Tyrosine Kinase Inhibitors. 12. Synthesis and Structure−Activity Relationships for 6-Substituted 4-(Phenylamino)pyrimido[5,4-*d*]pyrimidines Designed as Inhibitors of the Epidermal Growth Factor Receptor

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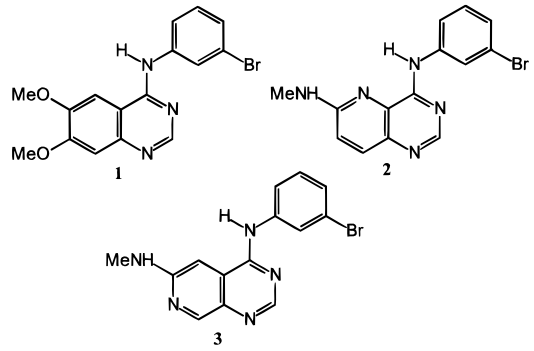
A series of 6-substituted 4-anilinopyrimido[5,4-*d*]pyrimidines has been prepared and shown to be potent inhibitors of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR). These compounds are structurally related to the pyrido[3,2-*d*]- and pyrido[3,4-*d*]-pyrimidines previously shown to be EGFR inhibitors. Their structure−activity relationships (SAR) for inhibition of the isolated enzyme more closely resemble those of the [3,2-*d*] than the [3,4-*d*] pyridopyrimidine isomers. This suggests the requirement of an aza atom in the 7- but not the 5-position (i.e., a carbon atom in the 5-position) for the enhanced potency shown by 6-N-methylated derivatives in each series. X-ray crystal structures were determined for the three NHMe derivatives **2**, **3**, and **5c** in the pyrido[3,2-*d*]-, pyrido[3,4-*d*]-, and pyrimido[5,4-*d*]-pyrimidine series, respectively. These show that a carbon rather than a nitrogen atom at the 5-position leads to significant conformational changes in the molecule (a longer C5a−C4 bond and a 30° out-of-plane rotation of the phenyl group), due to the requirement to relieve nonbonding interactions between the C5 and N9 protons. Pyrimido[5,4-*d*]pyrimidine analogues bearing bulky, weakly basic solubilizing side chains linked to the 6-position through a secondary amine generally retained potency both against the isolated enzyme and for inhibition of autophosphorylation of EGFR in intact A431 cells. This agrees with a recent binding model that suggests this general class of compounds binds to EGFR with the 6-position located in an area of comparative bulk tolerance at the entrance to the ATP-binding pocket. While these solubilized pyrimido[5,4-*d*]pyrimidine analogues were less potent than the NHMe derivative **5c** in the isolated enzyme assay, some were considerably superior to **5c** (and among the most potent ever reported) as inhibitors of EGFR autophosphorylation in cellular assays.

The epidermal growth factor (EGFR) and related enzymes such as erb-B2 are overexpressed in a high percentage of clinical cancers, with high levels of the enzymes associated with poor prognosis and response to treatment.[1−4] Inhibitors of the signaling functions of EGFR are therefore of interest as potential anticancer drugs, and monoclonal antibodies are in clinical trial.[5] We[6−10] and others[11,13] have reported that small molecule compounds belonging to the broad class of 4-(phenylamino)quinazolines (e.g., **1**) bind competitively to the ATP site of EGFR and are potent and highly selective inhibitors of its ability to phosphorylate a variety of substrates. Substitution in the 6- and/or 7-positions of the bicyclic chromophore with electron-donating groups was desirable, as was substitution in the 3′-position of the anilino ring with small lipophilic groups, particularly Br. We have recently also shown, in a comparative study of several isomeric series of pyridopyrimidines, that an aza atom was beneficial in the 6-position, acceptable in the 5- and 7-positions, but dystherapeutic in the 8-position.[14] Thus the [3,2-*d*] and particularly [3,4-*d*] isomers (e.g., **2** and **3**) were found to be potent and selective inhibitors both of the ability of the isolated EGFR enzyme to phosphorylate a PLCγ-based substrate and of its autophosphorylation in A431 cells in culture following stimulation by EGF.[14]



We have recently proposed[15] a model for the binding of the 4-(phenylamino)quinazolines to the ATP site of the EGFR, based on structural information for the catalytic subunit of the cAMP-dependent protein kinase.[16] The ATP-binding site is structurally similar among the entire protein kinase family, located in a cleft between the N- and C-terminal lobes and recognized by several highly-conserved residues including the two β-strands which form the phosphate binding "glycine loop". This model accommodates a large body of structure−activity relationship (SAR) data which have accumulated on this family of inhibitors. It predicts that quite large substituents at the 6- and 7-positions of the quinazolines and pyrido[*d*]pyrimidines can be tolerated without a major loss of affinity, as they project outward toward the exterior of the enzyme, from the narrow hydrophobic pocket in the N-terminal domain of EGFR

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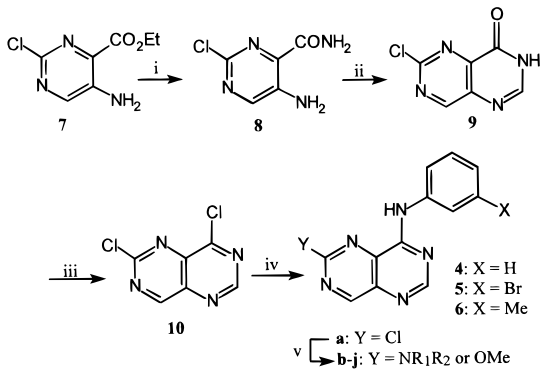
Scheme 1[a]



[a] (i) NH_3/EtOH/100 °C/2 days (pressure vessel); (ii) diethoxymethyl acetate/100 °C/6 h; (iii) $SOCl_2$/DMF (trace)/reflux/30 min; (iv) $ArNH_2$/2-propanol/reflux/10 min; (v) R_1R_2NH/DMSO/100 °C or MeOH/Et_3N/reflux.

tyrosine kinase (TK) which is the binding site of the adenine base of ATP.

Following on from these results, we now report the synthesis of and structure−activity relationships for a new class of 6-substituted 4-anilinopyrimido[5,4-*d*]pyrimidines (**4**−**6**) as EGFR inhibitors. These compounds contain aza atoms at both of the positions (5 and 7) found acceptable in the pyrido[3,2-*d*]- and pyrido[3,4-*d*]pyrimidines and an electron-donating N or O substituent at the 6-position, where such groups were found to be beneficial in the various pyridopyrimidine series. These compounds make possible a comparative study of compounds in the three series, to determine the relative contribution of aza atom positioning to the activity. A recent report[17] has also described the synthesis of some pyrimido[5,4-*d*]pyrimidines and their evaluation as inhibitors of EGFR.

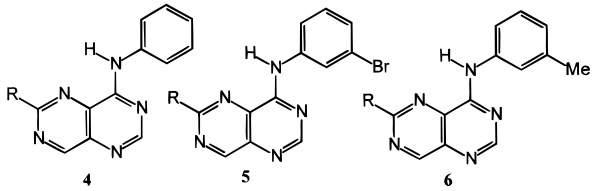
Chemistry

The compounds of Table 1 were prepared as shown in Scheme 1. Conversion of the known[18] amino ester **7** to the desired pyrimidinone **9** by direct reaction with formamidine acetate failed to occur cleanly, necessitating the intermediacy of the amide **8**. Reaction of **7** with ammonia in ethanol at 100 °C gave **8**, which could be converted to **9** in moderate yield using triethyl orthoformate and in much higher yield with diethoxymethyl acetate.[19−21] Reaction of **9** with $SOCl_2$ gave 4,6-dichloropyrimido[5,4-*d*]pyrimidine (**10**) which was not isolated but instead reacted directly with the appropriate aniline derivative in 2-propanol to give the 4-anilino-6-chloro derivatives **4a**, **5a**, and **6a**. Displacement of the chlorine atom, to give the corresponding 6-amino derivatives, occurred much more readily than with the analogous 6-fluoropyrido[3,4-*d*]pyrimidines,[14] and conversion to the methoxy derivative **5e** was achieved simply by heating **5a** in methanol containing triethylamine. It was also found that the methoxy group of **5e** could be displaced by reaction with amine nucleophiles, although this was not necessary for the present work.

Results and Discussion

The structures of the pyrimido[5,4-*d*]pyrimidines prepared are shown in Table 1. The ability of these compounds to inhibit the EGF-stimulated full-length EGFR enzyme, isolated from A431 cells, from tyrosine phosphorylation of a 14-residue polypeptide derived from

Table 1. 6-Substituted 4-(Phenylamino)-pyrimido[5,4-*d*]pyrimidines



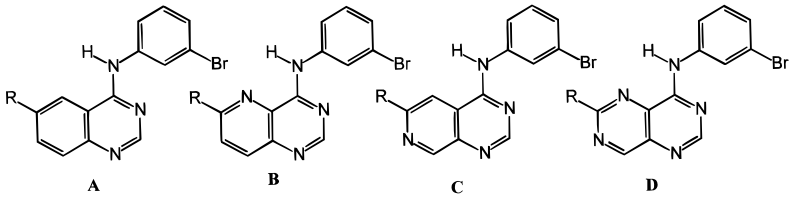
			IC$_{50}$ (nM)	
no.	R	mp (°C)	EGFR[a]	autophos[b]
	3-H Side Chain			
4a	Cl	130−131	2550	
4c	NHMe	195.5−196.5	13	
	3′-Br Side Chain			
5a	Cl	197.5−198	82	
5b	NH_2	280−282	1.5	
5c	NHMe	202.5−204	0.76	30
5d	NMe_2	179−180	0.95	22
5e	OMe	166−167	3.8	
5f	$NH(CH_2)_2NMe_2$	146−147	35	
5g	$NH(CH_2)_2$morpholide[c]	160−161	0.81	3.1
5h	$NH(CH_2)_3$morpholide	138−138.5	2.9	
5i	$NH(CH_2)_2$(4-imidazolyl)	209−210	0.25	5.1
5j	$NH(CH_2)_3$(1-imidazolyl)	192−194	2.3	
	3′-Me Side Chain			
6a	Cl	165−166	380	
6b	NH_2[c]	280−283	17	
6c	NHMe[c]	207−208	4.3	
6d	NMe_2[c]	122−122.5	4.0	
6g	$NH(CH_2)_2$morpholide[c]	170−172	2.3	
6i	$NH(CH_2)_2$(4-imidazolyl)	209−210.5	3.0	

[a] IC$_{50}$; concentration of drug (nM) to inhibit the phosphorylation of a 14-residue fragment of phospholipase Cγ1 by EGFR (prepared from human A431 carcinoma cell vesicles by immunoaffinity chromatography). See the Experimental Section for details. Values are the averages from at least two independent dose−response curves; variation was generally ±15%. [b] IC$_{50}$s for inhibition of phosphorylation of EGFR in A431 epidermoid carcinoma cells in culture. Values are the average of two experiments. See the Experimental Section for details. [c] Reference 17.

phospholipase Cγ1 was measured by reported methods.[6] At least two complete dose−response curves were determined for each compound, and averaged IC$_{50}$s are listed in Table 1. Selected compounds were also evaluated for their ability to inhibit autophosphorylation of the EGF receptor in A431 human epidermoid carcinoma cells. Compounds **5b**−**e** were prepared in order to compare a small range of compounds of the pyrimido[5,4-*d*]pyrimidine series with previously-reported quinazolines, pyrido[3,4-*d*]pyrimidines, and pyrido[3,2-*d*]pyrimidines with identical substituents in this position (Table 2).

As reported previously,[8] methylation of the 6-amino group in the quinazoline series leads to significant loss of inhibitory potency (IC$_{50}$s went from 0.78 nM for NH_2 to 84 nM for NMe_2; Table 2). A roughly similar, but less marked, effect was seen in the pyrido[3,2-*d*]-pyrimidines, with a slight loss of potency between the NH_2 and NMe_2 analogues (Table 2). However, a quite different pattern was seen with the corresponding pyrido[3,4-*d*]pyrimidines. Not only was the NH_2 analogue itself considerably more potent than in the other series (IC$_{50}$ 0.13 nM), but N-methylation provided large increases in potency (to 0.006 nM for the NMe_2 analogue; Table 2). This "supra-additive" effect shown by certain combinations of substituents has been suggested to be due to an induced conformational change to the enzyme, trapping the inhibitor in the hydrophobic core

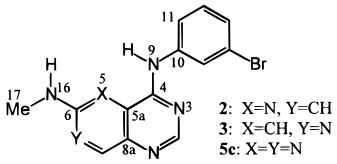
Table 2. Comparison of 6-Substituted 4-[(3-Bromophenyl)amino]quinazolines and Related Pyridopyrimidines and Pyrimido[5,4-*d*]pyrimidines: IC_{50} Values (nM) for Inhibition of PLCγ-Derived Substrate and Inhibition of Autophosphorylation of EGFR in EGF-Stimulated A431 Cells



	A,[a] quinazoline		B,[b] py[3,2-*d*]pyr		C,[b] py[3,4-*d*]pyr		D,[c] pyr[5,4-*d*]pyr	
R	isolated	autoph	isolated	autoph	isolated	autoph	isolated	autoph
NH_2	0.78		7.6	53	0.13	16	1.5	
NHMe	11		3.1	20	0.008	15	0.76	30
NMe_2	84		9.6	32	0.006	21	0.95	22
OMe	30		4.3		2.6	12	3.8	

[a] Data from refs 7 and 8. [b] Data from ref 14. [c] Data from present paper.

Table 3. Crystallographic Parameters for Compounds **2**, **3**, and **5c**



	2	**3**	**5c**
space group	monoclinic *C*2/*c*	monoclinic *P*21/*n*	triclinic *P*-1
unit cell *a* (Å)	14.902	13.571	7.326
b (Å)	14.542	9.189	7.638
c (Å)	14.252	14.772	12.486
α (deg)			78.82
β (deg)	117.90	103.46	76.99
γ (deg)			74.72
V (Å)^3	2729.4	1791.7	650.0
no. of reflections	1075	1399	1023
R-factor	0.168	0.068	0.043

of the enzyme/inhibitor complex and resulting in pseudoirreversible behavior.[8]

The pyrimido[5,4-*d*]pyrimidines studied here more closely resemble the [3,2-*d*] than the [3,4-*d*] isomers, with little difference in IC_{50}s between the NH_2- and NMe_2-substituted analogues, suggesting the requirement of an aza atom in the 7- but not the 5-position for the enhanced potency of the N-methylated derivatives. In order to compare the effect of different groups at the 3′-position, we also prepared and evaluated a subset of 3′-Me analogues, since this substituent group has also been found useful in anilinoquinazoline-type EGFR inhibitors.[12,22−24] The three compounds **(6b−d)** showed broadly similar trends to the corresponding 3′-Br analogues (**5b−d**) but were about 10-fold less effective on average. These compounds have recently been independently reported to be EGFR inhibitors.[17] The 3′-unsubstituted analogue **4c** had an IC_{50} of 13 nM compared with that of 0.76 nM for the 3′-Br analogue **5c**. The 17-fold increase in potency is in keeping with previous SAR indicating the importance of a 3′-lipophilic group.[7,12,22]

Assuming the inhibitory potencies for these compounds are directly proportional to their binding energies to the enzyme, as suggested previously,[8] the differences in IC_{50} values indicate an increase in binding energy of 1.8 kcal/mol between the NH_2 and NMe_2 pyrido[3,4-*d*]pyrimidine analogues, in contrast to a *loss* of 2.8 kcal/mol for the corresponding quinazolines. In order to see whether these different structure−activity relationships between the isomeric series could be related to subtle changes in conformations, X-ray crystal structures were determined for the three NHMe derivatives **2**, **3**, and **5c**. These are shown in Figure 1, with the basic crystallographic parameters in Table 3.

Figure 1. Superposition of the crystal structures of compounds **2** (blue), **3** (green), and **5c** (red). The superpositions were made by least-squares overlapping of the core atoms of the bicyclic rings using Quanta 96.

Table 4. Comparison of Key Bond Lengths and Torsion in Compounds **2**, **3**, and **5c**[a]

bond/angle	**2**	**3**	**5c**
	Distance (Å)		
C6−X5[b]	1.33(1)[c]	1.40(2)	1.357
X5−C5a	1.36(1)	1.38(3)	1.336
C5a−C8a	1.39(1)	1.38(1)	1.385
C5a−C4	1.43(1)	1.47(2)	1.432
C4−N9	1.34(1)	1.35(2)	1.366
N9−C10	1.399	1.41(2)	1.402
C4−N3	1.33(1)	1.29(2)	1.315
	Deviation from Coplanarity (deg)[d]		
C5a−C4−N9−C10	4.7	4.6	1.7
C4−N9−C10−C11	1.5	28.2	10.1
X5−C6−N16−C17	7.5	0.5	7.1

[a] See Table 3 diagram. [b] X = N for **2** and **5c**; X = CH for **3**. [c] Number in parentheses is estimated standard deviation in least significant digit. [d] Magnitude of difference of measured value from ±180°.

Table 4 provides comparative data for certain bond lengths and torsion angles of the three compounds. The

influence of the 5-hydrogen in **3** can be clearly seen. The C5a−C4 bond in **3** lengthened to 1.47 Å, compared to 1.43 Å in the 5-aza analogues **2** and **5c**, to relieve the nonbonded interaction between this and the amine hydrogen. The length of the C4−N9 bonds in all three analogues is quite short (1.34−1.37 Å), suggesting a considerable degree of conjugation and a consequent resistance to torsional twisting to relieve the nonbonded interaction between the C5 and N9 protons, as shown earlier with structurally-related anilinoacridines.[25] This is supported by the fact that even for **3** the C5a−C4−N9−C10 torsion angle is close to 180°, indicating an essentially coplanar geometry. However, the N9−C10 bonds are significantly longer (1.40−1.41 Å) in all three compounds, and in the case of **3** the torsion angle C4−N9−C10−C11 is nearly 30° out of plane, whereas in **2** and **5c** the corresponding torsion angles are 1.5° and 10°, respectively. Thus, compound **3** shows a considerable dihedral angle between the two ring systems, entirely explained by rotation about the N9−C10 bond, and the other two inhibitors are essentially planar. A loss of planarity between N9 and the phenyl ring might be expected to be reflected by a loss of bond order for N9−C10, leading to a longer C−N bond in **3** than in **2** and **5c**. However, although **3** does have the longest N9−C10 bond length of the three compounds (1.41 Å) it is only 0.01 Å longer than for the other compounds, suggesting a relatively minor perturbation in bonding orbitals. The superposition also shows that the C6−C16−N9 angle between the two side chain C−N bonds is expanded by about 5° in **3** with respect to its less potent analogues. The bromine atom in **3** is *anti* with respect to the the methylamino substituent, which in turn is *anti* to the aniline ring, whereas in **2** and **5c** these conformations are both *syn*. However, these represent alternative rotational isomers, likely to be of similar stability in solution and probably not of importance in explaining potency.

The combination of a longer C5a−C4 bond and the 30° out-of-plane rotation of the phenyl group in **3** suggests a minimum-energy conformation which positions the bromine atom quite differently with respect to the bicyclic chromophore than for **2** or **5c**. This can also be seen in Figure 1. However, there is little effect on the 6-NHMe group, which lies essentially coplanar to the ring in all cases (Table 4). Our binding model[15] incorporates an inhibitor with a dihedral angle between the two rings better than it incorporates a planar molecule. Although the model cannot be regarded as definitive, this does suggest a potential solution to the "supra-additive" phenomenon.[8] Either the enzyme could bind a planar molecule but in a less optimal mode than a bent one, or the enzyme could bind both in the same bent manner but would then have to pay an energy penalty for partial breaking of the N9−C10 π bond.

The pyrimido[5,4-*d*]pyrimidines **5c**,**d** showed similar potencies to the corresponding pyrido[3,2-*d*]- and pyrido-[3,4-*d*]pyrimidines for inhibition of autophosphorylation of EGFR in intact A431 cells (Table 2), with IC_{50}s of 20−30 nM. The high potency of these compounds in a cellular system and the indication of some steric tolerance at the substitution site prompted the evaluation of further analogues (**5f**−**j**) of higher aqueous solubility. These bear amine-containing side chains of varying base strength, linked through a secondary amine. The weakly basic derivatives **5g**−**j** generally retained potency against the isolated enzyme. The 4-imidazolyl analogue **5i** was even more effective (IC_{50} 0.25 nM), showing that a bulky, weakly basic solubilizing side chain can have a favorable influence on binding. In contrast, the more strongly basic (dimethylamino)ethyl analogue **5f** was less potent (IC_{50} 35 nM). Two 3′-Me analogues **6g**,**i** were somewhat less effective than the corresponding 3′-Br compounds (consistent with the above comparison between **5b**−**d** and **6b**−**d**). The morpholide analogues **5g** and **6g** have also been reported recently as EGFR inhibitors, but no biological data were given. The solubilized 3′-Br analogues **5g**,**i** were also evaluated for inhibition of autophosphorylation of EGFR in intact A431 cells and were considerably superior to the analogous NHMe compound **5c** (IC_{50}s 3.1 and 5.1 nM compared to 30 nM, respectively).

Conclusions

These results suggest that a key requirement in this class of compounds for very high potency against the isolated enzyme (equating to high binding energy) is a carbon atom in the 5-position. Thus the pyrimido[5,4-*d*]pyrimidines studied here more closely resemble the [3,2-*d*] than the [3,4-*d*] subclasses. The presence of a carbon rather than a nitrogen atom at the 5-position leads to significant conformational changes in the molecule, due to the requirement to relieve nonbonding interactions between the C5 and N9 protons. These changes (a longer C5a−C4 bond and a 30° out-of-plane rotation of the phenyl group) distinguish the pyrido[3,4-*d*]pyrimidines from the other subclasses. This requirement for a C5 bonding pair of electrons *peri* to the C4−N9 bond is seen in all of the (relatively few) other compounds that show the "supra-additive effect": quinazolines, imidazo[4,5-*g*]quinazolines, and more weakly basic pyrido[4,3-*d*]pyrimidines. Conversely, we have seen no "supra-additive effects" when the 5-position atom is a heteroatom and the *peri* pair of electrons is a lone pair. Within the pyrimido[5,4-*d*]pyrimidines, there is significant bulk tolerance at the 6-position, allowing the placement there of bulky, weakly basic solubilising groups. While these analogues were less potent than the NHMe derivative **5c** in the isolated enzyme assay, some (e.g., **5g** and **5i**) were considerably superior to **5c** in the autophosphorylation assay. These derivatives are among the most potent that we have seen as inhibitors of EGFR autophosphorylation in cellular assays and superior even to the pyrido[3,4-*d*]-pyrimidine **3**.

Experimental Section

Analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined using an Electrothermal Model 9200 digital melting point apparatus and are uncorrected. NMR spectra were measured on a Bruker AC-200 or DRX-400 spectrometer and referenced to Me_4Si. Mass spectra were recorded on a Varian VG 7070 spectrometer at nominal 5000 resolution. HPLC was carried out on a Waters system on a Bonclone 10 C18 column, using a Phillips PU4100M gradient elution pump and a Phillips PU 4120 diode array detector, and eluting with the appropriate ratios of 80% acetonitrile/20% water (solvent A) and ammonium formate buffer (solvent B; 28 g of ammonium formate + 2.55 mL of formic acid, made up to 1 L in deionized water, pH 4.5). Unless otherwise stated, amines were obtained from commercial sources and used without further purification.

Diethoxymethyl acetate was obtained from Aldrich Chemical Co., Inc., and used as received.

5-Amino-2-chloropyrimidine-4-carboxamide (8). A suspension of ethyl 5-amino-2-chloropyrimidine-4-carboxylate (**7**)[18] (5.7 g, 28 mmol) in EtOH (150 mL) was saturated with ammonia gas, and the mixture was heated in a sealed pressure vessel at 100 °C for 2 days. After cooling, the solid was collected and dried to give 5-amino-2-chloropyrimidine-4-carboxamide (**8**) (4.32 g, 89%): mp (EtOH) 247−248.5 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.40 (s, 1 H, H-6), 8.04 and 7.77 (2 br s, 2 H, exchangeable with D_2O, $CONH_2$), 7.01 (br s, 2 H, exchangeable with D_2O, NH_2); ^{13}C NMR δ 167.6 (s, CO), 151.2 (d, C-6), 143.5 (s), 142.0 (s), 134.5 (s). Anal. ($C_5H_5ClN_4O$) C, H, N.

6-Chloropyrimido[5,4-*d*]pyrimidin-4(3*H*)-one (9). A suspension of **8** (1.60 g, 9.3 mmol) in diethoxymethyl acetate (12 mL) was heated to 100 °C to give a clear solution which slowly deposited a white precipitate over time. Heating was continued for 6 h, and after cooling the solid was collected, washed with petroleum ether, and dried to give 6-chloropyrimido[5,4-*d*]pyrimidin-4(3*H*)-one (**9**) (1.11 g). Dilution of the mother liquors with petroleum ether gave crude material which was chromatographed on silica gel, eluting with CH_2Cl_2/EtOAc (3:2), to give a further 0.26 g of **9** (total yield 1.37 g, 81%): mp (EtOAc) 234 °C dec; ^{1}H NMR [$(CD_3)_2SO$] δ 13.02 (br s, 1 H, exchangeable with D_2O, NH), 9.32 (s, 1 H, H-8), 8.31 (s, 1 H, H-2); ^{13}C NMR δ 162.8 (d), 158.0 (s), 156.1 (s), 148.4 (d), 146.2 (s), 141.5 (s). Anal. ($C_6H_3ClN_4O$) C, H, N.

6-Chloro-4-(phenylamino)pyrimido[5,4-*d*]pyrimidine (4a). A suspension of **9** (0.37 g, 2 mmol) in 30 mL of $SOCl_2$ containing 1 drop of DMF was heated under reflux for 30 min to give a clear solution. The excess of $SOCl_2$ was then removed under reduced pressure, and the residue of crude 4,6-dichloropyrimido[5,4-*d*]pyrimidine (**10**) was treated immediately with a solution of aniline (0.47 g, 5 mmol) in 2-propanol. Precipitation of the product hydrochloride occurred rapidly, and after heating to reflux for 10 min the mixture was diluted with sufficient Et_3N to give a clear solution. After further dilution with water the solution was concentrated to give a crude solid which was purified by chromatography on silica gel, eluting with CH_2Cl_2/EtOAc (95:5), to give 6-chloro-4-(phenylamino)pyrimido[5,4-*d*]pyrimidine (**4a**) (0.41 g, 79%): mp (*i*-PrOH) 130−131 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.48 (br s, 1 H, exchangeable with D_2O, NH), 9.44 (s, 1 H, H-4), 8.75 (s, 1 H, H-6), 7.96 (d, J = 7.9 Hz, 2 H, H-2′,6′), 7.42 (dd, J = 8.1, 7.6 Hz, 2 H, H-3′,5′), 7.19 (t, J = 7.2 Hz, 1 H, H-4). Anal. ($C_{12}H_8ClN_5$) C, H, N.

Similarly prepared were the following compounds.

4-[(3-Bromophenyl)amino]-6-chloropyrimido[5,4-*d*]pyrimidine (5a): 80% yield; mp (*i*-PrOH) 197.5−198 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.62 (s, 1 H, exchangeable with D_2O, NH), 9.48 (s, 1 H, H-4), 8.83 (s, 1 H, H-6), 8.35 (br s, 1 H, H-2′), 8.40−8.00 (m, 1 H, H-6′), 7.41−7.36 (m, 2 H, H-4′,5′). Anal. ($C_{12}H_7BrClN_5$) C, H, N.

4-[(3-Methylphenyl)amino]-6-chloropyrimido[5,4-*d*]pyrimidine (6a): 71% yield; mp (*i*-PrOH) 165−166 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.37 (s, 1 H, exchangeable with D_2O, NH), 9.42 (s, 1 H, H-4), 8.76 (s, 1 H, H-6), 7.79 (br s, 1 H, H-2′), 7.76 (br d, J = 8.1 Hz, 1 H, H-6′), 7.30 (t, J = 7.8 Hz, 1 H, H-5′), 7.01 (d, J = 7.5 Hz, 1 H, H-4′), 2.35 (s, 3 H, CH_3). Anal. ($C_{13}H_{10}ClN_5$) C, H, N.

6-(Methylamino)-4-(phenylamino)pyrimido[5,4-*d*]pyrimidine (4c). A solution of **4a** (0.20 g, 0.78 mmol) and 40% aqueous $MeNH_2$ (3 mL) in 20 mL of DMSO was heated in a sealed pressure vessel at 100 °C for 2 h, and after cooling the mixture was diluted with water and extracted with EtOAc. Drying and removal of the solvent gave crude material which was chromatographed on silica gel, eluting with CH_2Cl_2/EtOAc (3:2), to give 0.16 g (82% yield) of 6-(methylamino)-4-(phenylamino)pyrimido[5,4-*d*]pyrimidine (**4c**): mp (*i*-PrOH) 195.5−196.5 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.38 (br s, 1 H, exchangeable with D_2O, NH), 9.00 (s, 1 H, H-4), 8.42 (s, 1 H, H-6), 7.98 (d, J = 7.9 Hz, 2 H, H-2′,6′), 7.80 (br, 1 H, exchangeable with D_2O, NH), 7.41 (dd, J = 8.1, 7.6 Hz, 1 H, H-5′), 7.13 (t, J = 7.3 Hz, 1 H, H-4′), 3.04 (d, J = 4.8 Hz, 3 H, CH_3). Anal. ($C_{13}H_{12}N_6$) C, H, N.

Similarly prepared were the following compounds.

6-Amino-4-[(3-bromophenyl)amino]pyrimido[5,4-*d*]pyrimidine (5b): 72% yield; mp (*i*-PrOH) 280−282 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.59 (s, 1 H, exchangeable with D_2O, NH), 9.06 (s, 1 H, H-4), 8.50 (s, 1 H, H-6), 8.42 (br s, 1 H, H-2′), 7.91 (br d, J = 8.3 Hz, 1 H, H-6′), 7.35 (t, J = 7.9 Hz, 1 H, H-5′), 7.29 (d, J = 7.5 Hz, 1 H, H-4′), 7.22 (br, 2 H, exchangeable with D_2O, NH_2). Anal. ($C_{12}H_9BrClN_6$) C, H, N.

4-[(3-Bromophenyl)amino]-6-(methylamino)pyrimido[5,4-*d*]pyrimidine (5c): 68% yield; mp (*i*-PrOH) 202.5−204 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.51 (s, 1 H, exchangeable with D_2O, NH), 9.01 (s, 1 H, H-4), 8.47 (s, 1 H, H-6), 8.38 (br s, 1 H, H-2′), 8.04 (br d, J = 7.0 Hz, 1 H, H-6′), 7.87 (br, 1 H, exchangeable with D_2O, NH), 7.34 (t, J = 8.0 Hz, 1 H, H-5′), 7.31 (d, J = 7.8 Hz, 1 H, H-4′), 3.06 (d, J = 4.4 Hz, 3 H, CH_3). Anal. ($C_{13}H_{11}BrN_6$) C, H, N.

4-[(3-Bromophenyl)amino]-6-(dimethylamino)pyrimido[5,4-*d*]pyrimidine (5d): 70% yield; mp (*i*-PrOH) 179−180 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.56 (s, 1 H, exchangeable with D_2O, NH), 9.09 (s, 1 H, H-4), 8.47 (s, 1 H, H-6), 8.35 (br s, 1 H, H-2′), 8.08 (br d, J = 7.5 Hz, 1 H, H-6′), 7.37 (t, J = 8.0 Hz, 1 H, H-5′), 7.32 (d, J = 7.8 Hz, 1 H, H-4′), 3.33 (s, 6 H, CH_3). Anal. ($C_{14}H_{13}BrN_6$) C, H, N.

4-[(3-Bromophenyl)amino]-6-methoxypyrimido[5,4-*d*]pyrimidine (5e): by reaction of **5a** in MeOH/Et_3N under reflux for 3 h, 100% yield; mp (*i*-PrOH) 166−167 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.00 (s, 1 H, exchangeable with D_2O, NH), 9.37 (s, 1 H, H-4), 8.70 (s, 1 H, H-6), 8.33 (br s, 1 H, H-2′), 8.04 (br d, J = 7.8 Hz, 1 H, H-6′), 7.40 (t, J = 7.9 Hz, 1 H, H-5′), 7.36 (d, J = 8.1 Hz, 1 H, H-4′), 4.19 (s, 3 H, CH_3). Anal. ($C_{13}H_{10}BrN_5O$) C, H, N.

4-[(3-Bromophenyl)amino]-6-[[2-(dimethylamino)ethyl]amino]pyrimido[5,4-*d*]pyrimidine (5f): 64% yield; mp (*i*-PrOH/H_2O) 146−147 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.50 (br s, 1 H, exchangeable with D_2O, NH), 9.02 (s, 1 H, H-4), 8.47 (s, 1 H, H-6), 8.36 (br s, 1 H, H-2′), 7.98 (br d, J = 8.3 Hz, 1 H, H-6′), 7.69 (br, 1 H, exchangeable with D_2O, NH), 7.37 (t, J = 8.0 Hz, 1 H, H-5′), 7.31 (d, J = 8.2 Hz, 1 H, H-4′), 3.37 (br, 2 H, CH_2), 2.49 (t, J = 6.6 Hz, 2 H, CH_2), 2.23 (s, 6 H, CH_3). Anal. ($C_{16}H_{18}BrN_7$) C, H, N.

4-[(3-Bromophenyl)amino]-6-[[2-(4-morpholino)ethyl]amino]pyrimido[5,4-*d*]pyrimidine (5g): 51% yield; mp (*i*-PrOH/H_2O) 160−161 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.50 (br s, 1 H, exchangeable with D_2O, NH), 9.02 (s, 1 H, H-4), 8.47 (s, 1 H, H-6), 8.32 (br s, 1 H, H-2′), 7.99 (br d, J = 7.7 Hz, 1 H, H-6′), 7.73 (br, 1 H, exchangeable with D_2O, NH), 7.38 (t, J = 8.0 Hz, 1 H, H-5′), 7.31 (d, J = 8.5 Hz, 1 H, H-4′), 3.69 (br, 2 H, NCH_2), 3.57 (t, J = 4.5 Hz, 4 H, OCH_2), 2.55 (t, J = 6.5 Hz, 2 H, NCH_2), 2.49 (m, 4 H, NCH_2). Anal. ($C_{18}H_{20}BrN_7O$) C, H, N.

4-[(3-Bromophenyl)amino]-6-[[3-(4-morpholino)propyl]amino]pyrimido[5,4-*d*]pyrimidine (5h): 92% yield; mp (CH_2Cl_2/hexane) 138−138.5 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.46 (br s, 1 H, exchangeable with D_2O, NH), 9.01 (s, 1 H, H-4), 8.47 (s, 1 H, H-6), 8.34 (br s, 1 H, H-2′), 8.00 (br d, J = 7.7 Hz, 1 H, H-6′), 7.95 (br, 1 H, exchangeable with D_2O, NH), 7.37 (t, J = 8.0 Hz, 1 H, H-5′), 7.31 (d, J = 8.1 Hz, 1 H, H-4′), 3.60 (br, 2 H, NCH_2), 3.57 (t, J = 4.5 Hz, 4 H, OCH_2), 2.42 (t, J = 6.8 Hz, 2 H, NCH_2), 2.37 (m, 4 H, NCH_2), 1.78 (pentet, J = 6.9 Hz, 2 H, CH_2). Anal. ($C_{19}H_{22}BrN_7O$) C, H, N.

4-[(3-Bromophenyl)amino]-6-[[2-(4-imidazolyl)ethyl]amino]pyrimido[5,4-*d*]pyrimidine (5i): 59% yield; mp (*i*-PrOH) 209−210 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.87 (br, 1 H, exchangeable with D_2O, NH), 9.59 (br s, 1 H, exchangeable with D_2O, NH), 9.02 (s, 1 H, H-4), 8.48 (s, 1 H, H-6), 8.38 (br s, 1 H, H-2′), 8.03 (m, 2 H, NH, H-6′), 7.58 (s, 1 H, H-2″), 7.39 (t, J = 8.0 Hz, 1 H, H-5′), 7.32 (d, J = 8.0 Hz, 1 H, H-4′), 6.89 (s, 1 H, H-5″), 3.75 (q, J = 6.4 Hz, 2 H, NCH_2), 2.86 (t, J = 7.1 Hz, 2 H, CH_2). Anal. ($C_{17}H_{15}BrN_8$) C, H, N.

4-[(3-Bromophenyl)amino]-6-[[3-(1-imidazolyl)propyl]amino]pyrimido[5,4-*d*]pyrimidine (5j): 93% yield; mp (CH_2Cl_2/hexane) 192−194 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.38 (br s, 1 H, exchangeable with D_2O, NH), 9.04 (s, 1 H, H-4), 8.48 (s, 1 H, H-6), 8.35 (br s, 1 H, H-2′), 8.06 (br, 1 H, exchangeable with D_2O, NH), 8.01 (br d, J = 7.0 Hz, 1 H, H-6′), 7.71 (s, 1 H, H-2″), 7.38 (t, J = 8.0 Hz, 1 H, H-5′), 7.32 (d, J = 8.4 Hz, 1 H,

H-4′), 7.25 (s, 1 H, H-5″), 6.92 (s, 1 H, H-4″), 4.11 (t, J = 7.0 Hz, 2 H, NCH_2), 3.53 (br, 2 H, NCH_2), 2.07 (pentet, J = 6.8 Hz, 2 H, CH_2). Anal. ($C_{18}H_{17}BrN_8$) C, H, N.

6-Amino-4-[(3-methylphenyl)amino]pyrimido[5,4-*d*]pyrimidine (6b): 54% yield; mp (*i*-PrOH) 280−283 °C (lit.[17] mp >260 °C); ^{1}H NMR [$(CD_3)_2SO$] δ 9.22 (s, 1 H, exchangeable with D_2O, NH), 9.03 (s, 1 H, H-4), 8.44 (s, 1 H, H-6), 7.79 (br s, 1 H, H-2′), 7.75 (br d, J = 8.1 Hz, 1 H, H-6′), 7.28 (t, J = 7.8 Hz, 1 H, H-5′), 7.24 (br, 2 H, exchangeable with D_2O, NH_2), 6.94 (d, J = 7.6 Hz, 1 H, H-4′), 2.34 (s, 3 H, CH_3). Anal. ($C_{13}H_{12}N_6 \cdot 0.25H_2O$) C, H, N.

6-(Methylamino)-4-[(3-methylphenyl)amino]pyrimido-[5,4-*d*]pyrimidine (6c): 61% yield; mp (MeOH/H_2O) 207−208 °C (lit.[17] mp 195−197 °C); ^{1}H NMR [$(CD_3)_2SO$] δ 9.30 (s, 1 H, exchangeable with D_2O, NH), 8.99 (s, 1 H, H-4), 8.42 (s, 1 H, H-6), 7.87−7.76 (br, 1 H, exchangeable with D_2O, NH), 7.82 (br d, J = 8.1 Hz, 1 H, H-6′), 7.78 (br s, 1 H, H-2′), 7.29 (t, J = 7.8 Hz, 1 H, H-5′), 6.96 (d, J = 7.4 Hz, 1 H, H-4′), 3.04 (d, J = 4.5 Hz, 3 H, NCH_3), 2.35 (s, 3 H, CH_3). Anal. ($C_{14}H_{14}N_6 \cdot 0.25H_2O$) C, H, N.

6-(Dimethylamino)-4-[(3-methylphenyl)amino]pyrimido[5,4-*d*]pyrimidine (6d): 92% yield; mp (*i*-PrOH) 122−122.5 °C (lit.[17] mp 120−121 °C); ^{1}H NMR [$(CD_3)_2SO$] δ 9.37 (s, 1 H, exchangeable with D_2O, NH), 9.07 (s, 1 H, H-4), 8.41 (s, 1 H, H-6), 7.83 (br d, J = 8.0 Hz, 1 H, H-6′), 7.77 (br s, 1 H, H-2′), 7.29 (t, J = 7.8 Hz, 1 H, H-5′), 6.96 (d, J = 7.7 Hz, 1 H, H-4′), 3.32 (s, 6 H, NCH_3), 2.35 (s, 3 H, CH_3). Anal. ($C_{15}H_{16}N_6$) C, H, N.

4-[(3-Methylphenyl)amino]-6-[[2-(4-morpholino)ethyl]-amino]pyrimido[5,4-*d*]pyrimidine (6g): 78% yield; mp (CH_2Cl_2/hexane) 170−172 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.29 (s, 1 H, exchangeable with D_2O, NH), 9.00 (s, 1 H, H-4), 8.42 (s, 1 H, H-6), 7.77 (br d, J = 8.1 Hz, 1 H, H-6′), 7.75 (br s, 1 H, H-2′), 7.70 (br, 1 H, exchangeable with D_2O, NH), 7.30 (t, J = 7.8 Hz, 1 H, H-5′), 6.96 (d, J = 7.4 Hz, 1 H, H-4′), 3.67 (br, 2 H, NCH_2), 3.60 (t, J = 4.5 Hz, 4 H, OCH_2), 2.56 (t, J = 6.6 Hz, 2 H, NCH_2), 2.50 (m, 4 H, NCH_2), 2.35 (s, 3 H, CH_3). Anal. ($C_{19}H_{23}N_7O$) C, H, N.

4-[(3-Methylphenyl)amino]-6-[[2-(4-imidazolyl)ethyl]-amino]pyrimido[5,4-*d*]pyrimidine (6i): 75% yield; mp (EtOAc/hexane) 198−200 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.87 (s, 1 H, exchangeable with D_2O, NH), 9.38 (s, 1 H, exchangeable with D_2O, NH), 8.99 (s, 1 H, H-4), 8.42 (s, 1 H, H-6), 7.99 (br, 1 H, exchangeable with D_2O, NH), 7.83 (br d, J = 7.4 Hz, 1 H, H-6′), 7.79 (br s, 1 H, H-2′), 7.56 (s, 1 H, H-2″), 7.30 (t, J = 7.8 Hz, 1 H, H-5′), 6.96 (d, J = 7.4 Hz, 1 H, H-4′), 6.89 (s, 1 H, H-5″), 3.74 (q, J = 6.1 Hz, 2 H, NCH_2), 2.86 (t, J = 6.9 Hz, 2 H, CH_2), 2.32 (s, 3 H, CH_3). Anal. ($C_{18}H_{18}N_8$) C, H, N.

X-ray Crystallographic Structure Determinations of 2, 3, and 5c. All inhibitors (2.0 mg) were crystallized in 2 mL microcentrifugation vials, by warming the solid until it dissolved and then allowing the capped vial to stand at room temperature until crystals appeared. If the crystallization was very rapid and produced rods too small for use, the warmed vial was allowed to cool slowly to room temperature in a small warm water bath. Compound **2** was obtained as rectangular yellow rods from DMF at a concentration of 500 mg/mL, using water bath-delayed cooling. Compound **3** was obtained from DMSO (as a DMSO solvate) at a concentration of 75 mg/mL as bright yellow anisotropic rods. These crystals readily effloresced DMSO, and the structure was therefore obtained in a sealed microcapillary tube containing 1 drop of DMSO. Compound **5c** was crystallized from DMF at a concentration of 160 mg/mL, using water bath-delayed cooling as light yellow rhombic rods. Data collection was carried out on an Enraf-Nonius CAD4 computer-controlled κ axis diffractometer, equipped with a graphite crystal incident beam monochromator using Cu Kα radiation (l = 1.541 84 Å).

Enzyme Assay. Epidermal growth factor receptor was isolated from human A431 carcinoma cell shed membrane vesicles by immunoaffinity chromatography as previously described,[26] and the assays were carried out as previously reported.[6] The substrate used was based on a portion of phospholipase Cγ1, having the sequence Lys-His-Lys-Lys-Leu-Ala-Glu-Gly-Ser-Ala-Tyr^{472}-Glu-Glu-Val. The reaction was allowed to proceed for 10 min at room temperature and then stopped by the addition of 2 mL of 75 mM phosphoric acid. The solution was then passed through a 2.5 cm phosphocellulose disk which bound the peptide. This filter was washed with 75 mM phosphoric acid (5×), and incorporated label was assessed by scintillation counting in an aqueous fluor. Control activity (no drug) gave a count of approximately 100 000 cpm. At least two independent dose−response curves were calculated, and the IC_{50} values were computed using the program CalcuSyn (Biosoft, Cambridge, U.K.). The reported values are averages; variation was generally ±15%.

EGF Receptor Autophosphorylation in A431 Human Epidermoid Carcinoma Cells. Cells were grown to confluency in 6-well plates (35 mm diameter) and exposed to serum-free medium for 18 h. They were then treated with **8** for 2 h and with EGF (100 ng/mL) for 5 min. The monolayers were lyzed in 0.2 mL of boiling Laemlli buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, and 50 mM Tris, pH 6.8), and the lysates were heated to 100 °C for 5 min. Proteins in the lysate were separated by polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. The membrane was washed once in 10 mM Tris, pH 7.2, 150 mM NaCl, and 0.01% azide (TNA) and blocked overnight in TNA containing 5% bovine serum albumin and 1% ovalbumin. The membrane was blotted for 2 h with antiphosphotyrosine antibody (UBI, 1 mg/mL in blocking buffer) and then washed twice in TNA, once in TNA containing 0.05% Tween-20 and 0.05% nonidet P-40, and twice in TNA. The membranes were then incubated for 2 h in blocking buffer containing 0.1 mCi/mL [^{125}I]protein A and then washed again as above. After the blots were dry they were loaded into a film cassette and exposed to X-AR X-ray film for 1−7 days. Band intensities were determined with a Molecular Dynamics laser densitometer.

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