Articles

Purine Derivatives as Competitive Inhibitors of Human Erythrocyte Membrane Phosphatidylinositol 4-Kinase

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The possibility of deriving a potent, cell-penetrating inhibitor of human erythrocyte PI 4-kinase, competitive with respect to ATP, has been investigated in a series of purine derivatives and analogues. The purine nucleus is not essential for binding to the ATP site but offers the advantage of synthetic accessibility to its derivatives. The optimum substitution pattern in purine was found to be an electron-releasing substituent in the 6-position (e.g. amino, as in adenine, 1) and a compact, lipophilic group in either the 8-position or, preferably, the 9-position, suggesting the importance of the N-1 lone pair and hydrophobic contributions of the 8- and 9-substituents to binding. The most potent inhibitor synthesized was 9-cyclohexyladenine (54), which has an apparent K_i value of 3.7 μM.

Introduction

In the sequence of events which lead to the generation of the intracellular second messengers D-*myo*-inositol 1,4,5-trisphosphate (I-(1,4,5)P_3) and diacylglycerol (DG), the membrane phospholipid phosphatidylinositol (PI) is phosphorylated sequentially at the 4- and 5-positions by specific kinases to give phosphatidylinositol 4,5-bisphosphate (PI-(4,5)P_2), which is cleaved by a phospholipase C on agonist stimulation of cell-surface receptors (Figure 1).[1] Both I-(1,4,5)P_3 and DG have been implicated as mediators in a range of physiological processes, including cell proliferation, smooth muscle contraction, inflammation, and secretion,[2,3] and consequently, efforts to find ways of controlling their production are receiving wide interest.

One possible approach is to limit the supply of the immediate precursor PI-(4,5)P_2 by inhibiting the phosphorylation of PI, and recent studies have shown that various derivatives of purine are competitive inhibitors of PI 4-kinase with respect to ATP, for example, adenosine, for which an IC_{50} of 25 μM has been reported.[4] In view of the high intracellular concentration of ATP, however, it was considered that a compound of greater potency, and the ability to readily penetrate cells would be essential.

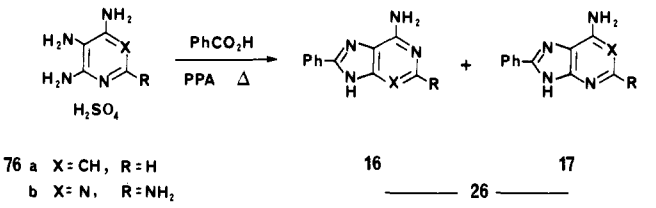
The purpose of this study was therefore to investigate in greater detail the structural requirements for binding to the ATP site of PI 4-kinase and to optimize inhibitory potency, to furnish tools for detailed biochemical study.

Chemistry

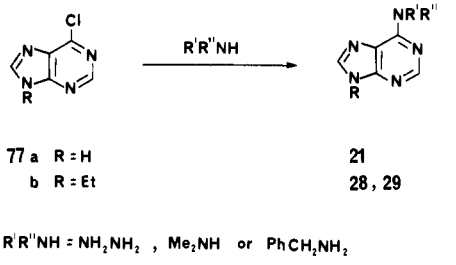
Cyclization of 2,3,4-triaminopyridine sulfate (**76a**) with benzoic acid by heating in polyphosphoric acid (PPA) gave a mixture of 2-phenyl-4-aminoimidazo[4,5-*c*]pyridine (**16**) and the isomeric 2-phenyl-7-aminoimidazo[4,5-*b*]pyridine (**17**), as shown in Scheme I. These two compounds were readily separated by chromatography. In an analogous way, 2,6-diamino-8-phenylpurine (**26**) was prepared by cyclizing 2,4,5,6-tetraaminopyrimidine sulfate (**76b**) with benzoic acid in PPA (Scheme I). The known 6-substituted aminopurines (**21**, **28**, and **29**) were prepared by the method of Montgomery and Temple,[5] as shown in Scheme II.

Most of the 8-substituted, 9-substituted, and 8,9-disubstituted adenines were prepared in a similar way to compounds **16**, **17**, and **26**, as shown in Scheme III. Cy-

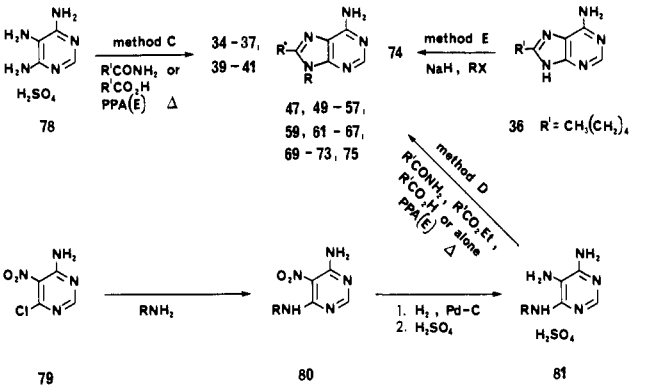
Scheme I. Method A



Scheme II. Method B



Scheme III[a]



[a] For definition of R and R' see Table III.

(1) Downes, C. P.; Michell, R. H. In *Molecular Aspects of Cellular Regulation, Vol 4. Molecular Mechanisms of Transmembrane Signalling*; Cohen, P.; Houslay, M. D., Eds.; Elsevier: Amsterdam, 1985; p 3.
(2) Berridge, M. J.; Irvine, R. F. *Nature (London)* **1984**, *312*, 315.
(3) Nishizuka, Y. *Nature (London)* **1984**, *308*, 693.
(4) Doctrow, S. R.; Lowenstein, J. M. *J. Biol. Chem.* **1985**, *260*, 3469.
(5) Montgomery, J. A.; Temple, C. *J. Am. Chem. Soc.* **1961**, *83*, 630.

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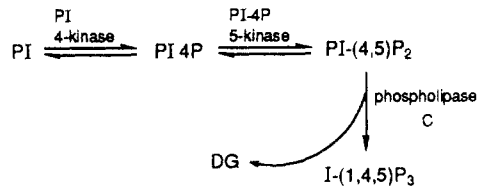
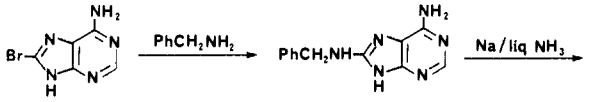
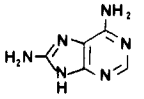


Figure 1.

Scheme IV. Method F



31 (base) 33 (base)



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clizations were performed with 4,5,6-triaminopyrimidine sulfate (**78**) and various 4-substituted amino analogues (**81**) by heating with formamide (to give the 8-unsubstituted compounds **47**, **49–57**, **59**, **61–67**, and **69**), acetamide (to give the 8-methylated compounds **34** and **70**), or a combination of a carboxylic acid or ester and PPA or its ethyl ester (PPE) (to give **35–37**, **39–41**, and **71–73**). The adenine 9-substituents were introduced at the pyrimidine 4-amino position of intermediates **81** by reacting 4-chloro-5-nitro-6-aminopyrimidine (**79**) with the appropriate amine, followed by catalytic reduction, by the procedure of Daly and Christensen.[6] Compound **75** was prepared in a similar way (Scheme III) but by carrying out a final intramolecular cyclization by heating 4-[(4-cyanobutyl)-amino]-5,6-diaminopyrimidine sulfate (**81**, R = 4-cyanobutyl) in PPE. In an alternative approach, 8,9-di-*n*-pentyladenine (**74**) was prepared by alkylation of the parent 8-substituted adenine **36**, using sodium hydride and bromopentane (Scheme III). This reaction gave a mixture containing polyalkylated derivatives which could be separated by chromatography.

Preparation of 6,8-diaminopurine (**32**) was achieved by reacting 8-bromoadenine with benzylamine to give intermediate **33** (base), followed by debenzylation in moderate yield using sodium in liquid ammonia, as shown in Scheme IV.

Enzyme Assay

The inhibitory activities of all the compounds included in this study were initially determined at a fixed concentration of 500 μM using the assay conditions described in the Experimental Section. IC_{50} values for compounds showing greater than 65% inhibition at this dose were then determined with single computer-fitted dose–response curves, and apparent K_i values were derived with the equation:

$$K_i = IC_{50}/(1 + [S]/K_m)$$

where the concentration of ATP substrate (S) in the assay is 100 μM, and its K_m value, calculated from standard Lineweaver–Burke analysis, is 221.5 μM. These results are presented in Tables I–III.

A more detailed analysis was conducted to ascertain the type of inhibition exhibited by five of the more active compounds (**1**, **23**, **42**, **48**, and **58**), chosen to typify the range of compounds included in the study. For each compound, the kinase was incubated with a range of ATP concentrations (0.50, 0.75, 1.5, and 10 × K_m) in the presence or absence of various concentrations of inhibitor (0, 1, 3, and 10 × K_i). A nonlinear-regression computer program was used to analyze the data, and the results were examined for fitting to equations for competitive and mixed inhibition. In each case, significant fits for competitive inhibition were obtained, and the parameters derived are shown in Table IV.

Because of their close structural similarity, all other inhibitors included in the study are assumed to act by a competitive mechanism with respect to ATP.

Results and Discussion

In accord with the results of a recent study,[7] adenosine (**42**) was found to be more potent as an inhibitor of PI 4-kinase than the adenine nucleotides **45** and **46**, perhaps suggesting some degree of electrostatic repulsion involving phosphate groups at the enzyme active site. Moreover, the observation that 2′,3′-*O*-isopropylideneadenosine (**43**) and 5′-iodo-5′-deoxyadenosine (**44**) (Table III) are at least as potent as adenosine implies that the hydroxyl functions in the ribose moiety have no specific role in binding, although adenine (**1**), which lacks this substituent, is a much weaker inhibitor. In order to assess the requirements for inhibition in greater detail, we have therefore made a systematic study of the effects of substitution at various positions around the purine nucleus and have also compared various alternative heterocyclic systems.

The purine nucleus is not an essential requirement for a competitive inhibitor of PI 4-kinase at the ATP binding site. The fused imidazole ring in adenine (**1**) can be replaced by a pyrazole, thiazole, or a benzene ring to give inhibitors of similar potency (**5**, **8**, and **11**, respectively) (Table I), while further simplification of the structure of **11**, by removing either the fused benzene ring or the ring nitrogen distal to the amino substituent, results in marked

(6) Daly, J. W.; Christensen, B. E. *J. Org. Chem.* **1956**, *21*, 177.
(7) Doctrow, S. R.; Lowenstein, J. M. *Biochem. Pharmacol.* **1987**, *36*, 2255.
(8) Cheng, C. C.; Robins, R. K. *J. Org. Chem.* **1956**, *21*, 1240.
(9) Elion, G. B.; Lange, W. H.; Hitchings, G. H. *J. Am. Chem. Soc.* **1956**, *78*, 2858.
(10) Fu, S. C. J.; Chinoporos, E.; Terzian, H. *J. Org. Chem.* **1965**, *30*, 1916.
(11) Sengupta, S. K.; Chatterjee, S.; Protopapa, H. K.; Modest, E. J. *J. Org. Chem.* **1972**, *37*, 1323.
(12) Radda, H. J. *J. Chem. Soc.* **1956**, 3509.
(13) Ochiai, E.; Kawazoe, Y. *Pharm. Bull. (Tokyo)* **1957**, *5*, 606.
(14) de Roos, K. B.; Salemink, R. K. *Rec. Trav. Chim. Pays-Bas* **1969**, *88*, 1263.
(15) Elion, G. B.; Burgi, E.; Hitchings, G. H. *J. Am. Chem. Soc.* **1952**, *74*, 411.
(16) Kuznetsov, V. S.; Remizov, A. L. *Khim. Geterotsikl. Soedin.* **1972**, *10*, 1430.
(17) Taylor, E. C.; Beardsley, G. P.; Maki, Y. *J. Org. Chem.* **1971**, *36*, 3211.
(18) Baker, B. R.; Schaub, R. E.; Joseph, J. P. *J. Org. Chem.* **1954**, *19*, 638.
(19) Kunieda, T.; Witkop, B. *J. Org. Chem.* **1970**, *35*, 3981.
(20) Koppel, H. C.; Robins, R. K. *J. Org. Chem.* **1958**, *23*, 1457.
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(22) Montgomery, J. A.; Temple, C. *J. Am. Chem. Soc.* **1958**, *80*, 409.
(23) Lewis, L. R.; Schneider, F. H.; Robins, R. K. *J. Org. Chem.* **1961**, *26*, 3837.
(24) Shaw, G. In *Comprehensive Heterocyclic Chemistry*; Potts, K. T., Ed.; Pergamon: Oxford, 1984; Vol. 5, p 499.

decreases in potency. Moreover, removal of the corresponding nitrogen atom in adenine (to give **15**) also leads to a reduction in potency.

The 6-amino substituent in adenine (**1**) makes a large contribution to inhibitory activity. Comparison of a small series of 6-substituted purine derivatives (**1**, **18–20**, and **22**) suggests that this effect is primarily electronic, and that the degree of inhibition is related to the pK_a value for dissociation of a proton from the conjugate acid, presumably protonated at N-1.[24] Thus, the order of decreasing inhibitory activity, 6-NH_2 > NMe_2 ≫ MeO ≈ Me ≈ H, runs parallel with the corresponding pK_a values, 4.25, 3.87, 2.21, 2.60, and 2.39.[25] Substitution of an amino group is somewhat less beneficial at the 2-position of purine (in **24**), while substitution at both the 2- and 6-positions results in a compound (**25**) of similar activity to adenine. Here again, pK_a (3.80 and 5.09, respectively[25]) appears to be reasonably well related to inhibitory activity; although, in these examples, protonation may not necessarily occur at N-1. These observations, taken in conjunction with the abolition of inhibitory activity caused by N-1 methylation (see compound **2**) (Table I) might infer that the availability of an electron pair on N-1 is important for binding to the enzyme. It might thus be argued that a minimum structural requirement for an effective inhibitor of PI 4-kinase, competitive with respect to ATP, is an aryl-fused 4-aminopyrimidine system, and adenine was consequently chosen for investigating the effects of further substitution because of its relative ease of structural modification.

Methylation of adenine at the N-1-, N-3-, N-7-, C-8-, and N-9-positions led to decreases in activity (see compounds **2–4**, **34**, and **47**), although these decreases were relatively small for C-8 and N-9 methylation. Like methyl, other lipophilic groups could be accommodated at the 8-position of adenine either to retain or even to enhance activity, as was found for pentyl, phenyl, and pyridyl (**36**, **38**, and **39**) (Table III), while pentadecyl (**37**) and 2-naphthyl (**41**) gave large reductions. Interestingly, 8-(1-naphthyl)adenine (**40**) is much more potent that its 2-naphthyl isomer, suggesting that the size and shape of the 8-substituent is important, perhaps for binding to a narrow hydrophobic cavity in the enzyme.

In agreement with the beneficial effect of phenylation at the 8-position of adenine, increases in inhibitory activity were also found on introducing a phenyl substituent at the corresponding position of **15** (to give **16**), and by direct fusion of a second benzene ring in 4-aminoquinazoline (**11**) at the appropriate position to give **10** (Table I), suggesting that these inhibitors bind to the active site in a similar manner. Unexpectedly, however, phenylation at the analogous 2-position of **8** gave a weaker inhibitor (**9**).

By far the most profitable position for substitution in adenine is the N-9-position (see Table III). Here, substitution was almost invariably found to be accompanied by an increase in inhibitory potency, being particularly marked with relatively compact, lipophilic groups. In this series of compounds, activity appears to be related to the bulk of the substituent, and a plot of activity against the steric substituent constant E_s' of Dubois et al.[26] (Figure 2) suggests that there is an optimum requirement for the size of a 9-substituent lying close to cyclohexyl. In adenosine (**42**), the steric bulk of the ribose moiety is presumably not far from ideal, and the similarity between the inhibitory activities of this compound and the tetrahydrofuryl analogue (**58**) suggests that the ribose hydroxyl

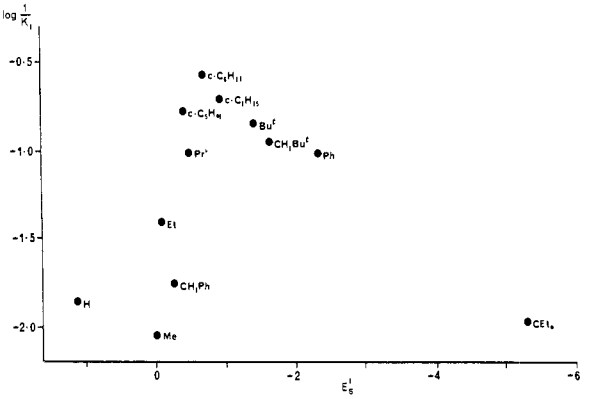

Figure 2. Relationship between PI 4-kinase inhibitory activity and the E_s' steric substituent constant for N-9-substituted adenines.

groups have no effect on binding. 9-Cyclohexyladenine (**54**) is the most potent PI 4-kinase inhibitor found to date and has an apparent K_i value of 3.7 μM. Further substitution in the phenyl ring of 9-phenyladenine (**60**) was also explored, but no significant improvement in inhibitory activity was found. In most cases activity fell, with the larger substituents giving the greatest reductions in potency, as expected from Figure 2 for adenine 9-substituents larger than phenyl.

By comprison, substitution at the 1-position of 4-aminopyrazolo[3,4-*d*]pyrimidine (**5**) by a phenyl group (to give **7**) (Table I) gave an increase in inhibitory activity consistent with its effect at the analogous 9-position of adenine.

In a small number of examples, the effects of combined substitution at the C-8 and N-9 positions of adenine were investigated (see compounds **70–75**) (Table III). As expected, substitution of a phenyl group at C-8 or N-9 consistently enhanced activity, while a methyl group made little or no contribution. In the case of 8,9-diphenyladenine (**72**), however, the effects of the two phenyl groups are not additive. This might suggest that the hydrophobic surface in the active site available for binding interactions is somewhat limited.

Combined substitution at other positions in adenine was also investigated. In contrast to the tolerance to introducing an 8-phenyl or a 9-methyl substituent in adenine, analogous substitution in 2,6-diaminopurine gave the weaker inhibitors **26** and **27**. Moreover, 9,6-disubstitution in adenine was poorly tolerated, as exemplified by 9-ethyl-6-(dimethylamino)purine (**28**). In these cases, it is possible that the inhibitors are binding to the enzyme in a different manner to that of their parent compounds.

The most potent inhibitor of PI 4-kinase found in the present study, 9-cyclohexyladenine (**54**), is about 5 times more potent than adenosine, described previously. Moreover, it is relatively lipophilic and therefore likely to cross cell membranes by passive diffusion, lending itself as a potential tool for the study of PI 4-kinase inhibition in intact cell preparations. This compound has recently been shown to have bronchodilator activity,[27] and it is probable that this might, at least in part, result from inhibition of PI 4-kinase.

Experimental Section

Chemistry. Proton magnetic resonance spectra (^{1}H NMR) were recorded on a Bruker AM250 (250-MHz) spectrometer, and

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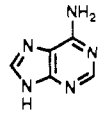
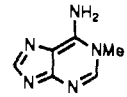
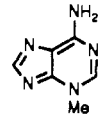
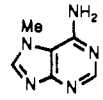
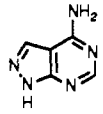
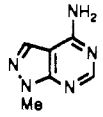
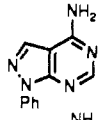
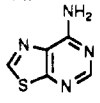
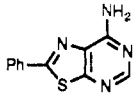
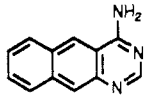
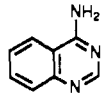
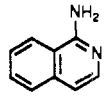
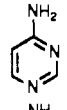
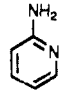
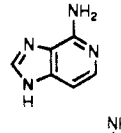
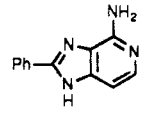
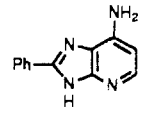
(26) MacPhee, J. A.; Panaye, A.; Dubois, J.-E. *Tetrahedron Lett.* **1978**, 3293; *Tetrahedron* **1978**, *34*, 3553.
(27) Naito, T.; Nakagawa, S.; Okita, T. A.; Yamashita, H.; Yamasaki, T.; Kamei, H.; Tomatsu, K.; Imanishi, H.; Kawaguchi, H. *Chem. Pharm. Bull.* **1982**, *30*, 2011.

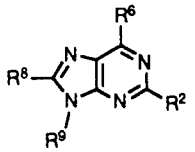
Table I. Substituted Adenines and Related Heterocyclic Systems

compd	structure	formula[a]	mp, °C	solvent	method[b]	% yield[c]	inhibition of PI 4-kinase[d]	
							IC_{50} (±SEM), μM	K_i, μM
1		$C_5H_5N_5 \cdot HCl$[e]	290–291 dec		*f*		182.6 ± 10.9	85.8[g]
2		$C_6H_7N_5$	>300		*f*		(inact at 500 μM)	
3		$C_6H_7N_5$[h]	subl >250		*f*		(58.8 ± 2.6% at 500 μM)	
4		$C_6H_7N_5$	>300		*f*		(27.3 ± 1.9% at 500 μM)	
5		$C_5H_5N_5$	>350	sublimed	*f*		144.9 ± 12.5	99.9
6		$C_6H_7N_5$	269–271	H_2O	*i*	55	82.5 ± 6.5	56.8
7		$C_{11}H_9N_5$	215–217		*f*		16.1 ± 2.0	11.0
8		$C_5H_4N_4S$	218–219	H_2O	*j*	75	116.9 ± 10.6	80.6
9		$C_{11}H_8H_4S$	262–267 dec	2 M HCl	*k*	37	(53.1 ± 5.6% at 500 μM)	
10		$C_{12}H_9N_3$[e]	339–340	sublimed	*l*	5	33.0 ± 1.6	22.7
11		$C_8H_7N_3$[m]	240 dec	MeOH	*n*	30	91.7 ± 5.2	63.4
12		$C_9H_8N_2$	115–120	H_2O	*o*	74	(28.4 ± 1.7% at 500 μM)	
13		$C_4H_5N_3$	154–156		*f*		(28.2 ± 1.4% at 500 μM)	
14		$C_5H_6N_2$	57–60		*f*		(7.1 ± 1.3% at 500 μM)	
15		$C_6H_6N_4$	229–230	EtOH	*p*	32	(35.7 ± 2.1% at 500 μM)	
16		$C_{12}H_{10}N_4$[q]	223–224	EtOH/EtOAc	A	14	(58.9 ± 2.3% at 500 μM)	
17		$C_{12}H_{10}N_4$[r]	294–295	MeOH	A	10	(48.3 ± 2.9% at 500 μM)	

Footnotes to Table I

[a] All compounds were analyzed for C, H, N (and Cl in 1) and are within 0.4% of theoretical values unless stated. [b] See the Chemistry section. [c] No attempts were made to optimize yields. [d] See the Experimental Section. IC_{50} values were determined from computer-fitted single dose–response curves, and K_i values were evaluated with the equation: $K_i = IC_{50}/(1 + [S]/K_m)$, where K_m refers to ATP (calculated as 221.5 μM) and [S] is its concentration (μM). [e] Contains 3.0% H_2O. [f] Commercial sample. [g] Investigated in further detail to confirm competitive inhibition. [h] Contains 7.0% H_2O. [i] Cheng and Robins[8] (mp 266–268 °C). [j] Elion et al.[9] (mp 211–212 °C). [k] Fu et al.[10] (mp 260–262 °C). [l] Sengupta et al.[11] (mp 337–338 °C). [m] Contains 3.0% HCl. [n] Radda[12] (mp 272–273 °C). [o] Ochiai and Kawazoe[13] (mp 120–121 °C). [p] de Roos and Salemink[14] (mp 237 °C). [q] Contains 0.7% EtOAc and 1.0% H_2O. [r] Contains 4.0% H_2O.

Table II. Substituted Purines



compd	R^2	R^6	R^8	R^9	formula[a]	mp, °C	solvent	method[b]	% yield[c]	inhibition of PI 4-kinase[d] IC_{50} (±SEM), μM	K_i, μM
18	H	H	H	H	$C_5H_4N_4$	218–219		*e*		(19.4 ± 3.4% at 500 μM)	
19	H	Me	H	H	$C_6H_6N_4$[f]	240–241 dec		*e*		(18.3 ± 2.5% at 500 μM)	
20	H	OMe	H	H	$C_6H_6N_4O$[g]	196–198		*e*		(20.0 ± 4.6% at 500 μM)	
21	H	$NHNH_2$	H	H	$C_5H_6N_6$[h]	242–244 dec[i]	H_2O	B	78	(30.7 ± 2.4% at 500 μM)	
22	H	NMe_2	H	H	$C_7H_9N_5$	265–265.5		*e*		218.0 ± 12.9	150.2
23	H	$NHCH_2Ph$	H	H	$C_{12}H_{11}N_5$	235–235.5		*e*		240.4 ± 16.2	199.0[j]
24	NH_2	H	H	H	$C_5H_5N_5$[k]	>280 dec		*e*		341.6 ± 21.0	235.4
25	NH_2	NH_2	H	H	$C_5H_6N_6 \cdot 0.5H_2SO_4$[l]	>300		*e*		146.8 ± 13.9	101.2
26	NH_2	NH_2	Ph	H	$C_{11}H_{10}N_6$[m]	303 dec[n]	*o*	A	10	199.5 ± 7.2	137.7
27	NH_2	NH_2	H	Me	$C_6H_8N_6$[p]	305–310	EtOH	*q*	6	(36.3 ± 3.2% at 500 μM)	
28	H	NMe_2	H	Et	$C_9H_{13}N_5$	81–83[r]	Et_2O/C_5H_{12}	B	46	(20.2 ± 2.2% at 500 μM)	
29	H	$NHCH_2Ph$	H	Et	$C_{14}H_{15}N_5$	146–147[s]	Et_2O	B	76	(7.3 ± 1.4% at 500 μM)	
30	H	NMe_2	H	CH_2Ph	$C_{14}H_{15}N_5$	120–121	EtOH	*t*	71	(13.4 ± 2.8% at 500 μM)	

[a] All compounds were analyzed for C, H, N (and S in **25**) and are within 0.4% of theoretical values unless stated. [b] See the Chemistry section. [c] No attempts were made to optimize yields. [d] See footnote *d*, Table I. [e] Commercial sample. [f] Contains 1.5% H_2O. [g] Contains 5.5% H_2O. [h] Contains 8.5% H_2O. [i] Literature[15] mp 244–245 °C. [j] Investigated in further detail to confirm competitive inhibition. [k] Contains 6.0% H_2O. [l] Contains 11.0% H_2O. [m] Contains 0.7% iPrOH and 2.3% H_2O. [n] Literature[16] mp for hydrochloride hydrate 342–343 °C. [o] Purified by chromatography (SiO_2, CH_2Cl_2/MeOH/NH_4OH). [p] Contains 3.5% H_2O. [q] Taylor et al.[17] (mp 314–316 °C). [r] Literature[18] mp 79–80 °C. [s] Literature[19] mp 141–144 °C. [t] Montgomery and Temple[5] (mp 131–132 °C).

mass spectra (EI) were obtained with a VG analytical 70-70F double-focusing spectrometer. Microanalytical data were within 0.4% of theoretical values except where noted, and melting points were determined on a Büchi 510 capillary apparatus and are uncorrected.

Compounds 1–**5**, **7**, **13**, 14, 18–**20**, **22**–**25**, **31**, **42**–**46**, and **48** were commercial samples and were purchased from Aldrich Chemical Co., except for **4** (Vega Biochemicals), **7** (Lancaster Synthesis), **31**, **43**, **48** (Sigma Chemical Co.), and **46** (Boehringer Mannheim). There were used as supplied, except **5**, which was resublimed, and **31**, which was converted to the hydrochloride salt. The other compounds listed in Tables I–III were either prepared by literature procedures (see the tables) or by the general methods given below.

Mammalian PI and [2-^{3}H]PI were obtained from Sigma Chemical Co. and Amersham International Plc, respectively.

Method A. 2-Phenyl-4-aminoimidazo[4,5-*c*]pyridine (16) and 2-Phenyl-7-aminoimidazo[4,5-*b*]pyridine (17). A mixture of benzoic acid (244 mg, 2.00 mmol) and **76a** (444 mg, 2.00 mmol) in PPA (10 g) was heated at 170 °C with stirring for 1 h. After being allowed to cool, the mixture was treated with ice and then neutralized with aqueous Na_2CO_3. The resulting precipitate was filtered off, washed with H_2O, and purified by chromatography (SiO_2, CH_2Cl_2/MeOH/NH_4OH 180:10:1) to give two crude products.

The more polar component (221 mg) was recrystallized from EtOH/EtOAC to give pale yellow plates of **16** (60 mg, 14%): mp 223–224 °C; NMR (DMSO-d_6) δ 6.16 (2 H, br s), 6.78 (1 H, d), 7.54 (3 H, m), 7.65 (1 H, d), 8.12 (2 H, d), 12.86 (1 H, v br s). Anal. ($C_{12}H_{10}N_4$) C, H. N.

The less polar component (96 mg) was recrystallized from MeOH to give colorless needles of **17** (40 mg, 10%): mp 294–295 °C; NMR (DMSO-d_6) δ 6.34 (3 H, m), 7.50 (3 H, m), 7.80 (1 H, d), 8.16 (2 H, d), 13.02 (1 H, v br s). Anal. ($C_{12}H_{10}N_4$) C, H, N.

Method B. 6-Substituted and 6,9-Disubstituted Purines. These were prepared from 6-chloropurine (**77a**) or 6-chloro-9-ethylpurine (**77b**) by reaction with the appropriate amine in methanolic solution according to the method of Montgomery and Temple.[5]

6-(Benzylamino)-9-ethylpurine (29). A solution of **77b** (365 mg, 2.00 mmol) in MeOH (10 mL) was treated with benzylamine (857 mg, 8.00 mmol), and the mixture was warmed at 50 °C for 3 h. After evaporating to dryness, the residue was shaken with a mixture of $CHCl_3$ and aqueous K_2CO_3, and the organic layer was separated, dried ($MgSO_4$), and evaporated to dryness. Recrystallization from Et_2O gave **29** (384 mg, 76%): mp 146–147 °C (lit.[19] mp 141–144 °C); NMR ($CDCl_3$) δ 1.53 (3 H, t), 4.24 (2 H, q), 4.90 (2 H, br s), 6.31 (1 H, br s), 7.23–7.43 (5 H, m), 7.68 (1 H, s), 8.43 (1 H, s). Anal. ($C_{14}H_{15}N_5$) C, H, N.

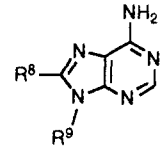
Method C. 8-Substituted Adenines. These compounds were prepared by heating 4,5,6-triaminopyrimidine sulfate (**78**) with either the appropriate carboxylic acid in excess PPA or PPE at 150 °C for 2–6 h or acetamide at 230 °C for 6 h (in the synthesis of **34**) by the procedure described previously.[28]

8-*n*-Pentyladenine (36). Hexanoic acid (1.78 g, 15.3 mmol) was reacted with **78** (3.62 g, 15.0 mmol) in PPA (50 g) at 150 °C for 2.5 h. The cooled mixture was poured into H_2O (1.5 L) and neutralized with $NaHCO_3$, and the resulting solution was evaporated to dryness. Purification by chromatography (SiO_2, $CHCl_3$/MeOH) gave a crude product which was recrystallized from aqueous EtOH to give **36** (1.29 g, 42%): mp 252–253 °C; NMR (DMSO-d_6) δ 0.72 (3 H, t), 1.15 (4 H, m), 1.60 (2 H, m), 2.61 (2 H, t), 6.87 (2 H, br s), 7.92 (1 H, s), 12.48 (1 H, br s); MS *m/e* 205. Anal. ($C_{10}H_{15}N_5$) C, H, N.

Method D. 9-Substituted and 8,9-Disubstituted Adenines. Cyclizations of 4-(substituted-amino)-5,6-diaminopyrimidine sulfates (**81**) were performed with the appropriate carboxamide (formamide for 8-unsubstituted adenines and acetamide for compound **70**) by heating at 230 °C for 1–6 h or with the appropriate carboxylic acid or ester (hexanoic acid or benzoic acid

(28) Yoshitomi Pharm. Ind. K. K. Jap. Pat. 62010085A, 1987.

Table III. Substituted Adenines



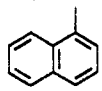
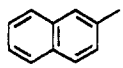
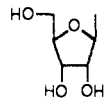
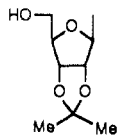
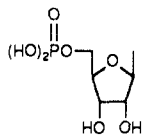
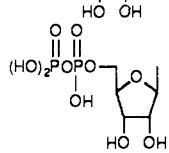
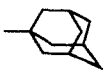
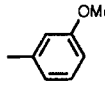
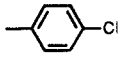
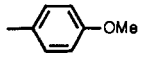
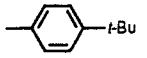
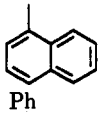
compd	R^8	R^9	formula[a]	mp, °C	solvent	method[b]	% yield[c]	inhibition of PI 4-kinase[d] IC_{50} (±SEM), μM	K_i, μM
31	Br	H	$C_5H_4BrN_5 \cdot 0.9HCl$	>264 dec	0.5 M HCl	*e*		215.3 ± 16.6	148.4
32	NH_2	H	$C_5H_6N_5 \cdot 2HCl$	>300	M HCl	F	40[f]	(61.9 ± 1.3% at 500 μM)	
33	$NHCH_2Ph$	H	$C_{12}H_{12}N_6 \cdot HCl$[g]	198–200	M HCl	F	63	133.0 ± 8.7	91.7
34	Me	H	$C_6H_7N_5 \cdot 2HCl$	270–272[h]	MeOH/Et_2O	C[i]	82	195.4 ± 10.1	134.3
35	tBu	H	$C_9H_{13}N_5$	>300	MeOH/EtOAc	C[j]	16	(64.8 ± 1.3% at 500 μM)	
36	$(CH_2)_4CH_3$	H	$C_{10}H_{15}N_5$	252–253	EtOH/H_2O	C	42	68.9 ± 6.1	47.5
37	$(CH_2)_{14}CH_3$	H	$C_{20}H_{35}N_5$	199–202	EtOAc	C	33	(inact at 500 μM)	
38	Ph	H	$C_{11}H_9N_5 \cdot HCl$	>300	2 M HCl	*k*	30	48.8 ± 2.0	33.6
39		H	$C_{10}H_8N_6$	355–356	MeOH	C	51	75.6 ± 8.6	52.3
40		H	$C_{15}H_{11}N_5$	>300	MeOH	C	33	129.8 ± 10.4	89.5
41		H	$C_{15}H_{11}N_5$	>350	MeOH	C	10	(23.0 ± 1.2% at 500 μM)	
42	H		$C_{10}H_{13}N_5O_4$	237–238		*l*		30.3 ± 5.0	19.2[m]
43	H		$C_{13}H_{17}N_5O_4$	222–223		*l*		12.1 ± 0.4	8.4
44	H		$C_{10}H_{12}IN_5O_3$	178–187		*l*		23.1 ± 0.9	15.9
45	H		$C_{10}H_{14}N_5O_7P$	200 dec		*l*		(31.3 ± 2.9% at 500 μM)	
46	H		$C_{10}H_{14}N_5O_{10}P_2K \cdot H_2O$			*l*		145.3 ± 18.8	100.1
47	H	Me	$C_6H_7N_5$	300–302[n]	MeOH/Me_2CO	D	23	161.0 ± 10.2	110.9
48	H	Et	$C_7H_9N_5$	196–197		*l*		32.4 ± 2.9	25.5[m]
49	H	iPr	$C_8H_{11}N_5$[o]	114–115[p]	$C_6H_{14}/CHCl_3$	D	7	14.9 ± 1.0	10.3
50	H	tBu	$C_9H_{13}N_5$	187–188	$C_6H_{14}/CHCl_3$	D	33	10.2 ± 0.6	6.9
51	H	CH_2-tBu	$C_{10}H_{15}N_5$	244–245	EtOH	D	20	12.8 ± 0.6	9.0
52	H	CEt_3	$C_{12}H_{19}N_5$	183–184	$C_6H_{14}/CHCl_3$	D	38	134.1 ± 17.8	93.7
53	H		$C_{10}H_{13}N_5$	155–156[q]	MeOH	D	6	8.7 ± 0.5	6.0
54	H		$C_{11}H_{15}N_5$[r]	190[s]	$C_6H_{14}/CHCl_3$	D	16	6.0 ± 0.7	3.7
55	H		$C_{12}H_{17}N_5$	190–192	$C_6H_{14}/CHCl_3$	D	33	7.2 ± 0.4	5.0
56	H		$C_{12}H_{17}N_5$[t]	173–174[u]	MeOH	D	42	7.2 ± 0.2	5.0
57	H		$C_{15}H_{19}N_5$	334–336	iPrOH	D	24	6.0 ± 0.7	4.1
58	H		$C_9H_{11}N_5O$	162.5–163.5	$CHCl_3$/pet. ether	*v*	42	28.2 ± 1.7	21.2[m]
59	H		$C_{10}H_8N_6$	296–299	MeOH	D	33	46.2 ± 2.0	31.8
60	H		$C_{11}H_9N_5$	245–246	EtOH/H_2O	*w*	48	15.4 ± 0.7	10.3

Table III (Continued)

compd	R^8	R^9	formula[a]	mp, °C	solvent	method[b]	% yield[c]	inhibition of PI 4-kinase[d] IC_{50} (±SEM), μM	inhibition of PI 4-kinase[d] K_i, μM
61	H		$C_{11}H_8FN_5$[x]	245–248	$MeOH/Me_2CO$	D	15	11.9 ± 0.6	8.3
62	H		$C_{12}H_{11}N_5$	213–215	MeOH	D	30	23.6 ± 1.8	16.5
63	H		$C_{11}H_8FN_5$	275–276	$MeOH/Me_2CO$	D	42	25.6 ± 1.5	17.9
64	H		$C_{12}H_{11}N_5O$	237–239	MeOH	D	22	22.9 ± 0.9	15.8
65	H		$C_{11}H_8ClN_5$	239–240	y	D	25	41.1 ± 4.2	28.2
66	H		$C_{12}H_{11}N_5O$[z]	225–227	y	D	52	21.9 ± 1.0	15.2
67	H		$C_{15}H_{17}N_5$	200–201	y	D	45	46.1 ± 2.8	31.7
68	H	CH_2Ph	$C_{12}H_{11}N_5$	233–237	$MeOH/H_2O$	aa	71	84.4 ± 7.3	57.9
69	H		$C_{15}H_{11}N_5$	247.5–248.5	DMF/H_2O	D	68	78.8 ± 5.7	54.4
70	Me	Ph	$C_{12}H_{11}N_5$	257–258	$C_6H_{14}/CHCl_3$	D	16	16.4 ± 1.1	11.0
71	$(CH_2)_4CH_3$	Ph	$C_{16}H_{19}N_5$	155–157	$C_6H_{14}/CHCl_3$	D	39	51.9 ± 4.3	35.8
72	Ph	Ph	$C_{17}H_{13}N_5$	247–248	$C_6H_{14}/CHCl_3$	D	42	16.4 ± 0.6	11.0
73	Ph	Me	$C_{12}H_{11}N_5$[bb]	230–231	$C_6H_{14}/CHCl_3$	D	24	34.9 ± 1.8	24.1
74	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	$C_{15}H_{25}N_5$	111–112	$C_6H_{14}/CHCl_3$	E	47	126.5 ± 9.7	
75	$-(CH_2)_4-$		$C_9H_{11}N_5$[bb]	272–273	$C_6H_{14}/CHCl_3$	D	11[cc]	52.4 ± 2.3	35.8

[a] All compounds were analyzed for C, H, N, (and Cl in **65**) and are within 0.4% of theoretical values unless stated. [b] See the Chemistry section. [c] No attempts were made to optimize yields. [d] See footnote *d*, Table I. [e] Prepared from commercial free base. [f] Crude yield. [g] Contains 2.0% H_2O. [h] Literature[20] mp for free base >300 °C. [i] Using $MeCONH_2$. [j] Using $tBuCO_2H$. [k] Fu et al.[10] (mp 310–311 °C). [l] Commercial sample. [m] Investigated in further detail to confirm competitive inhibition. [n] Literature[6] mp 300 °C. [o] Contains 2.4% H_2O. [p] Literature[21] mp for hydrochloride salt 235–236 °C. [q] Literature[22] mp 156 °C. [r] Contains 4.8% H_2O. [s] Literature[22] mp 199–200 °C. [t] Contains 1.0% MeOH. [u] Literature[21] mp 177 °C. [v] Lewis et al.[23] (mp 165–168 °C). [w] Daly and Christensen[6] (mp 235–238 °C). [x] Contains 1.5% H_2O. [y] Purified by chromatography (SiO_2, $CHCl_3$/MeOH). [z] Contains 4.0% $CHCl_3$. [aa] Daly and Christensen[6] (mp 224–225 °C dec). [bb] Contains 1.0% H_2O. [cc] Crude yield.

Table IV. Kinetic Constants of Representative Inhibitors of PI 4-Kinase

compd	V_{max}, cpm × 10^3	ATP K_m, μM	inhibitor K_i, μM
1	22.8	296.8	85.8
23	25.8	239.0	199.0
42	31.4	178.9	19.2
48	20.8	257.0	25.5
58	25.8	261.8	21.2

for compounds **71** and **72**, respectively, and ethyl benzoate for **73**) in PPE at 120–150 °C for 4–5 h or alone in PPE at 150 °C for 1 h (for compound **75**), by analogy with method C, as exemplified below. Intermediates (**81**) were prepared from **79** by the method described by Daly and Christensen.[6]

9-(1-Naphthyl)adenine (69). A mixture of **79** (2.00 g, 11.5 mmol) and 1-naphthylamine (6.59 g, 46.0 mmol) in nBuOH (50 mL) was heated to reflux for 2 h. The orange crystalline solid that formed on cooling was filtered off and recrystallized from nBuOH to give **80** (R = 1-naphthyl; 2.46 g, 76%): mp 266–267 °C; MS *m/e* 281.

This product (2.44 g, 8.67 mmol) was hydrogenated over Pd/C (10%, 1.0 g) at 50 psi in MeOH (200 mL) for 2 h. After filtering off the catalyst, the resulting yellow solution was treated with dilute H_2SO_4 (5%, 10 mL) to precipitate **81** (R = 1-naphthyl) as a colorless, crystalline solid (2.35 g, 78%), mp >300 °C.

The latter compound (2.30 g, 6.30 mmol) was mixed with excess $HCONH_2$ (5 mL) and heated at 230 °C for 1 h. After cooling, H_2O was added to deposit an impure, crystalline solid, which was purified by chromatography (SiO_2, $CHCl_3$/MeOH) and recrystallized from aqueous DMF to give **69** (1.11 g, 68%): mp 247.5–248.5 °C; NMR (DMSO-d_6) δ 7.28 (1 H, d), 7.47 (2 H, br s), 7.50–7.75 (4 H, br m), 8.07 (1 H, s), 8.09–8.21 (2 H, m), 8.43 (1 H, s). Anal. ($C_{15}H_{11}N_5$) C, H, N.

8-*n*-Pentyl-9-phenyladenine (71). A mixture of **81** (R = phenyl; 460 mg, 1.54 mmol) and hexanoic acid (206 mg, 1.77 mmol) in PPE (7.4 g) was heated, with stirring, at 120 °C for 3 h. The resulting syrup was dissolved in H_2O, neutralized with aqueous NaOH, and extracted with EtOAc. After drying ($MgSO_4$), the extract was evaporated to dryness to leave the crude product which was purified by chromatography (SiO_2, $CHCl_3$/MeOH) and recrystallized from $CHCl_3/C_6H_{14}$ to give **71** (166 mg, 39%): mp 155–157 °C; NMR ($CDCl_3$) δ 0.85 (3 H, t), 1.27 (4 H, m), 1.71 (2 H, m), 2.77 (2 H, t), 5.73 (2 H, br s), 7.38 (2 H, m), 7.56 (3 H, m), 8.30 (1 H, s). Anal. ($C_{16}H_{19}N_5$) C, H, N.

8,9-Tetramethyleneadenine (75). A mixture of **79** (1.18 g, 6.80 mmol) and 5-aminovaleronitrile (2.0 g, 20.3 mmol) in nBuOH (25 mL) was refluxed for 1.5 h and then cooled to 0 °C. The resulting crystalline solid was filtered off and washed with n-BuOH and Et_2O to give **80** (R = 4-cyanobutyl; 1.53 g, 95%), mp 190–192 °C.

The above product (1.46 g, 6.18 mmol) was dissolved in MeOH (250 mL) and hydrogenated over Pd/C (10%, 0.5 g) at 50 psi for 1.5 h. The resulting mixture was filtered and evaporated to dryness to give the free base of **81** (R = 4-cyanobutyl; 1.15 g, 91%) as an oil which crystallized to a light brown solid.

The latter compound (738 mg, 3.58 mmol) was cyclized by heating in PPE (10 g) at 150 °C for 1 h. After cooling, the mixture was neutralized with 40% aqueous NaOH, extracted with $CHCl_3$, dried, and evaporated to dryness to give a crude product (71 mg, 11%). A portion of this was purified by chromatography (SiO_2,

$CHCl_3$/MeOH) and recrystallized from $CHCl_3/C_6H_{14}$ to give **75**: mp 272–273 °C; NMR ($CDCl_3$) δ 2.08 (4 H, m), 3.05 (2 H, t), 4.16 (2 H, t), 5.56 (2 H, br s), 8.32 (1 H, s). Anal. ($C_9H_{11}N_5$) C, H, N.

Method E. 8,9-Di-*n*-pentyladenine (74). To a solution of **36** (500 mg, 2.44 mmol) in dry *N*-methylpyrrolidinone (1.5 mL) was added NaH (50%, 117 mg, 2.44 mmol), and the mixture was stirred for 10 min. Next, 1-bromopentane (368 mg, 2.44 mmol) was added dropwise with stirring over 0.5 h, and the reaction mixture was treated with H_2O, extracted with EtOAc, dried ($MgSO_4$), and evaporated to dryness. Purification by chromatography (SiO_2, CH_2Cl_2/MeOH) gave a crude product which was recrystallized from $CHCl_3/C_6H_{14}$ to give **74** (272 mg, 47%): mp 111–112 °C; NMR ($CDCl_3$) δ 0.91 (6 H, m), 1.37 (8 H, m), 1.82 (4 H, m), 2.83 (2 H, t), 4.12 (2 H, t), 5.64 (2 H, br s), 8.31 (1 H, s). Anal. ($C_{15}H_{25}N_5$) C, H, N.

Method F. 6,8-Diaminopurine (32). A solution of 31 free base (1.00 g, 4.67 mmol) in excess benzylamine (5 mL) was heated at 150 °C with stirring for 2 h. The resulting orange solution was poured into $CHCl_3$ (100 mL) to precipitate **33** free base as a fine, white solid (0.84 g, 75%). A sample of this product was treated with 1 N HCl in excess to give the crude hydrochloride, and recrystallized from H_2O to give **33**, mp 198–200 °C.

The free base of **33** (725 mg, 3.02 mmol) was dissolved in liquid NH_3 (250 mL) and small pieces of Na (2.84 g, 123 g-atom) were added over 3 h, with stirring until the color was no longer discharged. Excess NH_4Cl was added and the NH_3 was allowed to evaporate. The residue was chromatographed (SiO_2, $CHCl_3$/MeOH/NH_4OH 10:5:1) to give a yellow solid which was extracted into boiling EtOH, treated with activated charcoal, filtered, and evaporated to give a crude product (180 mg, 40%). This was dissolved in 1 N KOH and adjusted to pH 5 with 1 N HCl, filtered and cooled to give **32** as a white powder: mp >300 °C; NMR (DMSO-d_6) δ 6.51 (2 H, br s), 6.72 (2 H, br s), 7.96 (1 H, s). Anal. ($C_5H_8N_8 \cdot 2HCl$) C, H, N.

PI 4-Kinase Assay. Highly purified human erythrocyte plasma membranes were prepared by the method described by Hawkins et al.[29] Assays were carried out in a 200 μL final volume containing membranes (0.36 mg of membrane protein), PI (0.12 mM, 0.04 μCi [2-^{3}H]PI), ATP (100 μM), ouabain (100 μM), sodium metavanadate (100 μM), dithiothreitol (5 mM), Triton X-100 (0.6%), KCl (120 mM), NaCl (30 mM), EGTA (1 mM), $MgCl_2$ (5 mM), HEPES (50 mM, pH 7.4), plus inhibitor or vehicle. Mixtures were incubated for 10 min at 30 °C, and the reaction was terminated by the addition of 0.75 mL of $CHCl_3$/MeOH/concentrated HCl (40:80:1). Addition of 0.25 mL of $CHCl_3$ and 0.25 mL of 0.1 M HCl gave two phases, and the organic phase, containing the phospholipids, was separated, and made up to 1 mL with $CHCl_3$, to which 0.2 mL MeOH and 0.2 mL of 1 M NaOH in 5% v/v aqueous MeOH was added to initiate deacylation. After 20 min at room temperature 1 mL of $CHCl_3$, 0.6 mL of MeOH, and 0.6 mL of H_2O were added to the samples and they were mixed vigorously. The two phases were separated by centrifugation, and 1 mL of the upper aqueous phase containing the deacylated phospholipids was removed. This was made approximately neutral with 0.8 mL 1 M H_3BO_3 and brought up to 5 mL with $Na_2B_4O_7$ (5 mM).

Samples were loaded onto Dowex AG1-X8 columns (200–400 mesh, formate form). Next, 20 mL of 0.005 M $Na_2B_4O_7$/0.18 M HCO_2NH_4 was applied to the columns to leave 1-(*sn*-glycer-3-ylphosphoryl)-D-*myo*-inositol 4-phosphate and 1-(*sn*-glycer-3-ylphosphoryl)-D-*myo*-inositol 4,5-bisphosphate as the only deacylated ^{3}H-labeled phospholipids remaining on the columns. The former compound was specifically eluted with 10 mL 0.3 M HCO_2NH_4/0.1 M HCO_2H and 10 mL of Triton X-100/xylene scintillant was added prior to counting for ^{3}H radioactivity.

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Thermodynamics of the Interaction of Inhibitors with the Binding Site of Recombinant Human Renin

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The independent subsite model is widely used for the design of peptide inhibitors of enzymes with extended active sites. This model assumes that the subsites are independent of each other and that the free energies of binding contributed by the several subsites are additive. We questioned the strict application of this model for structure–activity studies, since one can, a priori, conceive of likely deviations from this model. Accordingly, we tested the independent subsite model by measuring the thermodynamic binding parameters of a series of peptide inhibitors of human renin. This enzyme–inhibitor system was chosen as a model by virtue of the high degree of specificity of renin for its natural substrate, angiotensinogen, and the availability of a large number of structurally similar peptide inhibitors. Although we found the general mode of binding of these renin inhibitors to be primarily hydrophobic, serious deviations from additivity and independent subsite model constraints were observed. We conclude that an important determinant of binding is most probably the conformation assumed by the peptide inhibitor in solution. Thus, we suggest that caution be exercised in using affinity constants to assess the interactions of peptide inhibitors with human renin and possibly with other enzymes having extended binding sites. Furthermore, the thermodynamic parameters of a class of compounds provide more information as to the mode of binding of ligands to their respective receptors than do dissociation constants.

Introduction

Traditionally, the interaction of substrates and inhibitors with the active site of enzymes and receptors is probed by measuring the changes produced upon varying the chemical structure of the ligand. This approach proved to be particularly fruitful in establishing the binding properties, and hence the specificity, of endopeptidases. These enzymes often possess an extended binding site ideally suited for positioning an intended cleavage site through recognition of several amino acid residues preceding and following the scissile bond. The results of investigations on protease specificity, particularly those concerning the in-

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