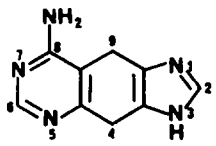
An extended, angular flavin analogue (105) has been synthesized by Shinkai, Ishikawa, and Manabe[91] that shows metal-coordinative ability. 2,4,7-Trimethylquino[8,7-g]pteridine-9,11(7H,10H)-dione (105) was made by the sequence shown in Scheme XIII (106 → 107 → 108 → 105). Complexed metal ions were capable of activating 105 as an oxidizing agent for dihydropyridine derivatives, phenylhydrazine, and L-ascorbic acid. Complexes with Zr^{4+} and Fe^{3+} oxidized primary and secondary alcohols and hydroquinone at ambient temperature. The tetrahedral coordination metal ions Cd^{2+}, Zn^{2+} and Co^{2+} gave the bent semiquinone radical of 105. The findings suggest that the metal ion complexes may be useful not only as model systems for the metalloflavoproteins but also as *in vitro* oxidation catalysts.

BENT HETEROCYCLIC BASES

Dihydrobenzene Ring Spacer

The 4,9-dihydro derivative (109) of *lin*-benzoadenine (2), the biochemically active analogue of adenine, should give a different type of information. Its bent (out of the plane) structure poses the question as to whether the contributing terminal pyrimidine and imidazole rings of adenine must be coplanar, especially in those systems where the reactivity of 2 has shown that they need not be contiguous. The 4,9-dihydro-*lin*-benzopurines bear the same relation to the corresponding *lin*-benzopurines that dihydroanthracene, with a 148° angle between the planes of the terminal rings, bears to anthracene. We have made the first representatives of the bent dihydro-*lin*-benzopurines, namely, 4,9-dihydroimidazo[4,5-g]quinazoline-2,8(1H,7H)-dione (110) and 4,9-dihydro-*lin*-benzouric acid (111) through the cycloaddition route shown in Scheme XIV.[94]

1,3-Diacetyl-4,5-dimethylimidazolin-2(1H)-one (112) was converted to the dibromo compound 113 with two equivalents of N-bromosuccinimide and free radical initiation, and 113 was treated directly under Finkelstein reaction conditions with sodium iodide and di-*t*-butyl acetylenedicarboxylate in dimethylformamide. The Diels-Alder cycloaddition product 114 was converted to the anhydride 115 by



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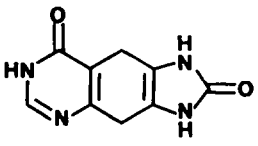

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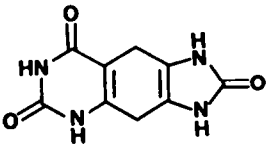
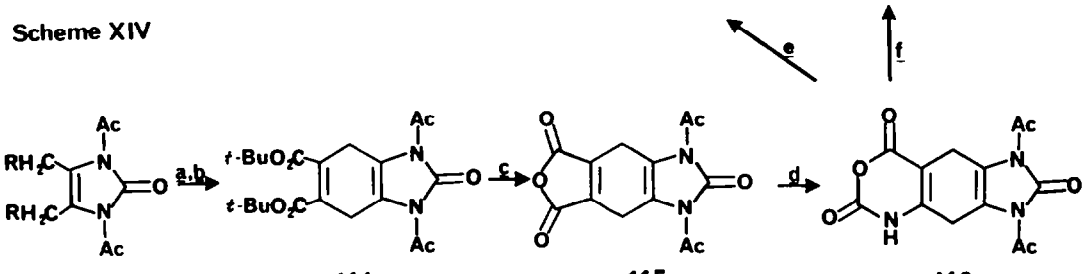


111

Scheme XIV



112, R = H
113, R = Br

114 115 116

a, NBS; b, NaI, DMF, t-BuOOC-C≡C-COOt-Bu; c, TsOH, toluene; d, $(CH_3)_3SiN_3$, CH_3CN; e, formamidine acetate; f, H_2NCONH_2

the simple expedient of heating 114 in benzene or toluene with a catalytic amount of p-toluenesulfonic acid. The Curtius rearrangement of 115 to the substituted dihydroisatoic anhydride 116 was effected with trimethylsilyl azide in refluxing acetonitrile. Compound 116 was the precursor of 110 by treatment with formamidine acetate in N-methylpyrrolidone in the absence of air and of 111, by brief melting with urea. Activity of 110 and 111 with xanthine oxidase, or lack thereof, is not available as yet.

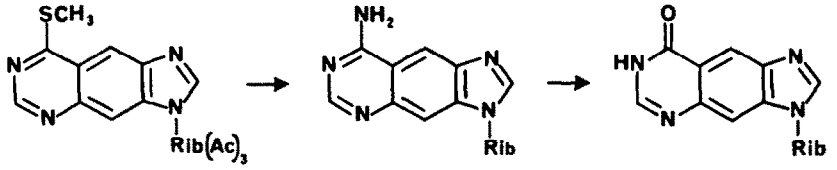
RIBOSYL DERIVATIVES

It will be recognized from the lengthy section that has preceded the discussion of ribosyl derivatives that major effort has been concentrated on the synthesis of new heterocyclic nuclei that are extended versions, linear or angular, of compounds possessing biological activity. As a class, the N-ribosyl derivatives of the nucleic acid bases and their analogues serve as enzyme substrates and are synthetic precursors of the broader general category of ribosyl 5'-mono-, di-, and triphosphates, including coenzymes (see below). For the establishment of the structures of N-ribosidated heterocycles, a number of avenues are open. First, the ribosyl, i.e., ß-D-ribofuranosyl, group may be in place already on the desired nitrogen when the analogue is elaborated from a naturally occurring ribonucleoside. This strategy is not always feasible. Second, alkylation on the new heterocycle may be used to scout the favored locus of ribosidation, along with unequivocal syntheses to determine the differences in properties among the various N-alkylated and N-ribosidated products. This classical procedure faces difficulties when there are many possible loci of alkylation/ribosidation and when syntheses of authentic models are uncharted or straightforward but time-consuming. We have introduced a third method that offers some simplicity and does not depend upon the synthesis of several isomers.[9a] It is a positive approach that depends upon ^{15}N-^{13}C spin-spin coupling in ^{13}C NMR for determination of the site of alkylation/ribosidation of nitrogen heterocycles. Most heterocyclic syntheses involve nitration or reaction with ammonia to introduce nitrogen into a developing ring system. Once

the heterocyclic synthesis is worked out, one can merely substitute $H^{15}NO_3$ or $^{15}NH_3$ at the appropriate stage. In the final ^{15}N-labelled, alkylated or ribosidated product, the ^{13}C NMR spectrum will reveal ^{15}N-^{13}C coupling for the carbon on the labelled N position, but only on that position. We illustrated this procedure with the confirmation of the structures of the benzylated and ribosidated products originating from 8-methylthioimidazo[4,5-*g*]quinazoline. This compound (3 in Scheme II, see earlier) was selected for ribosidation because the methylthio group, which would subsequently be replaceable by ammonolysis, deactivates the pyrimidine and favors benzylation and ribosidation on the imidazole ring. The ultraviolet spectra of the two separated 1- and 3-benzyl derivatives of 3 (see 2 for the numbering system) were distinctive[7] and matched the two ribosyl derivatives formed when compound 3 was treated with 2,3,5-tri-*O*-acetylribofuranosyl bromide and mercuric cyanide in anhydrous nitromethane.[18] The establishment of the identity and structure of 3-benzyl-8-methylthioimidazo-[4,5-*g*]quinazoline confirmed the structure of the other product of benzylation and differentiated between the structures of the two ribosyl derivatives (as the triacetates) obtained from 3. Synthesis of [1-^{15}N]-8-methylthioimidazo[4,5-*g*]-quinazoline and NMR spectral studies of the benzylated products verified the fact that benzylation of 3 occurs at the 1 position as well as the 3 position.[7a] Attachment at N-1 was shown by the ^{15}N-^{13}C coupling of 8.6 Hz in the ^{13}C NMR spectrum of the second benzyl isomer and by the ^{15}N-$C_{(8)}$-H coupling of 1.2 Hz in the ^1H NMR spectrum.[7a] Such relationships are general and definitive.

6,6,5-Ring Systems. Treatment of the compound verified as 8-methylthio-3-(β-*D*-tri-*O*-acetylribofuranosyl)imidazo[4,5-*g*]quinazoline (117) with ethanolic ammonia at 150 °C effected concomitant deblocking of the sugar groups and displacement of methylthio by amino (Scheme XV) to afford *lin*-benzoadenosine (118), or 3-β-*D*-ribofuranosyl-*lin*-benzoadenine.[18] Assignment of

Scheme XV



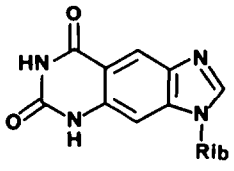
117 118 119

the β configuration was based on the ^1H NMR chemical shift difference observed for the methyl groups in the 2',3'-*O*-isopropylidene derivative of 118 and application of the correlations of Imbach and his coworkers.[7a,7d] The pK_a for *lin*-benzoadenosine was found to be 5.6 in water at 20 °C and 5.3 in 66% DMF, compared with the parallel figures for adenosine of 3.5 and 3.0. Thus, comparison of activities of an adenosine-related substrate with a *lin*-benzoadenosine-related substrate in enzyme systems should be run on the alkaline side where possible in order to ensure that both are unprotonated. The observed fluorescence lifetime of *lin*-benzoadenosine (3.7 nsec) and the fluorescence quantum yield (0.44) were constant for water, ethanol and dioxane.[18]

For the benzoadenine derivatives to be useful as fluorescent probes as well as dimensional probes for the study of biological activity, it was recognized as important that they be accepted as substrates or cofactors by a variety of enzymes. We found that *lin*-benzoadenosine (118) is converted to *lin*-

benzoinosine (119) by adenosine deaminase from calf intestinal mucosa at 85% of the V_{max} of the natural substrate adenosine, with a K_m value of the same order as that of adenosine.[18] Thus, the enzyme can accept a substrate of larger size, i.e., laterally extended by 2.4 A, than the normal substrate. The conformation of the ribosyl attachment is probably favored anti as in the case of adenosine, so that other features are unaltered. The condition that N-1 of lin-benzoadenosine is unsubstituted matches the substrate requirement of N-7 being unsubstituted in adenosine. The isomer of 118, 1-β-D-ribofuranosyl-lin-benzoadenine, which is the "stretched-out" analogue of 7-β-D-ribofuranosyl-adenine, a non-substrate, gave no detectable reaction with adenosine deaminase. The product of enzymatic deamination of 118, lin-benzoinosine (119), was also obtained by an independent synthetic route in this laboratory.[14] When lin-benzoadenosine was tested for possible inhibitory activity of ADP-induced human platelet aggregation, it was without effect at 1.42×10^{-4} M and gave 25% inhibition at 2.84×10^{-4} M against induction of aggregation by 8.9×10^{-4} M ADP. This behavior corresponds to a low order of inhibition.

Since inosine is not oxidized by xanthine oxidase, the enzymatic oxidation of lin-benzoinosine (119) to lin-benzoxanthosine (120) to the riboside of lin-benzouric acid (121) constituted a particularly unusual finding.[18] The relative rate of reaction of lin-benzoinosine with xanthine oxidase was at



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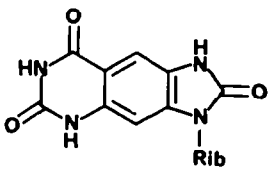


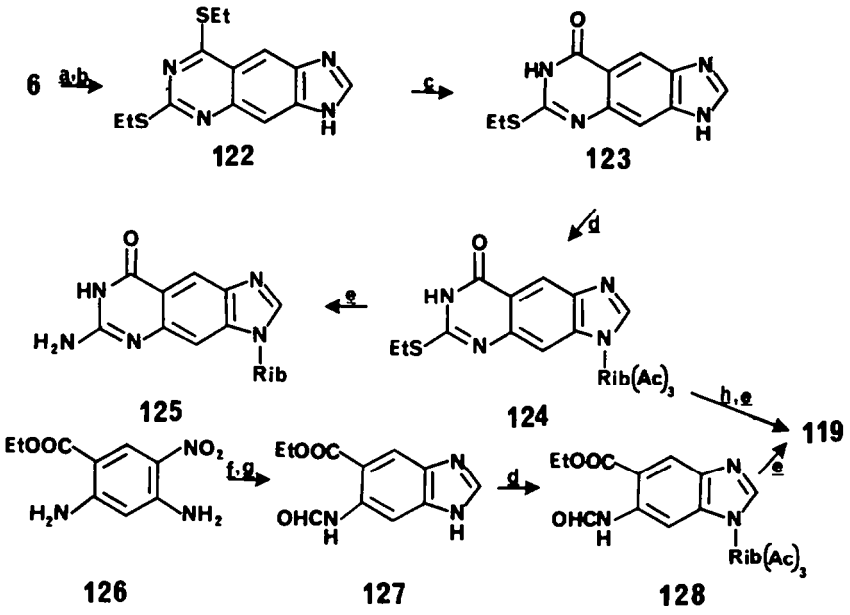
121

least as fast as that of hypoxanthine, the normal substrate. We concluded that when the apparently unfavorable steric interaction of the hydrophilic ribosyl group in inosine is displaced 2.4 A by the benzene-ring spacer in lin-benzoinosine, the possibilities of binding in the hydrophobic pocket of the enzyme and of oxidation are restored. By contrast, nucleoside phosphorylase, an enzyme that normally promotes cleavage of the glycosidic bond in ribofuranosyl and deoxy- ribofuranosyl derivatives of xanthine, hypoxanthine, and guanine, accompanied by phosphorylation of the departing ribosyl moiety, did not bring about any reaction with lin-benzoadenosine and lin-benzoinosine.[18]

Both chemical syntheses reported for lin-benzoinosine (119) required the separation of regioisomers with protected ribosyl groups attached to either nitrogen of the imidazole ring, that is, at stages 123 → 124 and 127 → 128 in Scheme XVI.[14] Intermediate 124 could be used for conversion to lin-benzoinosine (119) or to lin-benzoguanosine (125) with the reagents indicated. lin-Benzoxanthosine (120) could be prepared enzymatically from lin-benzoinosine as mentioned above.

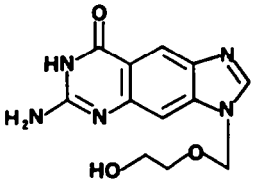
An acyclic nucleoside related to lin-benzoguanosine (125) has been made by

Scheme XVI



a, P_2S_5/pyridine; b, EtI, KOH; c, 20% KOH, t-BuOH, ; d, $(Ac)_3RibBr$, $Hg(CN)_2$; e, NH_3/CH_3OH; f, H_2, Pd/C, HCOOH; g, HCOOH, $PhCH_3$, ; h, Raney Ni, EtOH

Burroughs Wellcome scientists,[74a] namely, 6-amino-3-[(2-hydroxyethoxy)methyl]-imidazo[4,5-g]quinazolin-8(7H)-one (128a). Their synthesis involved the



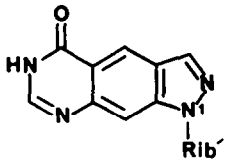
128a

silylation of lin-benzoguanine (5), alkylation with 2-(chloromethoxy)ethyl benzoate, chromatographic separation of the 3- and 7-substituted products, and liberation of 128a by short heat-treatment of the benzoate ester with aqueous methylamine. Compound 128a, which is the lin-benzo analogue of the potent anti-herpes drug acyclovir (Zovirax), showed no inhibition of herpes simplex virus-1 (HSV-1) thymidine kinase but did exhibit competition with acyclovir for binding to the enzyme.

In order to obtain 2'-deoxy-lin-benzoadenosine, the first extended analogue in the deoxyriboside series, we used 8-(methylthio)-3-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)imidazo[4,5-g] quinazoline (117)[73] or the corresponding tri-O-benzoyl derivative[74] as the precursor. Treatment of 117 with hydroxylammonium acetate afforded a mixture of the 3',5'- and 2',5'-di-O-acetyl nucleosides in ca. 4:1 ratio. This was converted to a separable mixture of diacetylthiobenzoyl ribonucleosides by treatment first with the chloroiminium chloride $PhClC{=}\overset{+}{N}Me_2Cl^-$ and then with H_2S/pyridine. The 3',5'-diacetyl-2'-thiobenzoyl isomer was subjected to free-radical deoxygenation with tri-n-butyltin hydride and with AIBN as an initiator. Replacement of the methylthio

group by means of ethanolic ammonia under pressure at 150 °C afforded 2'-deoxy-lin-benzoadenosine (118, with dRib at N-3). This linearly extended deoxyribonucleoside was phosphorylated sequentially to the 5'-triphosphate stage for study particularly with the enzyme DNA polymerase I (see later).

Synthesis of the various possible ribonucleosides of allopurinol (15), with attachment at N-1, N-2, and N-5, by Cuny and Lichtenthaler[77,78] preceded the ribosidation of lin-benzoallopurinol in the same laboratory.[79] In the lin-benzoallopurinol series, the proportions of the ribosidated isomers 129–131 (Rib' indicates tribenzoyl- or triacetylribosyl) obtained were functions of the



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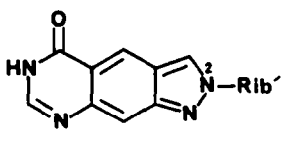

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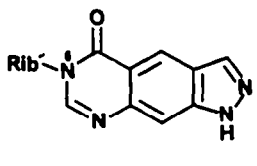
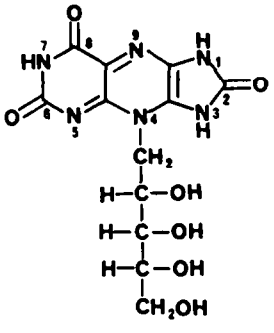


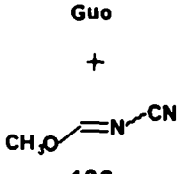
131

acyl protection and the reaction conditions, including temperature. The N-2 isomer was considered to be the product of kinetic control and the thermodynamically more stable N-1 and N-6 isomers, to result therefrom via transribosidation. The sites of ribosidation were assigned on the basis of optical rotational values and comparison of the ^{13}C and ^{1}H NMR spectra with those of the respective N-methyl derivatives of known structure.

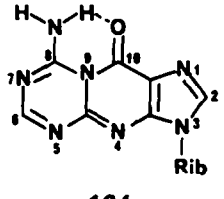
Naturally occurring lumazine (23) derivatives that have 6,7-diamino substitution have been found for the first time. Five yellow monomeric russpteridines (RP-yellow I–V) have been isolated by Iten, Maerki-Danzig, and Eugster[100] from Russula mushrooms by repeated chromatography on cellulose and sephadex gels. Of these, structure 132 may be taken as representative of the 4H-imidazo[4,5-g]-pteridines.



132



133

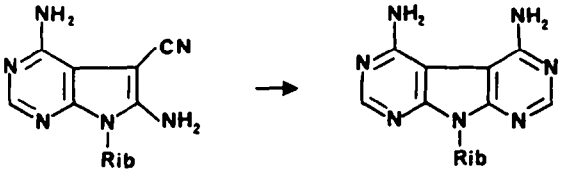


134

Finally, among the synthetic 6,6,5 ring systems, another method of generating a laterally extended adenosine analogue is by the reaction of guanosine with methyl N-cyanomethanimidate (133) and sodium methoxide in methanol.[101,102] Since the starting material is a bicyclic ribonucleoside and the product is a tricyclic ribonucleoside whose structure more closely resembles, in the periphery, that of adenosine, we have applied to 8-amino-9,10-dihydro-10-oxo-3-β-D-ribofuranosyl-3H-1,3,5-triazino[1,2-a]purine (134) the trivial name "IA'-metamorphosine." This name is intended to indicate, formally, the metamorphosis of an inosine (disconnection of the terminal ring) to an adenosine-like tricyclic molecule. The analytical and spectroscopic data were consistent with the proposed structure 134, but since the reactants offered the

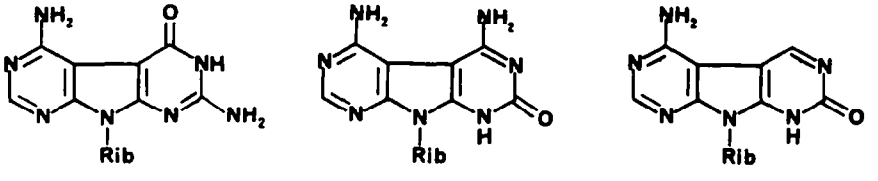
opportunity of different modes of condensation-cyclization, 1-N^{a} vs. N^{a}-3 in guanosine and C = N vs. C ≡ N in the reagent, we sought definitive means of distinguishing **134** from the three other possible isomeric products. We obtained confirmation of structure **134** by a pair of parallel spectroscopic matches involving **134** and its 2-bromo derivative with the product of 9-benzylguanine with **133** and its corresponding 2-bromo derivative. Single-crystal X-ray analysis of the last-mentioned compound of the group of four, which established the linear tricyclic ring structure of the series, showed that the central spacer was somewhat wider at the top than at the bottom, 2.4 vs. 2.2 Å.[101,102] In the ^{1}H NMR spectrum, the D_2O-exchangeable N-H's were nonidentical, as shown by the clearly differentiated δ values of 10.2 and 9.39, indicative of hydrogen bonding between the peri carbonyl and the proximate exocyclic N-H. In media of increasing viscosity at lower temperature, IA'- metamorphosine exhibits longer fluorescence lifetime and higher quantum yield, e.g.,at -38 °C in glycerol, τ = 1.31 nsec (by phase), τ = 1.38 nsec (by modulation) and Φ = 0.985, λ^{em}_{max} 444 nm. This behavior is probably due to a change in the facility of proton transfer from NH_a to the peri-carbonyl in the excited state as well as a decrease in energy release via vibrational processes. M, of adenosine with adenosine deaminase. It can also serve as a "protected" guanosine since it reverts to guanosine on treatment with 0.1 N NaOH at 20 °C within 5 min. The reaction of 9-substituted guanines with methyl N-cyanomethanimidate (**133**) is general. We have obtained similar fluorescent, tricyclic products from deoxyguanosine and from 9-(2-hydroxyethoxy)methylguanine (acyclovir).[103]

6,5,6-Ring Systems. Although they do not actually conform to the category of dimensional probes as defined here, some very interesting tricyclic ribonucleosides consisting of 6,5,6-ring systems have been constructed from naturally occurring or derived ribonucleosides by Townsend and his coworkers. For example, a double-headed diadenosine-like tricyclc nucleoside (**136**) has been prepared from 6-aminotoyocamycin (**135**) by a simple three-stage sequence with diethoxymethyl acetate, ethanolic ammonia, and 50% aqueous acetic acid.[104] Representatives of a large number of tricyclic ribonucleosides that have been

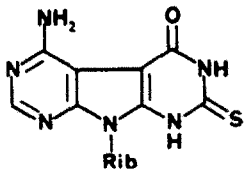


135 **136**

synthesized by annelation of a second pyrimidine ring onto a central five-membered ring are shown in formulas **136a-141**.[104,105] Some have shown moderate cytotoxicity in vitro against L-1210 mouse leukemia. For the synthesis of the



136A **137** **138**



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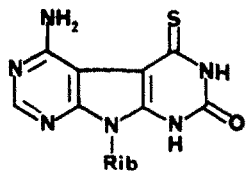

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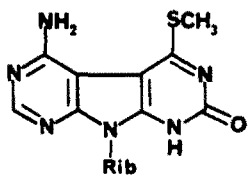
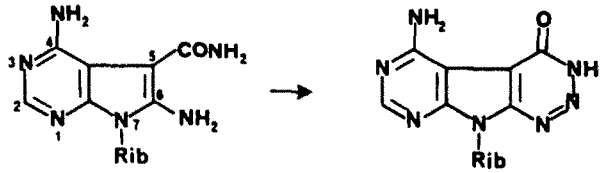


141

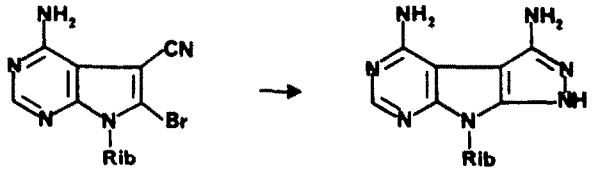
tricyclic ribonucleoside 143, which resembles adenosine at one end and a 2-azapurine nucleoside analogue at the other end, 6-aminosangivamycin (142) served as the starting material. Selective diazotization of the exocyclic amino



142 143

group at the 6 position of compound 142 led to ring closure with the formation of the 6,5,6-linear tricyclic ribonucleoside 143.[104,107]

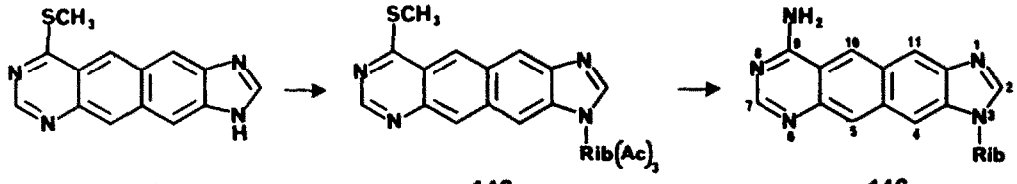
6,5,5-Ring System. An early representative of this series (145), made by heating a suspension of 6-bromotoyocamycin (144) in ethanol containing excess hydrazine at reflux,[108] was found to possess "good" anti-tumor activity.



144 145

6,6,6,5-Ring System. We synthesized lin-naphthoadenosine (146, Scheme XVII) in a manner similar to that used for lin-benzoadenosine (118, Scheme XV).[18] 9-(Methylthio)benzimidazo[5,6-g]quinazoline (147), which was also a precursor for lin-naphththoadenine (64),[46] was treated with 2,3,5-tri-O-acetylribofuranosyl bromide in the presence of mercuric cyanide using nitromethane/dimethylformamide as the solvent system. The two major products, protected 3- (148) and 1-β-D-ribofuranosyl derivatives of 147, were separated by chromatography and treated separately with ethanolic ammonia at 20 °C to effect removal of the acetyl groups and at 150 °C to effect displacement of the methylthio group. Structure assignments of the isomeric products, 9-amino-3-β-D-ribofuranosylbenzimidazo[5,6-g]quinazoline (or lin-naphthoadenosine) (146) and 9-amino-1-β-D-ribofuranosylbenzimidazo[5,6-g]quinazoline were made

Scheme XVII

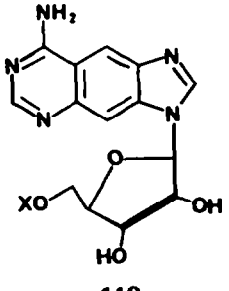


147 148 146

on the basis of chromatographic behavior and NMR and UV spectroscopic properties in parallel with those of the similar isomeric pairs in the lin-benzo series.[4a] The long wavelength absorption bands of lin-naphthoadenosine are related to those of lin-benzoadenosine as the long wavelength bands of naphthacene are related to those of anthracene. The group of three longer wavelength bands, 392-438 nm, in the electronic absorption spectrum of lin-naphthoadenosine (146) was not greatly affected by changing pH. By contrast, the second group of bands at intermediate wavelength, 340-370 nm, in its spectrum did show shifts to longer wavelength and greater absorbance upon protonation. There was no pronounced effect of the extra ring upon pK_a in going from 118 to 146 (both $\sim$ 5.6). The site of first protonation of lin-naphthoadenosine, as of lin-benzoadenosine, is predictably on the pyrimidine ring. Whereas lin-benzoadenosine was converted by adenosine deaminase from calf intestinal mucosa to lin-benzoinosine at a rate almost comparable to that for the conversion of adenosine to inosine,[18] lin-naphthoadenosine underwent no detectable deamination under identical reaction conditions, nor was it an effective inhibitor.[4a] Whereas lin-benzoadenine was (uniquely) an active substrate for adenosine deaminase, lin-naphthoadenine was inactive, like its ribosyl derivative. The absence of activity for 146 established that a lateral extension of 4.8 A from the normal substrate, adenosine, is too great for a satisfactory fit at the enzyme active site while at 2.4-A extension (118) of adenosine, binding to the enzyme and deamination of the substrate were efficient.

MONO-, DI-, TRIPHOSPHORIBOSYL DERIVATIVES

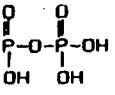
In terms of providing purine analogues that differ from the natural enzyme substrates or cofactors by defined dimensional changes, mono-, di-, and triphosphorylation at the 5'-hydroxyl of the ribosyl unit constitutes the third stage of experimentation that was mentioned in the introduction. The fourth and fifth stages, dealing with enzyme binding and activity and with spectroscopic analysis of binding, follow immediately upon the synthesis. In fact, they merge with the synthesis stage since enzyme methodology may be used to convert from one phosphorylated level to the next and to establish the integrity of structure at each phosphate level.[10a] We have been interested especially in phosphorylated derivatives (149, 150) of lin-benzoadenosine (118) since one-sixth of all known enzymes require ATP or a related adenine-containing

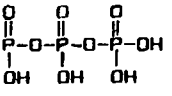


149

X

a 

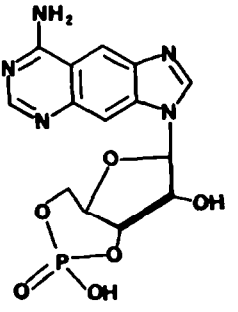
b 

c 

d lin-Benzoadenosine 5'-diphosphoryl

e Adenosine 5'-tetraphosphoryl

f Adenosine 5'-pentaphosphoryl



150

cofactor such as AMP, ADP, cyclic AMP, NAD^+, NADPH, FAD, or coenzyme A.[4-8] We have synthesized the fluorescent lin-benzo-AMP (149a), lin-benzo-ADP (149b), lin-benzo-ATP (149c), and lin-benzo-cAMP (150), inter alia,[110] and we used similar methodology for the synthesis of the fluorescent 5'-mono-, di-, and triphosphates of lin-benzoinosine (119), lin-benzoxanthosine (120), and lin-benzoguanosine (125).[111] The preferred route avoided the necessity of protection-deprotection steps. Unprotected lin-benzoadenosine (118) was converted to lin-benzo-AMP (149a) by reaction with pyrophosphoryl chloride in m-cresol according to the conditions described by Imai, Fujii, Takanohashi, Furukawa, Masuda, and Honjo.[112] The 5'-monophosphate was formed uniquely, as shown by HPLC, electrophoresis, microanalysis, ^{31}P NMR spectrum, and complete reconversion to lin-benzoadenosine on incubation with the specific enzyme 5'-nucleotidase.[109,110,113] For conversion of lin-benzo-AMP to lin-benzo-ADP (149b) and lin-benzo-ATP (149c), we used the phosphoromorpholidate method of Moffatt and Khorana.[114] Both the 5'-diphosphate and the 5'-triphosphate were characterized by TLC, HPLC, electrophoresis, and ^{31}P NMR spectra. Once we discovered that lin-benzo-ADP could be converted to lin-benzo-ATP with rabbit muscle pyruvate kinase and phosphoenolpyruvate, we used this covenient method to generate our supply of triphosphate and to recycle from diphosphate produced in various ATP- (and lin-benzo-ATP-) requiring enzyme systems (see below).[110]

Other phosphate derivatives were made by applying standard methodology. For example, lin-benzoadenosine cyclic 3',5'-monophosphate, lin-benzo-cAMP (150), was synthesized by the general procedure of Marumoto, Nishimura, and Honjo.[115] First, lin-benzoadenosine 5'-trichloromethylphosphonate was generated from lin-benzoadenosine (118) by treatment with trichloromethylphosphonic acid dichloride in triethyl phosphate at 0 °C. Then, cyclization was achieved with potassium t-butoxide in DMF at 25 °C, which generated the 3'-O anion for addition/displacement of the trichloromethyl group from phosphorus.[110,113,116] The purity and structure of this cAMP analogue were established by HPLC, electrophoresis, and ^{31}P NMR spectroscopy, and by ring-opening of the cyclic phosphate on incubation with beef heart nucleotide 3',5'-phosphodiesterase to lin-benzoadenosine 5'-monophosphate. lin-Benzoadenosine 3'(2'),5'-bisphosphate was prepared with pure pyrophosphoryl chloride at low temperature without solvent[109,117] in an application of a bisphosphorylation method that first appeared in 1963.[118,119] The symmetrical anhydride of lin-benzo-AMP, P^1,P^2-di-lin-benzoadenosine 5'-pyrophosphate (149d), was synthesized by reaction of a salt of lin-benzoadenosine 5'-phosphoromorpholidate with trifluoroacetic acid in anhydrous dimethylformamide and was isolated and purified by linear gradient chromatography.[11,110,113] The equivalence of the two phosphates and thus the symmetrical nature of the product, suggested by the synthesis, were confirmed by the singlet ^{31}P NMR signal. The unsymmetrical ribonucleoside oligophosphates 149e,f with lin-benzoadenosine at one terminus and adenosine at the other were synthesized from lin-benzoadenosine 5'-monophosphoromorpholidate and adenosine 5'-tri and tetraphosphates.[6-7,109,119] Enzymatic interconversions of some ribotides were possible. As in the case of lin-benzo-ADP to lin-benzo-ATP, conversion of the diphosphates of lin-benzoinosine (119), lin-benzo-xanthosine (120), and lin-benzoguanosine (125) was accomplished with pyruvate kinase and phosphoenolpyruvate.[111] The general reaction was driven to completion by the use of lactate dehydrogenase, with NADH present to consume the pyruvate formed in the reaction. The oxidation of lin-benzo-IMP to lin-benzo-

XMP with xanthine oxidase and oxygen was both useful and surprising, since it represents the first example of such a conversion occurring at the 5'-monophosphate level. Oxidation occurs in the pyrimidine ring, as in the case of lin-benzoinosine, whereas further oxidation in the imidazole ring, which occurs in the case of lin-benzoxanthosine, is prevented by the presence of the added phosphate group. While some enzyme reactions are surprisingly effective with the stretched-out ribotides, others fail to interconvert the phosphate levels of the analogues.[111] Thus, guanosine monophosphate kinase failed to convert lin-benzo-GMP to lin-benzo-GDP, and succinyl-CoA synthetase failed to convert lin-benzo-GDP to lin-benzo-GTP under normal assay conditions.

In the course of characterizing the various phosphate analogues 149a–f and 150 and the 3',5'- and 2',5'-bisphosphates, we observed some correlations in their ^{31}P NMR spectra that have the capability of further generalization[109,110,117,119] since the chemical shifts and P–O–P coupling constants show striking similarity between the adenine nucleotides and the corresponding lin-benzoadenine nucleotides. For example, when observed under similar conditions, the ^{31}P magnetic resonance for both cAMP and lin-benzo-cAMP is shifted upfield (−1.7 ppm) from 85% H_3PO_4, quite distinct from the chemical shift downfield (3.9 ppm) for the 5'-phosphate in both AMP and lin-benzo-AMP (149a). When complete relaxation of the NMR phosphorus signals is allowed, the ^{31}P relative chemical shifts are useful in structure assignment and in the quantitative estimation of proportions in mixtures such as those encountered in the bisphosphorylation reaction. With the 5'-P signal nearly constant at an average value of 3.88 ppm, the 3'-P and 2'-P signals can be differentiated readily, especially when the ribonucleoside 3',5'- and 2',5'-bisphosphates are present in different proportions. The 2'-P signals are consistently upfield from the 5'-P resonance and the 3'-P signals are downfield by ca. 0.20 ppm from the 5'-P average position. Thus, one can distinguish readily and in general among 5'-, 3'-, and 2'-phosphates on ribonucleosides. The ^{31}P shifts and coupling constants of lin-benzo-ADP (149b) (α, −10.3; β, −5.8; doublets, $^{31}J_{P-O-P}$ = 22 Hz) and lin-benzo-ATP (149c) (α, −11.1, d; β, −22.4, t; γ, −6.1, d; $J_{\alpha-\beta}$ = 20 Hz; $J_{\beta-\gamma}$ = 20 Hz) are similar to those of ADP and ATP, respectively, and therefore useful for characterization. The single ^{31}P chemical shift of lin-benzo-A(5')p_β(5')lin-benzo-A (149d) (−11.0) is like that of A(5')p_β(5')A (−11.1). For lin-benzo-A(5')p_α(5')A (149e) and lin-benzo-A(5')p_α(5')A (150e), the ^{31}P chemical shifts are centered at about −11.3, dd, for the α-P and −23.2, dd, for the β-P positions similar to those of the corresponding phosphates of A(5')p_α(5')A.

In the ^1H NMR spectra for the analogues 149a–d and 150, at dilutions approaching infinite, the 6-H becomes a monitor of charge (protonation takes place on the pyrimidine ring), the 4-H is sensitive to phosphate ionization and is therefore indicative particularly of the syn conformation that predominates under acidic conditions (pD ∿ 4.0) (the adenylates, of course, possess no equivalent proton), and the 2-H is responsive to the anti conformation (pD ∿ 8.5) of 149a–c and 150.[11,110] When the base is unprotected, these lin-benzoadenine nucleotides are vertically stacked in aqueous solution, with association constants of at least one order of magnitude greater than those of the corresponding adenine nucleotides.[11] Head-to-tail orientations of stacked lin-benzoadenine nucleotides in aqueous solution were indicated by the deuterium substitution effect on relaxation times (DESERT method). Head-to-tail alternate

stacking was found to be the intermolecular pattern in single crystals of the N^{6},N^{6}-dimethyl derivative of lin-benzoadenine by X-ray analysis.[6]

The pK_a values as determined by ultraviolet spectroscopy of lin-benzo-Ado, -cAMP, -AMP, -ADP, and -ATP responded to the presence and conformation of the phosphate side chain.[118,180] When no intramolecular interaction can occur between phosphate and base, as in lin-benzoadenosine (118) and lin-benzo-cAMP (150), the N^{+}-H pK_a value is 5.6, unchanged in 5 mM Mg^{2+}, whereas the values for 149a-c (7.6, 7.3, 7.1, respectively) indicate that the phosphates in these molecules are involved in the base protonation-deprotonation. The addition of divalent metal cations or quaternary ammonium micelles diminishes the direct intramolecular interaction between the phosphate(s) and bases and brings the pK_a values closer to that of lin-benzoadenosine. This information is germane to the discussion of the activity of the lin-benzoadenine nucleotides as substrates or cofactors with various enzymes. While there was hopeful anticipation, there was no a priori reason to expect enzyme activity with these unnatural substrates. We have already mentioned some of the fortuitous enzyme activities that were utilized in the synthesis of the various phosphate derivatives. In general, we have been rewarded with observations of a range of activity from natural substrate equivalence through intermediate activity to zero activity, all of which permitted positive conclusions to be drawn in practically all cases. It should be recognized that in no case is one of our totally synthetic stretched-out analogues contaminated with a natural nucleotide.

lin-Benzo-AMP. Adenylate kinase (rabbit or pig muscle) catalyzes the transfer of phosphate between Mg^{2+}-ATP plus AMP and Mg^{2+}-ADP plus ADP. The accumulated evidence with regard to the functioning of the enzyme supports a two-site model in which one site is specific for AMP or ADP and the other, less demanding, is specific for ATP or ADP. When we tried substituting lin-benzo-AMP (149a) for AMP in this system, no diphosphates were produced.[5,110] As we shall discuss below, the substitution of lin-benzo-ATP (149c) for ATP permits the enzyme to function, and both lin-benzo-ADP and ADP are formed. Our results support the two-site model in which the AMP binding site is highly specific. Since the sugar/base conformation of lin-benzo-AMP is anti, the requisite NH_2 is present in 149a,[121] and, in comparison, 3-iso-AMP will substitute satisfactorily for AMP in this system,[122] the extension of the heteroaromatic ring system probably accounts for its failure as a substrate for adenylate kinase. Our stretched-out model helps define the limiting size of the AMP hydrophobic binding pocket, at least for rabbit muscle adenylate kinase in solution.

lin-Benzo-cAMP. This analogue (150) of cAMP maximally activates protein kinase from brain and from skeletal muscle but at a higher concentration than cAMP.[114] Addition of low levels of lin-benzo-cAMP does not inhibit activation of protein kinase by cAMP, while lin-benzoadenosine (118) inhibits such activation slightly less than does adenosine. Thus, lin-benzoadenosine and its 3',5'-monophosphate interact with cAMP-dependent protein kinase, showing that the enzyme can accommodate the lateral extension of the purine ring by 2.4 Å.

lin-Benzoadenosine 3'(2'),5'-bisphosphate. This mixture, prepared as described earlier directly from lin-benzoadenosine, can be used to lengthen an oligonucleotide chain such as $(Ap)_2C$ in the presence of excess T4 RNA ligase and ATP. The 2',5'-bisphosphate is not utilized, and only the 3',5'-bisphosphate is incorporated at the 3' end of the tetranucleotide acceptor.[117] This 3',5'-bisphosphate and a variety of other analogues are nearly as good

substrates as their unmodified counterparts,[182] an observation consistent with the lack of specificity of T4 RNA ligase.

lin-Benzo-ADP. We have already mentioned that a satisfactory enzymatic synthesis of lin-benzo-ATP from lin-benzo-ADP (149b) was achieved with pyruvate kinase and phosphoenolpyruvate. The efficiency of this conversion also permits the use of a coupled assay for lin-benzo-ADP formation (from lin-benzo-ATP) that involves pyruvate kinase and lactate dehydrogenase.[110] If we return to a consideration of the enzyme adenylate kinase and the disproportionation of 2ADP into AMP and ATP,[8,110] we note that it should be possible to relate the highly specific AMP site to one of the ADP sites. This we have done by using a mixture of lin-benzo-ADP and ADP with adenylate kinase. lin-Benzo-ADP alone remains unchanged with the enzyme, while the mixture generates AMP, ATP, and lin-benzo-ATP, but no lin-benzo-AMP. Thus, one of the ADP binding sites is highly specific and corresponds to the AMP binding site. Neither lin-benzo-ADP nor lin-benzo-AMP can react at that site.

Although lin-benzo-ADP (149b) is not converted to lin-benzo-ATP by intact beef heart or rat liver mitochondria, submitochondrial particles or fragments will phosphorylate lin-benzo-ADP and will hydrolyze lin-benzo-ATP.[184] Purified mitochondrial ATPase is inhibited more potently by lin-benzo-ADP than by ADP (K_i 16 μM vs. 27 μM). The fluorescence quantum yield for lin-benzoadenosine (118) and its phosphate derivatives (149a-c) in aqueous solution is 0.40-0.44 upon excitation at 332 nm and the fluorescence lifetime is 3.7 nsec.[110,180] These properties are especially valuable for addressing the problems of enzyme binding. In this example, the fluorescence of 1 μM lin-benzo-ADP is strongly quenched (85%) by 5 μM mitochondrial ATPase, and the quenching is reversed by 50 μM ADP. The conclusion reached is that there is strong binding of the nitrogen-heteroaromatic ring system to the enzyme. The inactivity of the ADP analogue with intact mitochondria is due to its failure to be transported across the inner membrane at an appreciable rate. That is, it does not serve as a substrate for the adenine nucleotide carrier.

The folding or stacking of P^1,P^2-di-lin-benzoadenosine 5'-pyrophosphate (149d) in dilute aqueous solution was indicated by a number of its properties. The percentage of hypochromism in the UV spectrum of 149d, as determined by means of hydrolytic cleavage with snake venom phosphodiestrase, was found to be greater than that of P^1,P^2-diadenosine 5'-pyrophosphate.[112] The hydrolytic cleavage of 149d was accompanied by an increase in fluorescence intensity of approximately two orders of magnitude. The dramatic changes in UV and fluorescence are indicative of strong intramolecular stacking interactions between the two tricyclic rings of the anhydride (149d) of lin-benzo-AMP and are predictive of strong fluorescence quenching when the lin-benzoadenylate system is in close proximity to other moieties, including nucleic acid bases and aromatic amino acids. Accordingly, it was not surprising to find that the polymeric material, which was isolated by gel chromatography from the reaction of lin-benzo-ADP (149b) with primer-independent polynucleotide phosphorylase from Micrococcus luteus in the presence of Mn^{2+} at 37 °C, showed almost no fluorescence.[110] The observation was attributable to stacking interactions and a probably helical array in the poly(lin-benzoadenylic acid) of some 80 ± 5 nucleotide units. The structural integrity of the polymer was shown by its hydrolysis with the aid of a mixture of enzymes, micrococcal nuclease, snake venom phosphodiesterase, and alkaline phosphatase, or by alkali to

lin-benzoadenosine (118), with the return of fluorescence. The long-wavelength UV band of the polymer lacked the characteristic fine structure of the monomeric species and was broadened. Upon enzymatic or alkaline hydrolysis, the fine structure of the long-wavelength UV band reappeared. In the case of lin-benzo-IDP, polymerization with polynucleotide phosphorylase was possible in the presence of Mg^{2+} and a GpU primer and was accompanied by a strong hypochromic effect and loss of UV fine structure.[111] The extent of polymerization was not determined.

lin-Benzo-ADP was found to be a very mild aggregation inducer when added to human platelet-rich plasma. Comparative determinations showed lin-benzo-ADP to be active, but at least 200 times less active than ADP.[128]

The prediction of the facilitation of fluorescence polarization measurements[110] has been amply realized in the methodology that has emerged from Roskoski's laboratory.[124-130] The interaction of lin-benzoadenosine di- and triphosphates with the catalytic subunit and type II holoenzymes of adenosine cyclic 3',5'-monophosphate dependent protein kinase was investigated by steady-state kinetics and fluorescence spectroscopy. For the catalytic subunit which responds to ATP, lin-benzo-ADP (149b) is a competitive inhibitor with a K_i of 8.0 μM, similar to that for ADP (9.0 μM).[124] Thus, lin-benzo-ADP, which is 2.4 Å wider in the adenine moiety, binds to the active site of the catalytic subunit as well as does the natural reaction product, ADP. No change was observed in the fluorescence emission spectrum of lin-benzo-ADP in the presence of catalytic subunit, but binding to the enzyme was accompanied by an increase in polarization. Fluorescence-polarization titration was carried out by measuring the polarization (a) at constant lin-benzo-ADP concentration with increasing catalytic subunit concentration and (b) at constant catalytic subunit concentration with increasing lin-benzo-ADP concentration. The K_d of 9.0 for lin-benzo-ADP and the free catalytic subunit determined by this method agrees well with the kinetically determined K_i. Type II holoenzymes from skeletal muscle and brain gave K_d values of 3.5 and 3.4 μM, respectively, and both bound 2 moles of lin-benzo-ADP per mole of holoenzyme, in fact, with higher affinity than the free catalytic subunit. The average angle of rotation of bound lin-benzo-ADP during the lifetime of the excited state, calculated from the polarization data as 25° and 20° for the catalytic subunit and holoenzyme, respectively, are close to the theoretical values of 26° and 14° calculated from the Perrin equation. Thus, most, if not all, of the observed rotation is due to rotation of the protein itself, which indicates that both the catalytic subunit and type II holoenzyme bind lin-benzo-ADP rigidly, so that there is little or no rotation within the adenine binding site. Fluorescence-polarization titration was also useful in determining or confirming chemical modification of amino acid residues at or near the nucleotide binding site of the catalytic subunit.[124,128]

The measurement of fluorescence polarization was also used to determine the degree of displacement of lin-benzo-ADP from the catalytic subunit of bovine skeletal muscle type II protein kinase with increasing concentrations of selected competing nucleotides.[127] The K_d of each nucleotide was calculated from the decrease in fluorescence polarization of lin-benzo-ADP that accompanied its displacement from the catalytic subunit. By this fluorescence displacement titration technique, it is possible to map the exact requirements for the most tenacious binding of the nucleotide moiety, in this example by variants

including modification of the adenine ring, the ribosyl group, the phosphate multiplicity, and the 5'-terminal polarity. By using the same methodology, it is possible to determine which metal ions promote binding, and to what extent, of lin-benzo-ADP (and, therefore, presumably of ADP) to the catalytic subunit of cAMP-dependent protein kinase.[127] We have indicated earlier in this section that lin-benzo-cAMP can assume the role of cAMP at the regulatory site of this enzyme.

As in the case of cAMP-dependent protein kinase, cGMP-dependent protein kinase in the presence of phosphorylatable peptide was inhibited competitively by lin-benzo-ADP with respect to ATP with a K_i of 22 μM, similar to a K_m of 20 μM as determined by fluorescence polarization titration.[127] For the cGMP-dependent protein kinase, lin-benzo-ADP binds with over 6-fold tighter affinity than does ADP; i.e., there is a greater difference in binding affinity than in the case of the cAMP-dependent enzyme. The fluorescence polarization indicates that the lin-benzoadenine moiety is rigidly bound to the cGMP-dependent protein kinase within the limits of detection. Fluorescence polarization titration revealed that the binding of lin-benzo-ADP to this kinase was dependent upon a divalent cation and delineated which metal ions supported binding.

The presence of yeast hexokinase brought about a small increase in the fluorescence polarization of lin-benzo-ADP, which was raised further by the addition of D-lyxose, a competitive inhibitor of glucose phosphorylation.[8,130] This effect was indicative of enhanced binding of lin-benzo-ADP, as observable by other means with ADP. Fluorescence polarization using lin-benzo-ADP as the fluorescent ligand may be useful in estimating the extent of dissociation of dimeric into monomeric hexokinase under a variety of conditions.[130]

lin-Benzo-ATP. We found that lin-benzo-ATP (149c) substitutes with varying degrees of efficiency for a number of phosphoryl transfer enzymes, including acetate kinase, phosphofructokinase (PFK) phosphoglycerate kinase (PGK), adenylate kinase, and yeast hexokinase.[8,110] In all cases, utilization of lin-benzo-ATP represents the largest presently known deviation from the natural cofactor ATP. At higher concentrations of the stretched-out analogue, it is an allosteric inhibitor of phosphofructokinase, as is ATP. The structural requirements for inhibition are more stringent than those for activity at lower concentration, so the behavior of lin-benzo-ATP is unique among analogues. The ability of lin-benzo-ATP to phosphorylate 3-phosphoglyceric acid catalyzed by yeast 3-phosphoglycerate kinase, indicates that the enzyme conformation required for bringing the phosphoglycerate site and the ATP site together can accommodate the wider nucleus represented by lin-benzo-ATP but with lowered efficiency. It also permits exchange of the γ phosphate in the nucleoside triphosphate with $^{32}PO_4^{3-}$ in the presence of PGK, leading to [γ-^{32}P]-lin-benzo-ATP. Adenylate kinase has been discussed above with respect to lin-benzo-AMP, which is not a substrate, and lin-benzo-ADP, which does not function unless paired with ADP, in which case the lin-benzo-ADP disproportionates only in the direction of lin-benzo-ATP. lin-Benzo-ATP can substitute for ATP and, as long as AMP is present, it is converted to lin-benzo-ADP with adenylate kinase. The difference in specificity for the AMP and ATP sites, together with their complementary and separate ADP and ADP sites, is consistent throughout. In the phosphorylation of glucose catalyzed by yeast hexokinase, lin-benzo-ATP can replace ATP. The K_m values are in the same range, so that the stretched-out analogue binds satisfactorily, indicating that the site has at least an extra 2.4-A dimensional toler-

ance, but the substantial reduction in rate suggests that the juxtaposition of bound glucose and bound lin-benzo-ATP is not as favorable for the phosphate transfer as with ATP.

When lin-benzo-ATP and ATP were compared as substrates for hydrolysis by purified mitochondrial ATPase, they were found to be similar.[128a] Both compounds displayed negative cooperativity and activation by HCO_3^-. Moreover, the apparent Michaelis constants for lin-benzo-ATP and ATP determined in the presence of 10 mM HCO_3^- were equal. Like lin-benzo-ADP, lin-benzo-ATP displayed little, if any, activity as a substrate for the adenine nucleotide carrier. The allosteric activation of aspartate transcarbamylase (ATCase), which catalyzes the conversion of carbamyl phosphate and aspartate to carbamyl aspartate and phosphate, occurs with lin-benzo-ATP to the same extent that it does with ATP, and their association constants are similar.[131] The association constant for lin-benzo-ATP and ATCase was determined by fluorescence polarization. The fluorescence emission of lin-benzo-ATP is not quenched when bound to ATCase, which indicates the absence of π interactions between the activator and tyrosyl residues in the protein. Instead, the amino group plays a key role in the binding process, and the binding and activity of the extended ATP analogue are in accord with the London-Schmidt hypothesis for ATCase activation vs. inhibition.[132]

Uhlenbeck has shown that lin-benzo-ATP transfers phosphate to $(Ap)_3C$ as a substrate under catalysis by T4 polynucleotide kinase, but it does not serve as a catalyst for linking $(Ap)_3C$ and pCp.[133] In T7 phage DNA-directed RNA synthesis in vitro, lin-benzo-ATP does not replace ATP in RNA synthesis nor does it inhibit ATP incorporation into RNA.[110] Rowen and Kornberg have reported that lin-benzo-ATP does not get incorporated by primase, a rifampicin-resistant RNA polymerase, into a polynucleotide chain, the synthesis of which is template directed.[134] These experiments indicate that normal Watson-Crick base pairing is exacting in template-directed polymerization. With the rep protein, lin-benzo-ATP works in duplex strand separation in the role of ATP, according to Kornberg, Scott, and Bertsch.[135] We have shown lin-benzo-ATP to be an acceptable substrate for light production in the firefly luciferase - luciferin system.[136] This analogue displays strong enzyme binding and a reduced rate of enzyme catalysis compared with ATP. Variation in the color of the bioluminescence reaction suggests that a lateral extension in the purine base induces an incremental change in the conformation of luciferase in the vicinity of the excited light emitter.

We mentioned earlier Roskoski's methods of fluorescence-polarization titration and fluorescence displacement titration used for defining the ATP-catalytic sites in cAMP-and cGMP-dependent protein kinases by means of lin-benzo-ADP.[126,128,127] lin-Benzo-ATP is a good substrate for the phosphotransferase activities of cAMP-dependent protein kinase with peptides, water, or the type II regulatory subunit as phosphoryl acceptors. With Leu-Arg-Arg-Ala-Ser-Leu-Gly as phosphoryl acceptor, the observed K_m for lin-benzo-ATP was 11.3 μM, close to that of ATP, 11.9 μM. The V_{max} with lin-benzo-ATP was 20% of that with ATP, which makes lin-benzo-ATP the best nucleotide substrate, other than ATP, that has been reported for the catalytic subunit.[128a] lin-Benzo-[γ-^{32}P]ATP was synthesized from lin-benzo-ADP for use in determining the kinetic parameters of the catalytic subunit with lin-benzo-ATP as substrate.[128a] In the case of cGMP-dependent protein kinase with [Ala^{29}]histone H2B-(29-35) as the

phosphoryl acceptor; the observed K_m for lin-benzo-ATP was 29 μM, also close to that of ATP, 32 μM, but the V_{max} with lin-benzo-ATP was only 0.06% of that with ATP as the substrate.[108] Thus, while binding of lin-benzo-ATP to the adenine-binding region of cGMP-dependent protein kinase is equivalent to that of ATP, the conformational arrangement does not position the γ-phosphate for efficient transfer to the acceptor.

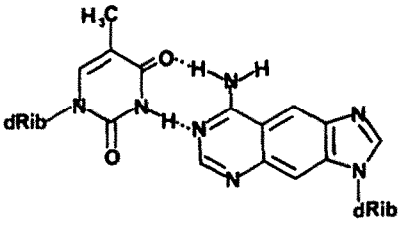
P¹,P⁴-Di-lin-benzoadenosine 5'-pyrophosphate. The strong intramolecular stacking interaction of bis-5',5'-[lin-benzo-AMP] was indicated by the ultraviolet and fluorescence spectroscopic evidence mentioned earlier.[110] This interaction has also been shown by the NMR chemical shifts of the 4-, 6-, 9-, and 1'-protons of bis-5',5'-[lin-benzo-AMP-2-d] at infinite dilution in water.[111] The base and anomeric protons are at higher field than those of the related monomer, lin-benzo-AMP, also at infinite dilution, which indicates that the diphosphate tether holds the two nitrogen-tricyclic rings in close enough association that the magnetic anisotropy is experienced.

P¹(lin-Benzo-5'-adenosyl)-P⁴-(5'-adenosyl)tetraphosphate and P¹-(lin-benzo-5'-adenosyl)-P⁵-(5'-adenosyl)pentaphosphate. The mixed dinucleoside oligophosphates lin-benzo-A(5')p_4(5')A and lin-benzo-A(5')p_5(5')A were made as dimensional probes to compare with known inhibitors of adenylate kinase A(5')p_4(5')A and A(5')p_5(5')A.[8,109,112] The stretched-out (at one end) oligophosphates are competitive with respect to ATP and AMP and are indeed potent inhibitors of porcine muscle adenylate kinase, with association constants of 2×10^4 M^{-1} for the tetraphosphate and 2×10^5 M^{-1} for the pentaphosphate as determined by kinetics and fluorescence experiments. Since the lin-benzo-A(5')p_4(5')A inhibition constant is intermediate between the values for A(5')p_4(5')A and A(5')p_5(5')A, the most effective inhibitor, it is suggestive that a qualitatively similar effect is produced whether the phosphate chain is lengthened (ca. 2.7 A from p_4 to p_5) or one terminal base is widened (2.4 A from A to lin-benzo-A). While these analogues are intramolecularly stacked when free in aqueous solution, as judged by their low fluorescence quantum yield and short fluorescence lifetime compared with lin-benzo-AMP, when adenylate kinase is present both yield and lifetime are increased. The reversal of fluorescence signifies that the intramolecular stacking has been broken and that these inhibitors are bound to the enzyme in an "open" or "extended" form of the oligophosphate chain.

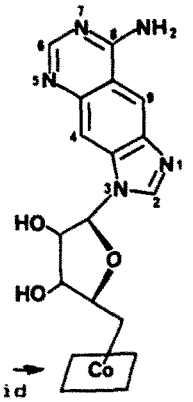
MONO-, DI-, TRIPHOSPHODEOXYRIBOSYL DERIVATIVES

As in the case of the lin-benzoadenosine phosphates (149a-c), the corresponding lin-benzodeoxyadenosine phosphates were made as follows: the mono, by chemical phosphorylation of 2'-deoxy-lin-benzoadenosine;[79,94] the di, by chemical phosphorylation of the monophosphate via the morpholidate;[95] and the tri, by the reaction of the diphosphate with pyruvate kinase and phosphoenolpyruvate.[95] 2'-Deoxy-lin-benzo-ADP binds to pyruvate kinase better than dADP, which is also true of lin-benzo-ADP, reflecting better accommodation of the extended aromatic lin-benzoadenine moiety in the hydrophobic region[137] of the active site. As dADP showed lower activity than ADP, so d-lin-benzo-ADP showed lower activity than lin-benzo-ADP with pyruvate kinase.[95] At the triphosphate level, when d-lin-benzo-ATP was tested in a standard nick-translation experiment with E. coli DNA polymerase I, a very low level of ^{32}P incorporation from [α-^{32}P]TTP into poly[d)AT)] was observed.[95] Nearest-neighbor analysis

indicated that the analogue was not significantly incorporated into internal positions within the polymer, while DNA-sequencing reactions showed that the analogue caused chain termination at adenine residues. Watson-Crick pairing of the incoming deoxynucleoside triphosphate to the corresponding base in the template strand is essential to selection by DNA polymerase I among natural deoxyribonucleotides and their analogues. The experiments show that d-lin-benzoadenosine can form a widened Watson-Crick base pair with thymidine (151). However, there would then be resulting distortion in the phosphodiester chain, mispositioning the 3'-hydroxyl of the terminating d-lin-benzo-A in the active site and thus preventing linkage with the α-5'-phosphate of the next incoming deoxyribonucleotide. An alternative explanation that cannot be ruled out on the basis of our experiments is that the distortion of the helix interferes with the next requisite A-T pairing.[88,98]



151



The corrin ring system is represented by the rhomboid

152

ANALOGUE OF COENZYME B_{12}

The analogue of coenzyme B_{12}, lin-benzoadenosylcobalamin (152), was synthesized in our laboratory from cob[I]alamin and 5'-chloro-5'-deoxy-lin-benzoadenosine.[138] The fluorescence of the lin-benzoadenosine moiety offered special advantage in the "profluorescent" coenzyme analogue.[7,138] Thus, compound 152 is nonfluorescent in solution but, on homolytic (light) or heterolytic (acid, cyanide) cleavage of the carbon-cobalt bond, forms fluorescent products. In addition, fluorescence is detectable on binding of the coenzyme analogue to ribonucleotide reductase, and the observed fluorescence polarization of the lin-benzoadenosyl moiety indicates that it is bound loosely to the enzyme when the coenzyme is partially dissociated. The stabilized radical pair, also detectable by EPR in the case of adenosylcobalamin, is considered to be the first intermediate in the ribonucleotide reductase reaction and is prerequisite for catalysis but does not guarantee that ribonucleotide reduction will be completed. Compound 152 was found to be an effective competitive inhibitor of ribonucleotide reductase from Lactobacillus leichmanii.

SUMMARY AND PROSPECTS

The concept of dimensional probes of binding and activity has stimulated the synthesis of analogues that differ from natural bicyclic bases, ribonucleosides, and ribonucleotides by defined dimensional changes. Linearly-extended heterocyclic bases have been made with carbocyclic or heterocyclic spacers formally inserted between terminal rings, and some of these have shown interesting activity in biological systems. The compounds with heterocyclic

ring spacers introduce not only definable dimensional changes but electronic and hydrogen-bonding changes. Angular tricyclic bases have also been made in which the dimensional change has been supplemented by an orientational change of peripheral substituents. These do not qualify as dimensional probes if the term is strictly applied. It is at the linearly-extended ribonucleoside and ribonucleotide levels, particularly in the lin-benzo series, that the dimensional probes have been and will probably continue to be most valuable. They help establish the spatial limitations of a substrate for the expression of activity or inhibition in enzyme systems. They delineate the factors responsible for inactivity/activity in complex biological systems, for example, with intact mitochondria versus submitochondrial particles. The fluorophore of the lin-benzoadenylates is sensitive to environmental conditions, including stacking, and the fluorescence yields and lifetimes are indicative of the mode of binding of activator or inhibitor to enzymes. The fluorescence polarization defines whether the activator or inhibitor rotates independently or with the enzyme and is the basis for the techniques of fluorescence-polarization titration and fluorescence displacement titration, for example, to map the exact requirements for binding of a nucleotide moiety at an active site. Additional probes with intermediate or further-extended dimensions constitute a synthetic challenge and offer the potential of fine-tuning the spatial requirements for binding and biological activity. The fluorescence behavior - yield, lifetime, polarization - of laterally extended, active analogues of the natural ribonucleotides and coenzymes will continue to provide information with respect to both analogue and natural substrate that must be incorporated in any total picture of enzyme-coenzyme or enzyme-substrate behavior.

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REFERENCES

[1]See: Burger's Medicinal Chemistry, Part II, 4th ed. (Edited by M. E. Wolff). Wiley-Interscience, New York (1979).

[2]See: B. R. Baker, Design of Active-Site-Directed Irreversible Enzyme Inhibitors. Wiley, New York (1967).

[3]G. Weber, Biochemistry 11, 864 (1972); see also Nature (London), 236, 326 (1972).

[4]N. J. Leonard, Heterocycles 12, 129 (1979).

[5]N. J. Leonard, Acc. Chem. Res. 15, 128 (1982).

[6]N. J. Leonard, in Biologically Active Principles of Natural Products (Edited by W. Voelter and D. G. Daves), pp. 237-249. Thieme-Stratton Inc., New York (1984).

[7]N. J. Leonard, Pure & Applied Chem. 56, 1025 (1984).

[8]N. J. Leonard, Biopolymers 24, 9-28 (1985).

[9]N. J. Leonard, A. G. Morrice, and M. A. Sprecker, J. Org. Chem. 40, 356 (1975).

[10]See: Symposium on 6-Mercaptopurine (Edited by C. P. Rhoads), Ann. N. Y. Acad. Sci. 60, 183 (1954).

[11]J. R. Barrio, F.-T. Liu, G. E. Keyser, P. VanDerLijn, and N. J. Leonard, J. Am. Chem. Soc. 101, 1564 (1979).

[12]N. J. Leonard, M. A. Sprecker, and A. G. Morrice, J. Am. Chem. Soc. 98, 3987 (1976).

[13]For review, see N. J. Leonard in The Chemistry and Biochemistry of Plant Hormones (Edited by V. C. Runeckles, E. Sondheimer, and D. C. Walton), pp 21-56. Academic Press, New York (1974).

[14]M. A. Sprecker, A. G. Morrice, B. A. Gruber, N. J. Leonard, R. Y. Schmitz, and F. Skoog, Phytochemistry 15, 609 (1976).

[15]G. E. Keyser and N. J. Leonard, J. Org. Chem. 41, 3529 (1976).

[16]G. E. Keyser and N. J. Leonard, J. Org. Chem. 44, 2989 (1979).

[17]S. W. Schneller and W. J. Christ, J. Org. Chem. 46, 1699 (1981).

[18]S. W. Schneller, A. C. Ibay, and W. J. Christ, J. Heterocycl. Chem. 21, 791 (1984).

[19]G. E. Keyser and N. J. Leonard, unpublished results.

[20]J. N. Wells, Y. J. Wu, C. E. Baird, and J. G. Hardman, Mol. Pharmacol. 11, 775 (1975).

[21]J. E. Garst, G. L. Kramer, Y. J. Wu, and J. N. Wells, J. Med. Chem. 19, 499 (1976).

[22]G. L. Kramer, J. E. Garst, S. S. Mitchel, and J. N. Wells, Biochemistry 16, 3316 (1977).

[23]We are indebted to Drs. K. Gerzon and M. J. Schmidt of Eli Lilly and Company for the data on phosphodiesterase inhibition and antiviral activity throughout.

[24]Compound 14 was characterized by elemental analysis (C, H, N), mass spectrum (m/e 272 for M^+), and ^1H NMR spectrum in $CDCl_3$ (δ 8.5, 8.2, 7.4, all s, arom H's; 8.5-7.5, br, 1, NH; 4.0, d, 2, CH_2; 3.5, s, 3, 7-CH_3; 2.2, m, 1, CH; 1.0, d, 6, $(CH_3)_2$).

[25]S. W. Schneller, A. C. Ibay, E. M. Martinson, and J. N. Wells, J. Med. Chem., in press. We appreciate receiving a preprint of this article from Professor Schneller for inclusion of the findings in this review.

[43]M. A. Alkhader, R. C. Perera, R. P. Sinha, and R. K. Smalley, J. Chem. Soc. Perkin Trans. I, 1056 (1979).
[44]S. Kumar, V. K. Kansal, and A. P. Bhaduri, Indian J. Chem. 20B, 1068 (1981).
[45]C. M. Gupta, A. P. Bhaduri, and N. M. Khanna, Indian J. Chem. 7, 1166 (1969).
[46]R. K. Robins, J. Am. Chem. Soc. 78, 784 (1956).
[47]P. Schmidt and J. Druey, Helv. Chim. Acta 39, 986 (1956).
[48]G. B. Elion, S. W. Callahan, H. Nathan, S. Bieber, R. W. Rundles, and G. H. Hitchings, Pharmacol. 12, 85 (1963).
[49]R. W. Rundles, J. B. Wyngaarden, G. H. Hitchings, G. B. Elion, and H. R. Silbermann, Trans. Assoc. Am. Physicians 76, 126 (1963).
[50]L. S. Goodman and A. Gilman, The Pharmacological Basis of Therapeutics, 5th ed., p. 352. Macmillan, New York (1975).
[51]E. K. T. Cuny and F. W. Lichtenthaler, U. S. Patent 4,223,143, September 16, 1980.
[52]R. H. Foster and N. J. Leonard, J. Org. Chem. 44, 4609 (1979).
[53]E. Cuny, F. W. Lichtenthaler, and A. Moser, Tetrahedron Lett. 21, 3029 (1980).
[54]E. Cuny, F. W. Lichtenthaler, and U. Jahn, Chem. Ber. 114, 1624 (1981).
[55]E. K. T. Cuny, F. W. Lichtenthaler, and A. R. Moser, Eur. Pat. Appl. EP 40,872, Dec. 2, 1981; U. S. Appl. 152,527, May 22, 1980.
[56]F. W. Lichtenthaler and A. Moser, Tetrahedron Lett. 22, 4397 (1981).
[57]T. Spector and D. G. Johns, J. Biol. Chem. 245, 5079 (1970).
[58]K. P. Moder and N. J. Leonard, J. Am. Chem. Soc. 104, 2613 (1982).
[59]M. El-Hashish, M. Mahmoud, and H. El-Fiky, Rev. Roumaine Chim. 24, 1191 (1979).
[60]S. W. Schneller and W. J. Christ, J. Heterocycl. Chem. 19, S-139 (1982).
[61]S. W. Schneller and W. J. Christ, J. Heterocycl. Chem. 18, 539 (1981).
[62]G. Y. Lesher, E. J. Froelich, M. D. Gruett, J. H. Bailey, and R. P. Brundage, J. Med. Pharm. Chem. 5, 1062 (1962).
[63]J. T. Smith, Pharm. Journal 233, 299 (1984).
[64]U. Jordis, G. Cai, and F. Sauter, personal communication. We appreciate the early notification of their research results, which we include here with their permission.
[65]Y. Tanaka and T. Nagata, Jap. Kokai 78, 28196 (1978); Chem. Abstr. 89, 109573c (1978).
[66]S. G. Cottis, P. B. Clark, and H. Tieckelmann, J. Heterocycl. Chem. 2, 192 (1965).
[67]M. A. Rossman, Ph.D. Thesis, University of Illinois, 1983.
[68]E. C. Taylor and W. R. Sherman, J. Am. Chem. Soc. 81, 2464 (1959).
[69]E. C. Taylor and M. Inbasekaran, Heterocycles 10, 45 (1978).
[70]I. J. Pachter and P. E. Nemeth, J. Org. Chem. 28, 1203 (1963).
[71]J. Clark and G. Hitiris, J. Chem. Soc. Perkin Trans. I, 2005 (1984).
[72]F. Yoneda, H. Yamoto, and M. Ono, J. Am. Chem. Soc. 103, 5943 (1981).
[73]F. Yoneda, T. Nagamatsu, and M. Takamoto, Chem. Pharm. Bull. 31, 344 (1983).
[74]Takeda Chemical Industries, Ltd., Jpn. Kokai Tokkyo Koho JP 58,109,485; Chem. Abstr. 99, 175792f (1983).
[75]W. Pfleiderer, Justus Liebigs Ann. Chem. 612, 173 (1958).
[76]N. M. Cherdantseva, W. M. Nesterov, and T. S. Safonova, Chem. Het. Compds. (Khim. Geter. Soedin) 19, 674 (1983).

[39]K. Hirota, K. Maruhashi, T. Asao, and S. Senda, Chem. Pharm. Bull. 30, 3377 (1982).

[40]W. Pfleiderer, K. Schündehütte, and H. Ferch, Justus Liebigs Ann. Chem. 615, 57 (1958).

[41]L. S. Tul'chinskaya, V. D. Klebanova, N. A. Polyakova, G. G. Dvoryantseva, and V. M. Berezovskii, Zh. Obshch. Khim. 44, 406 (1974).

[42]L. S. Tul'chinskaya, T. A. Zhilina, and V. M. Berezovskii, Zh. Obshch. Khim. 40, 868 (1970).

[43]F. Yoneda, K. Kuroda, M. Koga, and T. Ibuka, J. Chem. Soc. Chem. Commun. 872 (1984).

[44]V. Bakthavachalam, M. d'Alarcao, and N. J. Leonard, J. Org. Chem. 49, 289 (1984).

[45]M. d'Alarcao and N. J. Leonard, J. Am. Chem. Soc. 105, 5958 (1983).

[46]K. J. Gibson, M. d'Alarcao, and N. J. Leonard, J. Org. Chem. 50, 2462 (1985).

[47]M. d'Alarcao, V. Bakthavachalam, and N. J. Leonard, J. Org. Chem. 50, 2456 (1985).

[48]T. M. Stevenson and N. J. Leonard, J. Org. Chem. 49, 2158 (1984).

[49]T. A. Krenitsky, S. M. Neil, G. B. Elion, and G. H. Hitchings, Archiv. Biochem. Biophys. 150, 585 (1972).

[70]A. G. Morrice, M. A. Sprecker, and N. J. Leonard, J. Org. Chem. 40, 363 (1975).

[71]S. W. Schneller and A. C. Ibay, J. Org. Chem., in press. We appreciate receiving a preprint of this Note from Professor Schneller for inclusion of the findings in this review.

[72]E. P. Krebs and E. Bondi, Helv. Chim. Acta 62, 497 (1979).

[73]C. F. Spencer and H. R. Snyder, Jr., U. S. Patent 3,947,434, March 30, 1976; Chem. Abstr. 85, 33014r (1976).

[74]C. F. Spencer, H. R. Snyder, Jr., H. A. Burch, and C. J. Hatton, J. Med. Chem. 20, 829 (1977).

[75]H. Kasai, Z. Yamaizumi, K. Wakabayashi, M. Nagao, T. Sugimura, S. Yokoyama, T. Miyazawa, N. E. Spingarn, J. H. Weisburger, and S. Nishimura, Proc. Japan Acad. 56B, 278 (1980).

[76]H Kasai, S. Nishimura, K. Wakabayashi, M. Nagao, and T. Sugimura, Proc. Japan Acad. 56B, 382 (1980).

[77]H Kasai, Z. Yamaizumi, K. Wakabayashi, M. Nagao, T. Sugimura, S. Yokoyama, T. Miyazawa, and S. Nishimura, Chem. Lett. 1391 (1980).

[78]S. Yokoyama, T. Miyazawa, H. Kasai, S. Nishimura, T. Sugimura, and Y. Iitaka, FEBS Lett. 122, 261 (1980).

[79]N. E. Spingarn, H. Kasai, L. L. Vuolo, S. Nishimura, Z. Yamaizumi, T. Sugimura, T. Matsushima, and J. H. Weisburger, Cancer Lett. 9, 177 (1980).

[80]T. Sugimura and M. Nagao in Chemical Mutagens: Principles and Methods of Their Detection, Vol. 6, pp 41-60. Plenum Press, New York (1979).

[81]H. Kasai, Z. Yamaizumi, T. Shiomi, S. Yokoyama, T. Miyazawa, K. Wakabayashi, M. Nagao, T. Sugimura, and S. Nishimura, Chem. Lett. 485 (1981).

[82]R. H. Foster and N. J. Leonard, J. Org. Chem. 45, 3072 (1980).

[83]F. W. Lichtenthaler and E. Cuny, Heterocycles 15, 1053 (1981).

[84]T. Sandmeyer, Helv. Chim. Acta 2, 234 (1919).

[85]S. v. Niementowski, J. Prakt. Chem. 51, 564 (1895).

[86]T. M. Stevenson, F, Kazmierczak, and N. J. Leonard, J. Org. Chem. in press.

[87]I. H. Segel, Enzyme Kinetics. Wiley-Interscience, New York (1975), pp 793-813.

[88]S. W. Schneller and W. J. Christ, J. Heterocycl. Chem. 18, 653 (1981).

[89]S. W. Schneller and W. J. Christ, Lectures Heterocycl. Chem. 6, S-139 (1983).

[90]J. Johnson, E. F. Elslager, and L. M. Werbel, J. Heterocycl. Chem. 16, 1101 (1979).

[91]S. Shinkai, Y. Ishikawa, and O. Manabe, Bull. Chem. Soc. Jap. 56, 1694 (1983).

[92]D. F. Wiemer, D. I. C. Scopes, and N. J. Leonard, J. Org. Chem. 41, 3051 (1976).

[93]J. L. Imbach, J. L. Barascut, B. L. Kam, and C. Tapiero, Tetrahedron Lett. 129 (1974).

[94]J. L. Imbach, J. L. Barascut, B. L. Kam, B. Rayner, C. Tamby, and C. Tapiero, J. Heterocycl. Chem. 10, 1069 (1973).

[94a]L. M. Beauchamp, B. L. Dolmatch, H. J. Schaeffer, P. Collins, D. J. Bauer, P. M. Keller, and J. A. Fyfe, J. Med. Chem. 28, 982 (1985).

[95]R. A. Lessor, K. J. Gibson, and N. J. Leonard, Biochemistry, 23, 3868 (1984).

[96]R. A. Lessor and N. J. Leonard, J. Org. Chem. 46, 4300 (1981).

[97]E. Cuny and F. W. Lichtenthaler, Nucleic Acids Res., Spec. Pub. 1, s25 (1975).

[98]F. W. Lichtenthaler and E. Cuny, Chem. Ber. 114, 1610 (1981).

[99]E. Cuny, W. Eberhard, and F. W. Lichtenthaler, Nucleic Acids Res., Symp. Series 9, 77 (1981).

[100]P. X. Iten, H. Maerki-Danzig, and C. H. Eugster, Dev. Biochem. 4, in Chemistry and Biology of Pteridines (Edited by R. L. Kisliuk and G. M. Brown), pp 105-109 (1978), publ. 1979).

[101]N. J. Leonard, R. S. Hosmane, Y. S. Agasimundin, L. J. Kostuba, and F. T. Oakes, J. Am. Chem. Soc. 106, 6847 (1984).

[102]Y. S. Agasimundin, F. T. Oakes, L. J. Kostuba, and N. J. Leonard, J. Org. Chem. 50, 2468 (1985).

[103]F. T. Oakes and N. J. Leonard, unpublished results.

[104]L. B. Townsend, G. A. Bhat, F.-L. Chung, K. H. Schram, R. P. Panzica, and L. L. Wotring, INSERM Symp. 81, 37 (1979).

[105]F.-L. Chung, R. A. Earl, and L. B. Townsend, Tetrahedron Lett. 21, 1599 (1980).

[106]F.-L. Chung, R. A. Earl, and L. B. Townsend, J. Org. Chem. 45, 2532 (1980).

[107]K. H. Schram and L. B. Townsend, J. Chem. Soc., Perkin Trans. I, 1253 (1975).

[108]R. L. Tolman and L. B. Townsend, Tetrahedron Lett. 4815 (1968).

[109]N. J. Leonard, in Phosphorus Chemistry Directed Towards Biology (Edited by W. J. Stec), pp 85-94. Pergamon Press, Oxford and New York (1980).

[110]N. J. Leonard, D. I. C. Scopes, P. VanDerLijn, and J. R. Barrio, Biochemistry 17, 3677 (1978).

[111]N. J. Leonard and G. E. Keyser, Proc. Natl. Acad. Sci. U. S. A. 76, 4262 (1979).

[112]K.-I. Imai, S. Fujii, K. Takanohashi, Y. Furukawa, T. Masuda, and M. Honjo, J. Org. Chem. 34, 1547 (1969).

[113]D. I. C. Scopes, J. R. Barrio, and N. J. Leonard, Science 195, 296 (1977).

[114]J. G. Moffatt and H. G. Khorana, J. Am. Chem. Soc. 83, 649 (1961).

[115]R. Marumoto, T. Nishimura, and M. Honjo, Chem. Pharm. Bull. (Tokyo), 23, 2295 (1975).

[114]M. J. Schmidt, L. L. Truex, N. J. Leonard, D. I. Scopes, and J. R. Barrio, J. Cyclic Nucleotide Res. 4, 201 (1978).

[117]J. R. Barrio, M. del C. G. Barrio, N. J. Leonard, T. E. England, and O. C. Uhlenbeck, Biochemistry 17, 2077 (1978).

[118]M. Honjo, K. Imai, Y. Furukawa, H. Morijama, K. Yasumatsu, and A. Imada, Takeda Kenyuskko Nempo 22, 47 (1963).

[119]P. VanDerLijn, J. R. Barrio, and N. J. Leonard, Biochemistry 18, 5557 (1979).

[120]P. VanDerLijn, J. R. Barrio, and N. J. Leonard, Proc. Natl. Acad. Sci. U. S. A. 75, 4204 (1978).

[121]L. Noda, Enzymes, 3rd Ed., 8, 279 (1973).

[122]N. J. Leonard and R. A. Laursen, Biochemistry 4, 365 (1965).

[123]T. E. England and O. C. Uhlenbeck, Biochemistry 17, 2069 (1978).

[124]R. F. Kauffman, H. A. Lardy, J. R. Barrio, M. del C. G. Barrio, and N. J. Leonard, Biochemistry 17, 3686 (1978).

[125]We are indebted to Drs. K. Gerzon and E. W. Shuey of Eli Lilly and Company for the data on platelet aggregation.

[126]F. T. Hartl, R. Roskoski, Jr., M. S. Rosendahl, and N. J. Leonard, Biochemistry 22, 2347 (1983).

[127]D. Bhatnagar, R. Roskoski, Jr., M. S. Rosendahl, and N. J. Leonard, Biochemistry 22, 6310 (1983).

[128]D. Bhatnagar, F. T. Hartl, R. Roskoski, Jr., R. A. Lessor, and N. J. Leonard, Biochemistry 23, 4350 (1984).

[129]D. Bhatnagar, D. B. Glass, R. Roskoski, Jr., R. A. Lessor, and N. J. Leonard, Biochemistry 24, 1122 (1985).

[130]R. N. Puri, D. Bhatnagar, and R. Roskoski, Jr., unpublished results.

[131]P. VanDerLijn, J. R. Barrio, and N. J. Leonard, J. Biol. Chem. 253, 8684 (1978).

[132]R. E. London and P. G. Schmidt, Biochemistry 11, 3136 (1972).

[133]O. Uhlenbeck, University of Illinois, private communication.

[134]L. Rowen and A. Kornberg, J. Biol. Chem. 253, 758 (1978).

[135]A. Kornberg, J. F. Scott, and LeR. L. Bertsch, J. Biol. Chem. 253, 3298 (1978).

[136]M. S. Rosendahl, N. J. Leonard, and M. DeLuca, Photochem. Photobiol. 35, 857 (1982).

[137]D. L. Sloan and A. S. Mildvan, J. Biol. Chem. 251, 2412 (1976).

[138]M. S. Rosendahl, G. M. Omann, and N. J. Leonard, Proc. Natl. Acad. Sci. U. S. A. 79, 3480 (1982).

[139]T. M. Stevenson, Ph.D. Thesis, University of Illinois, 1983.