Studies on Condensed Pyrimidine Systems. XXV. 2,4-Diaminopyrido[2,3-*d*]pyrimidines. Biological Data

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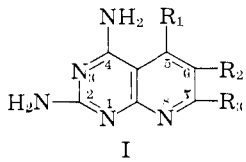
Structure–activity relationships have been examined in a series of 2,4-diaminopyrido[2,3-*d*]pyrimidines bearing alkyl and aralkyl substituents in the pyridine moiety. In common with other 2,4-diaminopyrimidines, these substances are inhibitors of dihydrofolate reductases with considerable species specificity of action. Significant antibacterial and antiprotozoal effects are exhibited by various members of the series, and sulfonamide potentiation is a general feature of their action. The largest chemotherapeutic indices against gram-negative bacteria were found in the subgroup possessing a 5-methyl and a 6-branched-alkyl group of four or five carbon atoms and unsubstituted in the 7 position.

It was recognized some time ago that various 2,4-diaminopyrimidines and condensed pyrimidine systems show selective toxicities toward different organisms which reflect selective binding at a locus concerned with the utilization of folates.[1] It was postulated that minor changes in the fine structure of these compounds adapt specific compounds to a close approximation of the protein surfaces of particular species.[1c] Developments of the general aspects of this problem have led to the identification of the locus of action as the enzyme, dihydrofolate reductase. Although this enzyme appears to be present in all living cells, interspecific differences in the individual enzymes have been found.[2c] In pursuit of various aspects of the problem, a large numer of 2,4-diaminopyrimidine and condensed pyrimidine derivatives have been prepared in these laboratories.[2a]

Prior to 1958 a number of pyrido[2,3-*d*]pyrimidines were prepared.[3] The preceding papers[4] describe the syntheses of 2,4-diaminopyrido[2,3-*d*]pyrimidines of structure I. The derivatives prepared earlier[3] were 7-substituted, and analogy with the folic acid molecule suggested that derivatives with substituents only in the 5 and 6 positions would be more active. This paper presents pertinent data on the antimicrobial activities



I

R_1, R_2, R_3 = H, alkyl, aryl, aralkyl

of several subgroups of the pyridopyrimidines from which certain structure–activity relationships can be deduced. It seems probable that selected members of the series may have antibacterial activity of useful dimensions, either alone or, more probably, as potentiators of sulfonamides.

Methods and Results

All of the compounds reported in this study were assayed in the agar plating test described previously.[5] Table I shows the zones of inhibition obtained for each compound against four typical organisms. Organisms other than those given in Table I were also tested by this method. *Salmonella typhosa* and *Aerobacter aerogenes* are similar to *Proteus vulgaris* in their sensitivity to these compounds. Only trace activities for any of the compounds against *Pseudomonas aeruginosa* were found in this test. When the test was modified by the inclusion of low levels (25 μg/ml) of sulfadiazine in the medium, some of the compounds did show activity against *Ps. aeruginosa*. The concentration of sulfadiazine used would not alone inhibit growth of *Ps. aeruginosa* on the plates. A comparison of the zones of inhibition of *Ps. aeruginosa* caused by a number of compounds with and without sulfadiazine is given in Table II. It was found that these compounds were very nearly as effective against a penicillin-resistant strain of *Staphylococcus aureus* (S.a./r.) as against the penicillin-sensitive strain (S.a./s.) shown in Table I. Table III illustrates this comparison for six of the more active compounds.

The minimum inhibitory concentrations (MIC) for most of the compounds listed in Table I against a number of bacteria were also determined as previously described.[5] The lowest concentration at which growth of the organism was inhibited was recorded. Table IV shows the MIC values of 41 of the 2,4-diaminopyrido-[2,3-*d*]pyrimidine derivatives against the same organisms that were used in the agar plate test. This test was run with other organisms, and Table V shows the MIC values of five of the most active derivatives against 18 organisms.

Based on the *in vitro* tests, a number of the more active derivatives were chosen for *in vivo* assay. In this assay, groups of eight or ten mice were inoculated

(1) (a) G. H. Hitchings, G. B. Elion, H. VanderWerff, and E. A. Falco, *J. Biol. Chem.*, **174**, 765 (1948); (b) G. H. Hitchings, E. A. Falco, G. B. Elion, S. Singer, G. B. Waring, D. J. Hutchison, and J. H. Burchenal, *Arch. Biochem. Biophys.*, **40**, 479 (1952); (c) G. H. Hitchings, E. A. Falco, H. VanderWerff, P. B. Russell, and G. B. Elion, *J. Biol. Chem.*, **199**, 43 (1952).

(2) (a) G. H. Hitchings, and J. J. Burchall, *Advan. Enzymol.*, **27**, 417 (1965); (b) J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, **1**, 126 (1965); (c) G. H. Hitchings and J. J. Burchall, *Fed. Proc.*, **25**, 881 (1966).

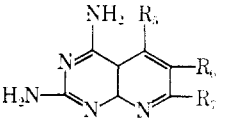
(3) R. K. Robins and G. H. Hitchings, *J. Am. Chem. Soc.*, **80**, 3449 (1958).

(4) (a) B. S. Hurlbert, K. W. Ledig, P. Stenbuck, B. F. Valenti, and G. H. Hitchings, *J. Med. Chem.*, **11**, 703 (1968); (b) B. S. Hurlbert and B. F. Valenti, *ibid.*, **11**, 708 (1968).

(5) B. Roth, E. A. Falco, and G. H. Hitchings, *ibid.*, **5**, 1103 (1962).

TABLE I

STRUCTURE OF 2,4-DIAMINOPYRIDO[2,3-*d*]PYRIMIDINE DERIVATIVES AND ZONES OF INHIBITION IN THE AGAR PLATING TEST



Compd	R_5	R_6	R_7	Zone size, mm[a]		
				S.a. s.[b]	*S. faecalis*	*E. coli*	*P. vulgaris*
1	H	H	H	0	13p	0	0
2	H	H	CH_3	0	0	0	0
3	H	H	$CH_2CH(CH_3)_2$	...	...	...	...
4	H	H	C_6H_5	0	0	0	Tr
5	H	H	*p*-C_6H_4Br	Tr	0	Tr	0
6	H	H	*p*-C_6H_4Cl	11	...	0	...
7	H	CH_3	CH_3	14p	24	16	20p
8	H	CH_3	C_2H_5	17	0	24p	Tr
9	H	CH_3	C_3H_7	24, 28p	15	17, 28p	Tr
10	H	CH_3	C_6H_5	17	13p	23p	Tr
11	H	C_2H_5	C_3H_7	28	22	22	Tr
12	H	C_2H_5	C_6H_5	22	24p	19p	0
13	H	C_2H_5	*p*-C_6H_4Cl	14	12p	0	0
14	H	C_3H_7	H	27p	22	21p	0
15	H	C_3H_7	C_4H_9	25	17	Tr	0
16	H	C_3H_7	C_6H_5	20	...	0	...
17	H	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	16	...	19	...
18	H	C_4H_9	H	32	24	23	0
19	H	C_4H_9	C_6H_5	0	...	0	...
20	H	$CH_2CH(CH_3)_2$	H	30	22	19p	0
21	H	C_5H_{11}	H	27	20	21	0
22	H	C_6H_{13}	H	26	24	12p	0
23	H	C_9H_{19}	H	15p	15	12p	0
24	H	C_6H_5	H	25	25	12	0
25	H	$CH_2C_6H_5$	H	34	34	26	Tr
26	H	*p*-$CH_2C_6H_4OCH_3$	H	28	26	15	0
27	H	*p*-$CH_2C_6H_4CH_3$	H	31	27	21	0
28	H	*p*-$CH_2C_6H_4Cl$	H	29	34	14p	Tr
29	H	*o*-$CH_2C_6H_4Cl$	H	33	34	17	0
30	H	*p*-$CH_2C_6H_4NO_2$	H	27	23	0	0
31	H	$CH_2CH_2C_6H_5$	H	30	30	0	13
32	H	-$(CH_2)_6$-		0	0	20	Tr
33	CH_3	H	H	0	17p	0	0
34	CH_3	H	CH_3	17	...	0	...
35	CH_3	CH_3	H	0	...	16p	0
36	CH_3	CH_3	CH_3	13	22	23	0
37	CH_3	C_3H_7	H	18p	50	29	29p
38	CH_3	C_4H_9	H	20	...	31	25
39	CH_3	$CH(CH_3)C_2H_5$	H	24	61	34	26p
40	CH_3	C_5H_{11}	H	23	42	29	23p
41	CH_3	$CH_2CH_2CH(CH_3)_2$	H	25	46	26	21p
42	CH_3	$CH(CH_3)C_3H_7$	H	30	45	40p	30p
43	CH_3	C_6H_{13}	H	22p	32	23p	Tr
44	CH_3	C_7H_{15}	H	17	27	18p	0
45	CH_3	$CH_2C_6H_5$	H	24	41	31	24p
46	CH_3	*p*-$CH_2C_6H_4CH_3$	H	21	36	25	0
47	CH_3	*p*-$CH_2C_6H_4Cl$	H	22	29	21	18p
48	CH_3	*p*-$CH_2C_6H_4OCH_3$	H	24	38	25	0
49	CH_3	*o*-$CH_2C_6H_4Cl$	H	25	42	27	27p
50	CH_3	*o*-$CH_2C_6H_4OCH_3$	H	20	40	20	19p
51	C_2H_5	$CH_2C_6H_5$	H	0	40	23p	0
52	C_3H_7	H	H	0	24p	16p	Tr
53	C_3H_7	C_2H_5	H	0	30	0	0
54	C_3H_7	$CH_2C_6H_5$	H	0	30	0	0
55	C_6H_5	H	H	0	0	0	0
56	C_6H_5	H	C_6H_5	0	...	0	...
57	C_6H_5	$CH_2C_6H_5$	H	0	0	0	0
58	-$(CH_2)_4$-		H	16	51	29	19p

[a] A p following the number indicates a zone of partial inhibition; Tr = trace activity. [b] S.a. s. = *Staphylococcus aureus*, penicillin-sensitive strain.

TABLE II

COMPARISON OF ZONES OF INHIBITION OF *Pseudomonas aeruginosa* IN AGAR PLATING TEST BY SEVERAL DERIVATIVES WITH AND WITHOUT SULFADIAZINE (SD)

Compd	Zone of inhib, mm: Alone	With SD[a]
14	0	0
18	0	11
21	0	0
22	0	0
33	0	0
37	15	23
38	0	21
40	0	20
42	18	28
43	0	12

[a] 25 μg/ml of sulfadiazine was added to the medium. This level of sulfadiazine alone does not inhibit growth in the medium.

TABLE III

COMPARISON OF ZONES OF INHIBITION IN AGAR PLATING TEST OF PENICILLIN-SENSITIVE AND PENICILLIN-RESISTANT STRAINS OF *Staphylococcus aureus*

Compd	Av zone size, mm[a]: S.a./s[b]	S.a./r[b]
18	32	29
22	26	28
25	34	34
38	20	16p
43	22p	22p
45	24	24

[a] A p following the number indicates a zone of partial inhibition. [b] S.a./s = *Staphylococcus aureus*, penicillin-sensitive strain; S.a./r = penicillin-resistant strain.

intraperitoneally with 0.5 ml of a suspension (in 5% mucin) of the test organism. The size of the inoculum was chosen to be sufficient to cause 100% mortality within 1 day. In each test, one group of mice served as an untreated control. The treated mice were given a single dose of drug *per os* immediately after inoculation with the bacterium. The 2,4-diaminopyrido[2,3-*d*]pyrimidine derivatives were given both alone and in combination with doses of sulfadiazine which alone would give less than 20% survival. Table VI shows the results in terms of survivals at the end of 7 days of tests of several derivatives against four organisms. Single-dose, toxic levels of the compounds were determined approximately and are also shown in Table VI.

TABLE IV

MINIMUM INHIBITORY CONCENTRATIONS OF A NUMBER OF DERIVATIVES

Compd	MIC, μg/ml: *S. faecalis*	*S. aureus*	*S. typhosa*	*E. coli*	*Ps. aeruginosa*	*P. vulgaris*	*A. aerogenes*
5		250		>1000		>1000	
6		12.5	>100	>100	>100	>100	
7		0.4	1.6	0.8	>100	>100	
8		0.2	6.2	6.2	>100	50	
9		0.5	8.0	16.0	>1000	250	
11		0.2	0.8	1.6	>100	12.5	
12		0.2	6.2	3.2	>100	25	
13		0.2	12.5	>100	>100	>100	
14		0.25–0.6	31	16–62	>1000	125	
15		0.125	8–16	125	>500	>500	
16		0.2–2.5	25	25	>100	>100	
18		0.125–0.25	2–16	16–31	>1000	250–500	
20		0.25	16	16–31	500–1000	125–250	
21				31	250–1000	>125	
22		0.03–0.3	12–16	50–>1000	1000	1000	
23		25	>100	1000	>1000	>1000	
24		16	>1000	>1000	1000	>1000	
25		0.07–0.2	0.7	6–10	1000	62	
26	0.3	0.25		20	>50	>50	>50
27	0.1	0.12		9	>50	50	50
28	0.1	0.12		12.5	>50	>50	>50
29	0.1	0.1		6	>50	50	25
30	1.6	0.5		>50	>50	>50	>50
31		2	250	125–1000	1000	>1000	
32		0.8	1.6	1.6	>50	1.6	
33		125	62	62–125	250–1000	250–1000	
35				2	62	4–8	
37		1	0.25	0.25–0.5	16–250	1	
39	0.006	0.25–0.6	0.02–0.06	0.1–0.5	8–62	0.12–1.2	0.5
40	0.004	0.125–0.25	0.01	0.5	250–1000	2–8	
41				1–2	62	8–16	0.5
42		0.2–2.5	0.1	0.25–0.4	4–100	1.0	
43				0.5	1000	4–8	
44				2.0	>125	8–16	
45		4	1	16	62–250	2–4	
46	0.02	1		1.6	>50	12	4
47	0.02	0.5		1.6	>50	12	4
48	0.02	0.4		2	>50	6	5
49	0.01	0.25		1	>50	3	3
50	0.02	0.5		1.6	>50	8	2
53		500	125	250	>1000	>1000	

TABLE V

ACTIVITY OF FIVE OF THE MORE ACTIVE DERIVATIVES IN TERMS OF MINIMUM INHIBITORY CONCENTRATIONS

Organism	CN no.[c]	MIC, μg/ml				
		18	**25**	**39**	**40**	**45**
Streptococcus pyogenes	10	0.002–0.1	0.01	0.002	0.002	0.006
Streptococcus pneumoniae	33		0.25	0.06	0.01	0.06
Streptococcus faecalis	478	0.125–0.25	0.3	0.006	0.004	0.06
Staphylococcus aureus	491		0.2	0.25	0.25	0.5
Corynebacterium diphtheriae	83		0.1	0.2	0.25	0.05
Corynebacterium pyogenes	1856	2–1000	0.2	0.06	0.025	0.06
Escherichia coli	314	16–31	6	0.1	0.5	0.5
Aerobacter aerogenes	345		25	0.5	0.5	2
Klebsiella pneumoniae	3632		25	0.5	0.5	2
Salmonella typhi	512	2–16[a]	6	0.25	0.8	0.8
Shigella dysenteriae	1513	16–31	4	0.1	0.3	0.3
Vibrio comma	248	4–8	1	0.06	0.06	0.1
Pasteurella septica	1066	1–4[b]	1	0.01	0.02	0.06
Haemophilus influenzae	1714		1	0.06	0.06	0.06
Moraxella lacunata	5119		6	0.02	0.1	0.1
Pseudomonas aeruginosa	200	>1000	1000	16–62	250–1000	62–250
Proteus vulgaris	329	250–500	25	1	2	2
Neisseria gonorrhoeae	503		9	2	6	4

[a] *S. typhosa.* [b] *P. boviseptica.* [c] Strain reference number, culture collection, Wellcome Research Laboratories, Beckenham.

The derivatives were also tested in a system which has been developed in order to detect dihydrofolate reductase inhibitors.[6] In this test the wild strain of *Lactobacillus casei*, which required exogenous folic acid, was grown in the presence of two levels of folate, one just sufficient for half-maximal growth, and one containing an excess of the vitamin. Folate antagonism by added drug could be detected by specific reversal of growth inhibition in the medium containing the higher level of folate. The results of such tests on a number of pyrido[2,3-*d*]pyrimidine derivatives are recorded in Table VII.

The isolation of dihydrofolate reductases from bacterial and mammalian liver cells has been described.[2b] A number of 2,4-diaminopyrido[2,3-*d*]pyrimidine derivatives were assayed for their inhibition of the enzymes isolated from rat liver, *Escherichia coli*, and *Ps. aeruginosa*. The results of this test are reported in Table VIII as the molar concentration of drug giving a 50% inhibition of enzyme activity.

Some of the 2,4-diaminopyrido[2,3-*d*]pyrimidines have been tested for their effects on organisms other than bacteria. Five compounds (**14**, **25**, **37**, **40**, **43**) were found to be effective against malaria (*Plasmodium gallinaceum*) in chicks, having minimum effective doses of 0.6–4.0 mg/kg. Compound **39** was an effective inhibitor of coccidiosis (*Eimeria tenella*) in chicks and **25** and **40** showed slight activity below toxic levels. Compounds **14** and **23** were inactive against coccidiosis. A number of the compounds showed significant activity against *P. gallinaceum* in chicks and *Plasmodium berghei* in mice. Compounds showing minimum effective doses below 5 mg/kg × 7 were **12**, **13**, **14**, **25**, **37**, **39**, **40**, **43**. Various derivatives were ineffective against the intestinal parasites *Nippostrongylus braziliensis* and *Hymenolepis nana* and against a number of fungi. With one exception there was no effect on Adenocarcinoma 755 and Sarcoma 180 in mice. At near toxic doses compound **47** showed some inhibition of Sarcoma 180.

(6) G. H. Hitchings, G. B. Elion, E. A. Falco, P. B. Russell, M. B. Sherwood, and H. VanderWerff, *J. Biol. Chem.*, **183**, 1 (1950).

Discussion

The *locus* of action of the 2,4-diaminopyrimidines and related substances has been established by a variety of evidence [1b,1c,2a] culminating in studies of their binding to isolated dihydrofolate reductases.[2b] Their mode of action as chemotherapeutic agents depends to a considerable extent on the difference in binding to host and parasite enzyme, respectively. In this regard, the pyrido[2,3-*d*]pyrimidines fall short of the extremely favorable binding ratios exhibited by some of the 5-benzylpyrimidines.[2c] Nevertheless, significantly tighter binding to microbial than to rat liver enzymes is exhibited by a number of the derivatives (Table VIII), and their utility in chemotherapeutic trials has been demonstrated (Table VI).[5]

I has been shown[7] that bacterial inhibition by 2,4-diaminopyrido[2,3-*d*]pyrimidine derivatives occurs at the level of reduction of folic acid. Growth of *Streptococcus faecalis* in the presence of folic acid or leucovorin is inhibited by **25**. However, a 100 times greater concentration of the inhibitor is required to block growth in the presence of the reduced cofactor (leucovorin) than in the presence of folic acid, indicating that the former essentially bypasses the primary inhibition mechanism.

The results of tests of the 2,4-diaminopyrido[2,3-*d*]pyrimidines in combination with sulfadiazine are pertinent to a consideration of the mechanism of action. Most bacteria synthesize dihydrofolic acid *de novo* from *p*-aminobenzoic acid and a pteridine (a phosphate ester of 2-amino-3,4,7,8-tetrahydro-6-hydroxymethyl-4-oxopteridine) and subsequently reduce it. Sulfadiazine competes with *p*-aminobenzoic acid in the first reaction, and the 2,4-diaminopyrido[2,3-*d*]pyrimidine derivatives are inhibitors of the second. Since the resultant blocks lie in sequence on the same biochemical pathway, the use of the two antimetabolites in combi-

(7) (a) G. H. Hitchings, T. A. Herrmann, B. S. Hurlbert, and S. R. M. Bushby, Proceedings of the IIIrd International Congress of Chemotherapy, Stuttgart, July 1963, p 1363; (b) G. H. Hitchings, J. J. Burchall, and R. Ferone, 16th Symposium of the Society for General Microbiology, Biochemical Studies of Antimicrobial Drugs, London, 1966, p 294.

TABLE VI

RESULTS OF TREATMENT OF BACTERIAL INFECTIONS IN MICE WITH SEVERAL DERIVATIVES AND SULFADIAZINE (SD) AND ACUTE TOXICITY TO MICE

Compd	Organism	Dose, mg/kg		Survival, %[b]	Av days survival[c]	LD_{50}, mg/kg *po* (ip)
		Compd	SD[a]			
14	*S. pyogenes*	100	0	33	4.1	380
	P. vulgaris	100	0	17	1.1	
	P. vulgaris	100	100	17	2.0	
15	*P. vulgaris*	200	0	0	<1	>750
	P. vulgaris	100	100	25	2.1	
	S. pyogenes	250	0	35		
18	*S. pyogenes*	200	0	84	5.7	>600
	P. vulgaris	200	0	0	<1	
	P. vulgaris	100	100	58	4.4	
22	*P. vulgaris*	200	0	0	<1	>1200
	P. vulgaris	100	100	25	2.5	
24	*P. vulgaris*	200	0	0	<1	
	P. vulgaris	100	100	25	2.1	
25	*S. pyogenes*	200	0	33	3.5	>870 (610)
	P. vulgaris	200	0	0	<1	
	P. vulgaris	100	100	25	2.7	
26	*S. aureus*	21	0	12		>1000 (420)
	S. aureus	68	0	24		
	S. aureus	215	0	12		
	S. aureus	0	43	12		
	S. aureus	21	43	75		
	S. aureus	68	43	87		
	S. aureus	215	43	87		
27	*S. aureus*	21	0	0		>1000 (105)
	S. aureus	68	0	12		
	S. aureus	215	0	12		
	S. aureus	0	43	12		
	S. aureus	21	43	87		
	S. aureus	68	43	87		
	S. aureus	215	43	100		
32	*P. vulgaris*	200	0	8		>1000 (450)
	P. vulgaris	100	100	58	4.5	
39	*S. pyogenes*	25	0	84	5.8	57
	E. coli	25	0	17	2.5	
	P. vulgaris	50[d]	0	10	1.4	
	P. vulgaris	25	0	60	4.3	
	P. vulgaris	12.5	0	5	<1	
	P. vulgaris	50[d]	100	10	1.5	
	P. vulgaris	25	100	92	6.5	
	P. vulgaris	12.5	100	78	5.7	
	P. vulgaris	5	100	60	4.3	
	P. vulgaris	0	100	15	1.2	
40	*S. pyogenes*	100	0	100		450
	P. vulgaris	100	0	8		
	P. vulgaris	100	100	100		

[a] SD = sulfadiazine, given at dose levels which alone will achieve less than 20% survial. [b] Inoculum of infection is sufficient to cause 100% mortality within 1 day. [c] Based on a 7-day test. [d] Toxic dose.

nation gives potentiative effects.[2a] This is shown in both *in vitro* and *in vivo* tests. At the concentrations used in the agar plating test, only minimal activities *vs. Ps. aeruginosa* can be detected, yet in the presence of subinhibitory amounts of sulfadiazine, a number of derivatives give zones of inhibition (Table II).

The derivatives alone are effective antibacterial agents *in vivo*. When used in combination with subinhibitory levels of sulfadiazine they effect cures at lower dose levels than when used alone. This potentiation is quite striking in some cases as is shown in Table VI. A dose of 43 mg/kg of sulfadiazine gave 12% cures of an *S. aureus* infection in mice. A dose of 215 mg/kg of **27** also gave 12% cures of the same infection, yet a combined dose of 43 mg/kg of sulfadiazine and 215 mg/kg of **27** gave 100% cures.

Final confirmation that the primary locus of activity of the 2,4-diaminopyrido[2,3-*d*]pyrimidine derivatives is the inhibition of dihydrofolate reductase activity came from the studies with the isolated dihydrofolate reductase enzymes. The derivatives were found to bind to and inhibit the activity of the isolated enzymes from a number of sources as shown in Table VIII. Comparison of the results in Table VIII with those in Table I shows that the spectrum of inhibition of a bacterial enzyme by a number of derivatives is strongly correlated with the spectrum of activity against the intact source organism. The concentration of drug required for inhibition of growth of the intact cell is always considerably higher than that required for inhibition of the isolated enzyme. Table VIII shows that the compounds inhibit the enzymes isolated from

TABLE VII

INHIBITION OF *Lactobacillus casei* GROWTH

Compd	Concn, μg/ml	% change in growth[a] OFA	FA+	Compd	Concn, μg/ml	% change in growth[a] OFA	FA+
1	50	−24	0	27	1000	−97	−95
	1000	−92	−56		50	−84	−90
2	100	−15	0	29	1	−91	−92
3	50	−73	+12		0.1	−90	−8
	1000	−89	−92	30	1	−88	−51
4	50	−55	0	32	10	−76	0
	1000	−95	−98		100	−94	−91
5	10	−30	0	33	50	−93	−94
	100	−95	−97		10	−91	−29
6	50	−97	−98	34	50	−92	−23
	5	−26	0		1000	−93	−98
7	100	−89	0	35	10	−86	−72
9	10	−72	−66	36	10	−68	0
	100	−92	−80		100	−94	−96
10	50	−88	0	37	10	−96	−97
	1000	−95	−98		1	−95	−80
11	10	−73	0	38	10	−97	−98
	100	−94	−79		1	−96	−84
12	10	−82	0		0.5	−94	−93
	100	−95	−92		0.05	−76	−11
13	10	−61	0	40	0.5	−97	−90
	100	−92	0		0.05	−90	0
14	1000	−92	−93	41	1.0	−100	−94
	50	−92	−70		0.5	−98	−85
15	10	−83	0	42	0.5	−95	−97
16	10	−93	−31		0.05	−89	−49
	100	−93	−27	43	0.5	−93	−95
17	10	−53	0		0.05	−79	−19
	100	−90	−80	44	0.5	−95	−91
18	1000	−95	−94		0.05	−77	−17
	50	−95	−88	45	1.0	−88	−98
	10	−91	−85		0.5	−88	−48
19	10	−11	0	46	1.0	−97	−97
21	10	−91	−93		0.1	−92	−83
	1	−71	−29	47	0.1	−91	−88
22	50	−94	−98	48	1.0	−91	−95
23	1	−92	−96		0.1	−90	−59
25	5	−99	−100	52	1.0	−86	−35
	1	−100	−90	53	10	−78	−10
	0.1	−78	−56	56	50	−29	−29
26	50	−91	−93		1000	−97	−98

[a] OFA = medium containing sufficient folate for half-maximal growth; FA+ = medium containing excess folate.

Ps. aeruginosa to about the same extent as they inhibit the enzymes from *E. coli*. The much lower *in vitro* activity *vs. Ps. aeruginosa* is probably due to relatively poor absorption of the drug by the living *Ps. aeruginosa* cells.[7b] Enzyme inhibition by the 5-methyl-6-alkyl series of derivatives increased with increasing 6-alkyl chain length. However, the increased enzyme inhibition observed in the long-chain members of this series was not reflected in greater *in vitro* inhibition as shown in Table I. Compound **38** gave the largest zone of inhibition of *E. coli* growth although the pentyl to heptyl homologs were all more inhibitory to the enzymes. Poorer transport of the higher homologs across the cell walls is the probable cause of this phenomenon.

The structure–activity relationships among the 2,4-diaminopyrido[2,3-*d*]pyrimidine derivatives have been developed *in vitro* in plating and MIC tests and in studies of isolated enzymes and *in vivo* in chemotherapeutic trials in mice. In general the various sets of data agree closely. The parent, unsubstituted compound has only meager activity, none of the derivatives being less active. The most critical position of substitution is the 6 position; derivatives unsubstituted in this position have activities approximately equal to those of the parent compound. The activities in a series of derivatives having the same 5 and 7 substituents increase regularly with increasing size of the 6 substituent, reaching a maximum against intact cells with a 6-alkyl group having five or six carbon atoms or with a 6-benzyl group, *e.g.*, **33**, **35**, **37–45** in Table I. A compound having a branched alkyl group in the 6 position is more active than its straight-chain isomer (*cf.* **38** and **39** and **40–42**).

The presence of a small substituent on the benzene ring of a 6-benzyl derivative has relatively little effect on activity (*cf.* **45–50** and **25–29**). Activity is least sensitive to changes in the 7 position but generally is diminished with increasing size of this substituent. The most active compounds are unsubstituted in the 7 position, but 7 substituents tend to increase the effective size of the substituent in the 6 position. In the series of 5-unsubstituted 7-phenyl derivatives (**4**, **10**, **12**, **16**, **19**) activity reaches a peak in **12** (R_6 = C_2H_5) whereas in the series of 5,7-unsubstituted

TABLE VIII

MOLAR CONCENTRATIONS FOR 50% INHIBITION OF DIHYDROFOLIC ACID REDUCTION BY THREE ISOLATED ENZYMES

Compd	50% inhibitory concn ×10⁸, M — Rat liver	*E. coli*	*Ps. aeruginosa*
1	20,000	14,000	
7	4,400	140	
12	280	14	
14			100
15	42	1.9	14
18	46	50	70
20			30
21			37
22			40
23			600
24			700
25	25	40	46
31	42		160
33	2,200	270	320
35	180	23	85
37	7.0	1.5	3.0
38	26	2.0	2.0
39		0.20	0.80
40	6.0	0.38	1.2
41			0.70
42			0.40
43	3.0	0.92	1.4
44	3.5	0.78	1.8
45	4.0	1.0	0.23
53	25		
54	6.0	120	
58	1.5		

derivatives (**14**, **18**, **21**, **22**, **23**) activity reaches a peak in **18** ($R_6 = C_4H_9$).

For antibacterial activity, the size of the 5 substituent is quite limited since only 5-methyl and 5-unsubstituted compounds show appreciable activity. This is illustrated in the series of 6-benzyl-7-unsubstituted compounds (**25**, **45**, **51**, **54**, **57**). As the group in the 5 position increases above methyl, activity is sharply decreased. The rat liver enzyme is not as sensitive to changes in the 5 substituent as are the bacterial enzymes. As is shown in Table VIII, **54** is nearly as inhibitory as **45** to the rat liver enzyme, whereas **54** is only $^1/_{100}$ as inhibitory as **45** toward the *E. coli* enzyme. There is a notable change in the spectra of activity between the 5-unsubstituted and 5-methyl series of derivatives which has been documented previously,[7] and which can be seen in Tables I and IV. Generally the 5-unsubstituted derivatives are more active against *S. aureus* than against *E. coli* or *P. vulgaris* and the 5-methyl derivatives are more active against *E. coli* and *P. vulgaris* than against *S. aureus* (compare **18** with **38**, and **25** with **45**).

An over-all picture has been developed of a 2,4-diaminopyrido[2,3-*d*]pyrimidine derivative having no substituent in the 7 position, no substituent or a methyl group in the 5 position, and a substituent of medium bulk, consisting of an alkyl group of four or five carbon atoms or a benzyl group, in the 6 position. Such derivatives are potent inhibitors of bacterial dihydrofolate reductases and thus have general antibacterial activity. The spectrum of this activity depends primarily on the substituent in the 5 position. The action of the derivative is potentiated by sulfonamides and combinations of the two drugs have useful chemotherapeutic indices for the treatment of bacterial infections in mammalian species.

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Synthesis and Biological Activity of Some N⁶-Alkyladenosines

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The synthesis of N⁶-isoamyl-, N⁶-*n*-propyl-, N⁶-isopropyl-, and N⁶-allyladenosine and N⁶-isoamyladenine was carried out by a general method involving the condensation of 6-chloropurine riboside or its base with an excess of the appropriate amine in an alcoholic medium in the presence of calcium carbonate. By this method essentially pure products were readily obtained. The biological effects of these compounds were examined with cell cultures of Sarcoma 180 and its subline AH/S and with cultures of *Streptococcus faecalis* and *Escherichia coli*. All the compounds synthesized showed growth inhibitory activity in one or the other of these test systems.

N⁶-(Δ^2-Isopentenyl)adenosine[1,2] (IPA) is a very potent cytokinin with activity equal to or better than kinetin and zeatin in the bud tests of Wickson and Thimann.[3] This compound was also shown to inhibit the growth of human and mouse tumor cells *in vitro*.[4] Because of this biological activity of IPA and because several N⁶-alkylpurine bases have shown kinetin activity in tobacco callus growth tests,[5] it was of interest to determine whether other N⁶-alkyl derivatives of adenosine would also show biological activity. For this reason the following adenosine derivatives were prepared and their biological activity was examined: N⁶-isoamyl- (I), N⁶-*n*-propyl- (II), N⁶-isopropyl- (III), N⁶-allyl- (IV), N⁶-isoamyl- (V), and N⁶-methyladenosine. Compounds I–IV have not been reported thus far, while V has been studied by Strong[5a] and has been shown to possess kinin activity; however, the method of

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