Ethenesulfonamide Derivatives, a Novel Class of Orally Active Endothelin-A Receptor Antagonists

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In the present article we wish to report the discovery of a novel class of ET_A-selective endothelin (ET) receptor antagonists through the modification of the ET_A/ET_B non-selective antagonist, Ro47-0203 (Bosentan, 1). Replacement of the benzenesulfonamide group of 1 with a 2-phenylethenesulfonamide group gave compound 5a and resulted in improvement in ET_A-selectivity. Optimization of the alkoxy side chain attached to the core pyrimidine ring yielded the 2-fluoroethoxy derivative (5n) with further improvement of ET_A-selectivity. [IC_{50}=2.1 nM for ET_A receptor, ET_B/ET_A ratio=1200]. After oral administration, compound 5n inhibited the big ET-1 induced pressor response in pithed rats with a DR_2 value of 2.6 mg/kg and also exhibited a potent antagonistic activity in conscious rats.

Key words endothelin antagonist; ET_A-selective; ethenesulfonamide

Endothelin (ET), isolated from a conditioned medium of cultured porcine vascular endothelial cells in 1988, is a highly potent vasoconstrictive 21-amino acid peptide.[3] There are three isoforms (ET-1, ET-2, ET-3). ET-1 is the predominant component of the three ET-isopeptides and derived from precursor big ET-1.[4] Largely because of its ability to constrict vascular and nonvascular smooth muscle, ET-1 has been believed to be implicated in the pathogenesis of the various diseases such as myocardial infarction, hypertension, heart failure, atherosclerosis, cerebral and coronary vasospasm, renal failure and asthma. In fact, it has been reported that plasma ET-1 levels are increased in these diseases[5] and ET receptor antagonists have proven to be efficacious in some animal models,[6] suggesting that ET receptor antagonists will be useful as a therapeutic agent.

Two subtypes of receptors for ETs, termed ET_A receptor and ET_B receptor, have been cloned and stably expressed in mammals, and it appears that the ET_A receptor exhibits affinity for ET-1 and ET-2 over ET-3, whereas the ET_B receptor has nearly equipotent affinity for these three ETs.[7]

Although a number of ET_A-selective and ET_A/ET_B non-selective non-peptide antagonists have been reported for a decade,[8] which type of antagonist would be better might depend on the target diseases. However, some literature sources have indicated that the ET_A-selective antagonists are the promising candidates for the treatments of cardiovascular diseases such as hypertension and heart failure.[9]

We decided that our first goal was to discover ET_A-selective antagonists. At the beginning of our research, it seemed that Ro47-0203 (Bosentan) **1**,[8g] a potent, orally active and ET_A/ET_B non-selective non-peptide antagonist , was an attractive compound as a lead. Compound **1** possesses a central pyrimidine nucleus bearing the pharmacophores indispensable for activity and notably an acidic sulfonamide group adjacent to the large hydrophobic 4-*tert*-butylphenyl group. While the acidic function in this area of the molecule seems to be necessary to maintain activity,[8,10] the role of the 4-*tert*-butylphenyl group in the binding to the receptor remains uncertain.[11,12]

First, we focused on the replacement of the 4-*tert*-butylbenzenesulfonamide group by an alternative large hydrophobic group. We postulated that the vinylogous styryl group could be designed as a surrogate for the phenyl group.[13] Furthermore, since the SAR of the 6-position of the core pyrimidine ring was not apparent at the beginning of our research, we expected that judicious variations of the substituents of this position could result in more ET_A selective compounds. Herein we report the discovery of a novel series of potent and orally active nonpeptide ET_A selective antagonists derived from **1**.

Chemistry Charts 1 and 2 show the syntheses of 2-phenylethenesulfonamide.

The starting compound (**2**) was prepared according to the method reported by Burri *et al.*[11] Nucleophilic substitution of the pyrimidine derivative **2** with (*E*)-2-phenylethenesulfonamide (**3**) resulted in the chloropyrimidine (**4**) in 88% yield. The (*E*) form of **4** was confirmed by the coupling constant between vinyl protons (J=19.5 Hz) in the ^{1}H-NMR spectrum. The chloropyrimidine **4** was treated with alkoxide in the corresponding alcohol by heating to give the hydroxyalkoxy analogues (**5a**—**c**). When **4** was treated with 2-aminoethoxide in 2-aminoethanol by heating, the 2-hydroxyethylamino derivative (**5d**) was obtained instead of the 2-aminoethoxy derivative. On the other hand, **4** and 2-aminoethanol afforded the 2-aminoethoxy derivative (**5e**) at ambient temperature using sodium hydride. It is suggested that the 2-hydroxyethylamino derivative **5d** might be derived first by nucleophilic substitution of the chlorine group with the alkoxide to give the 2-aminoethoxy derivative **5e**, followed by nucleophilic substitution of the alkoxy group with the amino group. In fact, heating the 2-aminoethoxy derivative **5e** in 2-aminoethanol at 80 °C afforded hydroxyethyl amino derivative **5d**.

Treatment of the amine **5e** with acetylchloride gave the acetamide (**5f**). Sulfonylation of the amine **5e** with BOC-$NHSO_2Cl$ followed by deprotection with trifluoro acetic acid (TFA) gave the sulfamide (**5g**). Compound **5e** was also treated with the isopropylisocyanate to give the urea (**5h**) (Chart 1).

Nucleophilic substitution of the chloropyrimidine **4** with

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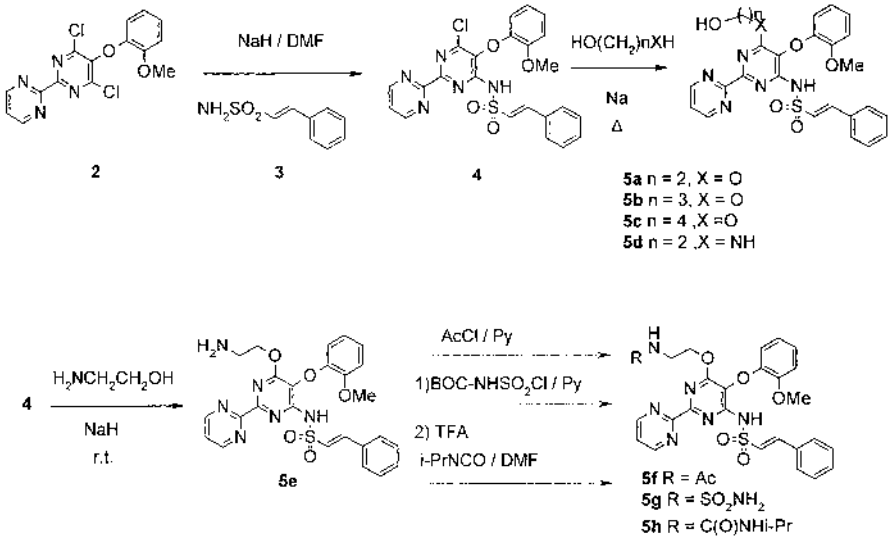
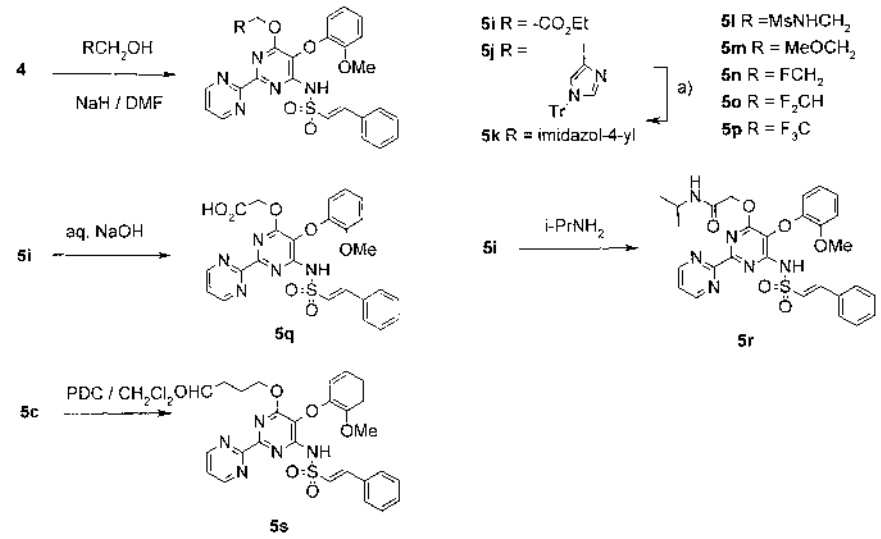


Reagents: BOC=*tert*-butyloxycarbonyl, TFA=trifluoroacetic acid

Chart 1



Reagents: a) TFA. Tr=triphenylmethyl

Chart 2

various alkoxides in DMF gave **5i**, **j** and **5l**—**p** in 20—72% yield. The triphenylmethyl group in **5j** was removed using TFA to give the imidazole (**5k**) in 42% yield. The carboxylic acid (**5q**) was generated by saponification of the corresponding ester **5i** using 1 N NaOH in 63% yield. The ester **5i** was treated with isopropylamine to give the amide (**5r**) in 46% yield. Oxidation of the alcohol **5c** using PDC gave the aldehyde (**5s**) in 59% yield (Chart 2).

The (*E*) form of all compounds was also confirmed by the coupling constant between vinyl protons ($J>15$ Hz) in the ^{1}H-NMR spectrum.

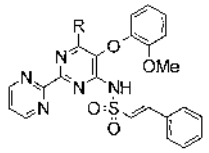
Results and Discussion

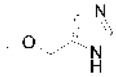
Compounds have been evaluated *in vitro* for their affinity for cloned human ET_A and ET_B receptors expressed in COS-1 cell by employing receptor-binding assays. Functional vascular ET-1 antagonism was determined *in vitro* for the ability to inhibit the ET-1 induced contraction of the ring preparation sample of rat aorta. Some compounds were further examined *in vivo* for their ability after intravenous or oral administration to inhibit an increase in mean arterial blood pressure (MABP) due to the administration of exogenous big ET-1 to pithed or conscious rats.

The structure–activity relationships (SARs) of our novel series of endothelin receptor antagonists are summarized in Tables 1 and 2.

Replacement of the benzenesulfonamide group of **1** with the 2-phenylethenesulfonamide group was investigated. The 2-phenylethenyl analogue **5a** showed about 4-fold improvement in binding affinity at the ET_A receptor compared to **1** and had an IC_{50} at the ET_A receptor of 1.6 nM. This modification also led to 6-fold increase in ET_A selectivity over that of the parent compound **1**; the selectivity *versus* the ET_B receptor of **5a** was found to be 230-fold. In order to confirm the

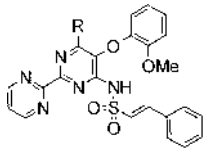
Table 1. ET_A and ET_B Receptor Binding Affinities for Ethenesulfonamide Derivatives



Compound	R	IC_{50} (nM)[a]		Selectivity for ET_A[c]
		ET_A[b]	ET_B[b]	
1[d]		6.6	250	38
5a[e]	$-OCH_2CH_2OH$	1.6	370	230
5b	$-OCH_2CH_2CH_2OH$	11	990	90
5c[e]	$-OCH_2CH_2CH_2CH_2OH$	80	1000	13
5e	$-OCH_2CH_2NH_2$	100		
5k		30		
5q	$-OCH_2COOH$	370		
5r	$-OCH_2CONH$*i*-Pr	200		
5f	$-OCH_2CH_2NHAc$	120		
5l	$-OCH_2CH_2NHMs$	180		
5g	$-OCH_2CH_2NHSO_2NH_2$	520		
5h	$-OCH_2CH_2NHCONH$*i*-Pr	71		
5d	$-NHCH_2CH_2OH$	24		

a) $n=2$ except **1** ($n=7$). *b*) Cloned human receptor binding. Blank space: not tested. *c*) Expressed as ET_B IC_{50}/ET_A IC_{50}. *d*) Sodium salt. *e*) Pottasium salt.

Table 2. ET_A and ET_B Receptor Binding Affinities for Ethenesulfonamide Derivatives



Compound	R	IC_{50} (nM)[a]		Selectivity for ET_A[c]
		ET_A[b]	ET_B[b]	
1[d]		6.6	250	38
5a[e]	$-OCH_2CH_2OH$	1.6	370	230
5i	$-OCH_2CO_2Et$	100		
5s[e]	$-OCH_2CH_2CH_2CHO$	130		
5m	$-OCH_2CH_2OMe$	4.8	2000	417
5n	$-OCH_2CH_2F$	2.1	2500	1200
5o	$-OCH_2CHF_2$	120		
5p	$-OCH_2CF_3$	280		

a—*e*) See footnotes in Table 1.

acidity of the sulfonamide moiety at the 4-position of the central pyrimidine ring, p*K*a values were measured for **1** and **5a**. Compound **5a** showed no significant difference in p*K*a value from **1** (**5a**, 4.7 *vs.* **1**, 5.0). This result imply that the space of the phenyl group or the size of the 4-substituent of the pyrimidine ring may participate in determining the affinity for both ET_A and ET_B receptors.

Compound **5a** also blocked the contractions caused by ET-1 in an isolated rat aorta in a concentration dependent fashion with a pA_2 value of 7.0. (pA_2; see experimental section and Table 3).

Intrigued by this observation, we then focused on the modification of the hydroxyethoxy side chain at the 6-position of the core pyrimidine ring of **5a**. There were few reports of SARs around this side chain of **1** at the beginning of our research.[12]

Table 3. Functional Vascular ET-1 Antagonism *in Vitro*; Inhibition of the ET-1 Induced Contraction of Ring Preparation Sample of Rat Aorta

Compound	pA_2	n[a]
1	6.7	19
5a	7.0	21
5n	6.9	19

a) Values are the means of the indicated number of experiments (n).

Lengthening the distance between the alcohol oxygen and the pyrimidine ring of **5a** by one or two carbon units (**5b**, **c**) resulted in decreases in ET_A activity by 7-fold and 50-fold, respectively. Replacement of the hydroxyl group in compound **5a** with an amino group led to a large loss of binding affinity (**5e**). The imidazole derivative **5k** and the acetic acid derivative **5q** were also less active than **5a**, indicating that basic or strong acidic groups were not well tolerated at this position.

We further investigated the surrogates of the hydroxyl group in compound **5a** because of concern for the metabolism *in vivo* of the alkyl alcohol moiety.[14] We synthesized the amide analogues **5r** and **5f**, the sulfonamide analogue **5l**, the sulfamide analogue **5g** and the urea analogue **5h**. These derivatives have hydrogen bond donors in the side chain as the hydroxyl analogue **5a**. These were generally found to have much lower binding affinity than **5a**.

Replacement of the hydroxyethoxy group in **5a** with the hydroxyethylamino group gave **5d** and resulted in reduced activity by 15-fold. None of the investigated substituents mentioned above resulted in improved ET_A binding. These SARs of the side chain led us to focus our attention on replacing the hydoroxyethoxy side chain in **5a** with groups bearing no hydrogen bond donor, but a hydrogen bond acceptor (Table 2).

Although the ester analogue **5i** and the aldehyde analogue **5s** were significantly less active, the replacement of the hydroxyl group with a methoxy group (**5m**) was well tolerated in ET_A binding affinity and yielded the improvement in the ET_A selectivity. The replacement of the hydroxyl group with a fluorine group (**5n**) led to a decrease in ET_B affinity (from 370 to 2500 nM) with little change in ET_A affinity (from 1.6 to 2.1 nM); the net result is a 5-fold improvement in ET_A selectivity. The ET_A selectivity *versus* the ET_B receptor was found to be 1200-fold (**5n**), which was the best of the series. These results indicated that removal of a hydrogen bond donor in the side chain lead to improved ET_A selectivity.

Compound **5n** also blocked the contractions caused by ET-1 in an isolated rat aorta in a concentration-dependent fashion with $pA_2=6.9$ (Table 3). Further introduction of fluorine atoms to the side chain, affording **5o** and **5p**, caused reduced binding affinity for the ET_A receptor.

Table 4 and Fig. 2 highlight our *in vivo* studies of the selected compounds.

Compounds with a 2-phenylethenesulfonamide moiety (**5a**, **n**) inhibited the increase in MABP due to the administration of exogenous big ET-1 to anesthetized pithed rats after intravenous administration or oral administration (Table 4).

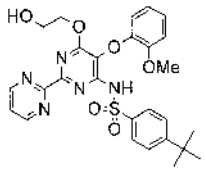


Fig. 1. Structure of R047-0203 (Bosentan, **1**)

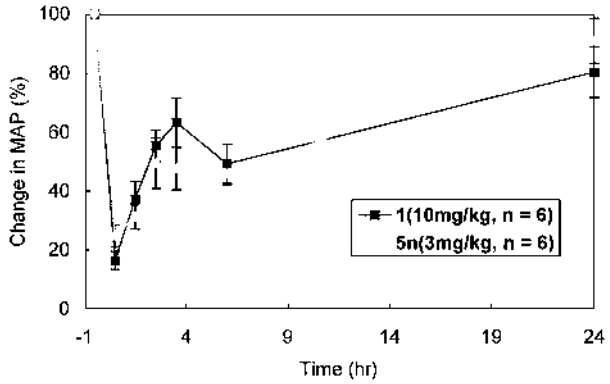


Fig. 2. Effect of *p.o.* Administration of the 2-Phenyethenesulfonamide Derivative **5n** and **1** on Pressor Response to Big ET-1 in Conscious Normotensive Rats

Change in MAP (%): Increase in mean arterial pressure (MAP) in conscious rats elicited by intravenous administration of big ET-1 (0.5 nmol/kg). **1**: Sodium salt.

Table 4. Effects of *p.o.* and i.v. Administration of the 2-Phenyethenesulfonamide Derivatives on Pressor Response to Big ET-1 in Pithed Rats

Compound	DR_2 (mg/kg)[a]			
	i.v.	n[b]	*p.o.*	n[b]
1	4.6	12	32	11
5a	3.0	10	5.6	14
5n	1.6	19	2.6	16

a) DR_2 value was defined as the dose of tested compounds which was required to produce a 2-fold rightward shift of dose–response curves of big ET-1 in diastolic blood pressure (DBP). *b*) Values are the means of the indicated number of experiments (n).

In this study, the DR_2 value was defined as the dose of the test compound which was required to produce a 2-fold rightward shift of the dose–response curves of big ET-1 in diastolic blood pressure (DBP) (details are described in the experimental section). Compounds **5a** and **5n** showed excellent inhibitory activities after oral administration with DR_2 values of 5.6 and 2.6 mg/kg, respectively. The oral activity of compound **5n** was 12-fold more potent than that of **1**. The calculated i.v./*p.o.* ratio from these DR_2 values of **5n** was 0.62.

Compounds **1** and **5n** were tested for their antihypertensive activity in conscious rats after oral administration. Compound **5n** also showed potent activity with a duration of action>6 h (Fig. 2). Maximum inhibition of the pressor effect of big ET-1 after oral administration was 83% for **1** (10 mg/kg) and 75% for **5n** (3 mg/kg).

These results indicated that our modification led to improvement in not only ET_A selectivity *in vitro* but also oral antagonistic activity *in vivo.*

Conclusion

Replacement of the benzenesulfonamide group of ET_A/ET_B non-selective antagonist Ro47-0203 (Bosentan) **1** with 2-phenylethenylsulfonamide led to the discovery of a new class of ET_A selective endothelin antagonists. Among this series, **5n** (YM-62899) showed the highest ET_A selectivity with potent affinity for ET_A receptor. Compound **5n** also had good oral activity in the inhibition of the pressor response caused by a big ET-1 infusion in both pithed and conscious rats. Compound **5n** is a suitable tool for investigating the therapeutic effects of an ET_A selective antagonist in various disease models. Further modification of **5n** will be reported elsewhere.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus without correction. ^{1}H-NMR spectra were recorded on a JNM-LA400, LA500, and A500 spectrometer using tetramethylsilane as an internal standard. MS spectra were determined with a Hitachi M-80 or JEOL JMS-DX300 spectrometer. Elemental analysis data were within±0.4% of the calculated values unless otherwise noted. All organic extracts were dried over anhydrous $MgSO_4$. Chromatographic purification was performed on Merck KGaA Silica gel 60 (0.040—0.063 mm).

(*E*)-*N*-[6-Chloro-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (4) To an ice-cooled solution of (*E*)-2-phenylethenesulfonamide (**3**) (36.7 g, 200 mmol) in *N*,*N*-dimethylformamide (DMF) (400 ml) was added 60% sodium hydride in mineral oil (17.6 g, 441 mmol), and the mixture was stirred for 30 min at room temperature. To the mixture 4,6-dichloro-5-(2-methoxyphenoxy)pyrimidine (**2**) (70.0 g, 200 mmol) was added, and the mixture was stirred for 2 h at room temperature. It was poured into ice-water and 1 N HCl, and the resulting precipitate was collected by filtration and washed with water and ethanol (EtOH) to give **4** (92.2 g, 93%), ^{1}H-NMR (DMSO-d_6) δ: 3.80 (3H, s), 6.81—6.91 (2H, m), 7.06—7.16 (2H, m), 7.43—7.48 (3H, m), 7.70—7.80 (3H, m), 7.84—7.94 (2H, m), 9.10 (2H, d, J=6.0 Hz). FAB-MS m/z: 496 (M^++1). ^{1}H-NMR (DMSO-d_6) at 90 °C δ: 3.79 (3H, s), 6.84—6.90 (2H, m), 7.06—7.14 (2H, m), 7.40—7.46 (3H, m), 7.62—7.68 (3H, m), 7.72 (1H, d, J=19.5 Hz), 7.79 (1H, d, J=19.5 Hz), 9.03 (2H, d, J=6.0 Hz).

Potassium (*E*)-*N*-[6-(2-Hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamidate (5a) Sodium (230 mg, 10.0 mmol) was added to ethyleneglycol (5.6 ml, 100 mmol) and stirred at 60 °C until all the sodium was dissolved. (*E*)-*N*-[6-chloro-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (**4**) (495 mg, 1.00 mmol) was added to the solution and stirred at 80 °C for 3 h. It was poured into ice-water and 1 N HCl, and the resulting precipitate was collected by filtration. The solid was chromatographed over silica gel using 20 : 1 $CHCl_3$–methanol (MeOH) to give (*E*)-*N*-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (500 mg, 96%), ^{1}H-NMR (DMSO-d_6) δ: 3.48—3.56 (2H, m), 3.83 (3H, s), 4.34—4.42 (2H, m), 4.68 (1H, m), 6.72—6.88 (2H, m), 7.00—7.14 (2H, m), 7.38—7.50 (3H, m), 7.62—7.78 (3H, m), 7.82 (1H, d, J=15.5 Hz), 7.97 (1H, d, J=15.5 Hz), 9.07 (2H, d, J=4.5 Hz), 11.34 (1H, s). FAB-MS m/z: 522 (M^++1). *Anal.* Calcd for $C_{25}H_{23}N_5O_6S$: C, 57.57; H, 4.44; N, 13.43; S, 6.16. Found: C, 57.50; H, 4.40; N, 13.31; S, 6.13.

(*E*)-*N*-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (104 mg, 0.200 mmol) was added to a 0.1 N potassium hydroxide (KOH) solution in EtOH (2 ml, 0.200 mmol) and stirred at room temperature for 30 min. The mixture was concentrated *in vacuo.* The residue was crystallized from ether (Et_2O) to give **5a** (84 mg, 75%). ^{1}H-NMR (DMSO-d_6) δ: 3.52—3.60 (2H, m), 3.85 (3H, s), 4.26—4.36 (2H, m), 4.82—4.94 (1H, m), 6.40—6.52 (1H, m), 6.72—6.80 (1H, m), 6.84—6.92 (1H, m), 7.01 (1H, d, J=8.0 Hz), 7.08—7.20 (1H, m), 7.38—7.44 (3H, m), 7.54—7.68 (3H, m), 8.20 (1H, d, J=17 Hz), 9.03 (2H, d, J=5.0 Hz). FAB-MS m/z: 560 (M^++1). *Anal.* Calcd for $C_{25}H_{22}N_5O_6SK \cdot 0.8H_2O$: C, 52.31; H, 4.14; N, 12.20; S, 5.59. Found: C, 52.50; H, 4.40; N, 12.08; S, 5.43.

In the same manner, compounds **5b** and **5c** were synthesized.

(*E*)-*N*-[6-(3-Hydroxypropoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (5b) 1.82 g (88%). mp 190—191 °C. ^{1}H-NMR (DMSO-d_6) δ: 1.75—195 (2H, m), 3.54—3.60 (2H, m), 3.98 (3H, s), 4.80 (2H, t, J=5.5 Hz), 5.14—5.30 (1H, m), 6.89 (1H, t, J=7.5 Hz), 7.00 (1H, d, J=7.0 Hz), 7.04—7.16 (2H, m), 7.32—7.42 (4H, m), 7.52—7.60 (2H, m), 7.62 (1H, d, J=15.5 Hz), 8.04 (1H, d, J=16 Hz),

8.81 (1H, s), 8.95 (2H, d, J=4.5 Hz). FAB-MS m/z: 536 (M^++1). *Anal.* Calcd for $C_{26}H_{25}N_5O_6S \cdot 0.1Et_2O$: C, 58.40; H, 4.83; N, 12.90; S, 5.91. Found: C, 58.44; H, 4.75; N, 12.61; S, 5.80.

Potassium (*E*)-*N*-[6-(4-Hydroxybutoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl]-2-phenylethenesulfonamidate Monohydrate (5c) 88 mg (72%). mp 141—142 °C. ^{1}H-NMR (DMSO-d_6) δ: 1.20—1.36 (2H, m), 1.50—1.56 (2H, m), 3.20—3.30 (2H, m), 3.85 (3H, s), 4.25 (2H, t, J=7.0 Hz), 4.31 (1H, t, J=5.5 Hz), 6.35—6.50 (1H, m), 6.68—6.80 (1H, m), 6.82—6.92 (1H, m), 7.01 (1H, d, J=3.8 Hz), 7.06—7.20 (1H, m), 7.28—7.48 (3H, m), 7.50—7.70 (3H, m), 8.20 (1H, d, J=17 Hz), 9.02 (2H, d, J=4.5 Hz). FAB-MS m/z: 550 (M^++1). *Anal.* Calcd for $C_{27}H_{26}N_5O_6SK \cdot H_2O$: C, 53.54; H, 4.66; N, 11.56; S, 5.29; K, 6.45. Found: C, 53.53; H, 4.63; N, 11.62; S, 5.30.

(*E*)-*N*-[6-(2-Hydroxyethylamino)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (5d) Sodium (69.3 mg, 30.2 mmol) was added to 2-aminoethanol (18.2 ml, 302 mmol) and stirred at 60 °C until all the sodium was dissolved. Compound **4** (3.00 g, 6.03 mmol) was added to the solution and stirred at 60 °C for 1 h and 80 °C for 2 h. It was poured into ice-water and 1 N HCl and neutralized with $NaHCO_3$. The mixture was then extracted with EtOAc. The organic layer was washed with brine, dried, and concentrated *in vacuo.* The residue was chromatographed over silica gel using 20 : 1 $CHCl_3$–MeOH to give a solid. The solid was washed with Et_2O to give **5d** (2.40 g, 76%). ^{1}H-NMR (DMSO-d_6) δ: 3.40—3.64 (4H, m), 3.85 (2.1H, s), 3.88 (0.9H, s), 4.74—4.90 (1H, m), 6.72 (1H, t, J=7.5 Hz), 6.87 (1H, d, J=8.0 Hz), 6.94 (1H, t, J=7.5 Hz), 7.05 (1H, d, J=8.0 Hz), 7.34—7.40 (2H, m), 7.40—7.60 (3H, m), 7.60—7.74 (1H, m), 7.78—7.86 (0.7H, m), 8.00 (1H, d, J=19 Hz), 9.02 (0.6H, d, J=4.0 Hz), 9.15 (1.4H, d, J=4.0 Hz), 10.74 (0.3H, s), 13.31 (0.7H, s). FAB-MS m/z: 521 (M^++1). *Anal.* Calcd for $C_{25}H_{24}N_6O_5S \cdot 0.3H_2O$: C, 57.09; H, 4.71; N, 15.98; S, 6.10. Found: C, 59.96; H, 4.44; N, 15.76; S, 6.10.

(*E*)-*N*-[6-(2-Aminoethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)-pyrimidin-4-yl]-2-phenylethenesulfonamide (5e) To a solution of 2-aminoethanol (1.23 g, 20.2 mmol) in DMF (10 ml) was added 60% sodium hydride in mineral oil (1.13 g, 28.2 mmol), and the mixture was stirred for 30 min at room temperature. To the mixture, **4** (2.00 g, 4.03 mmol) was added and the mixture was stirred for 1 h at room temperature. It was poured into ice-water and neutralized with 1 N HCl. The mixture was then extracted with $CHCl_3$. The organic layer was washed with brine, dried, and concentrated *in vacuo.* The residue was crystallized from EtOH to give **5e** (1.32 g, 63%), mp 187—189 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.80 (2H, t, J=5 Hz), 3.84 (3H, s), 4.36—4.40 (2H, m), 6.48 (1H, d, J=8.0 Hz), 6.73 (1H, t, J=7.5 Hz), 6.89 (1H, t, J=7.5 Hz), 7.01 (1H, d, J=8.0 Hz), 7.18 (1H, d, J=15 Hz), 7.31 (1H, t, J=7.5 Hz), 7.36—7.42 (2H, m), 7.60 (2H, d, J=7.5 Hz), 7.65 (1H, t, J=5.0 Hz), 8.21 (1H, d, J=15 Hz), 9.04 (2H, d, J=5.0 Hz). FAB-MS m/z: 521 (M^++1). *Anal.* Calcd for $C_{25}H_{24}N_6O_5S \cdot 1.25H_2O$: C, 55.29; H, 4.92; N, 15.47; S, 5.90. Found: C, 55.02; H, 4.78; N, 15.60; S, 5.86.

(*E*)-*N*-[2-({5-(2-Methoxyphenoxy)-6-[(2-phenylethenesulfonyl)amino]-2-(pyrimidin-2-yl)pyrimidin-4-yl}oxy)ethyl]acetamide (5f) To an ice-cooled solution of **5e** (275 mg, 0.528 mmol) in pyridine (5 ml) was added acetylchloride (50 mg, 0.634 mmol), and the mixture was stirred for 2.5 h at room temperature. The mixture was concentrated *in vacuo.* The residue was chromatographed over silica gel using 20 : 1 $CHCl_3$–MeOH to give an oil. The oil was crystallized from Et_2O to give **5f** (180 mg, 61%), mp 186—188 °C. ^{1}H-NMR (DMSO-d_6) δ: 1.70 (3H, s), 3.16—3.28 (2H, m), 3.80 (3H, s), 4.30—4.42 (2H, m), 6.74—6.88 (2H, m), 7.00—7.14 (2H, m), 7.38—7.52 (3H, m), 7.60—7.74 (5H, m), 7.83 (1H, d, J=15 Hz), 7.98 (1H, d, J=15 Hz), 9.08 (2H, d, J=4.0 Hz), 11.40 (1H, s). FAB-MS m/z: 563 (M^++1). *Anal.* Calcd for $C_{27}H_{26}N_6O_6S$: C, 57.64; H, 4.66; N, 14.94; S, 5.70. Found: C, 57.30; H, 4.67; N, 14.78; S, 5.75.

(*E*)-*N*-{5-(2-Methoxyphenoxy)-2-(pyrimidin-2-yl)-6-[2-(sulfamoylamino)ethoxy]pyrimidin-4-yl}-2-phenylethenesulfonamide (5g) To an ice-cooled solution of **5e** (375 mg, 0.720 mmol) in pyridine (5 ml) was added *N-tert*-butyloxycarbonylsulfamoylchloride (69.2 mg, 3.24 mmol) and the mixture was stirred for 12 h at room temperature. The mixture was concentrated *in vacuo.* The residue was chromatographed over silica gel using 20 : 1 $CHCl_3$–MeOH to give (*E*)-*N*-(6-{2-[(*N'-tert*-butoxycarbonylsulfamoyl)-amino]ethoxy}-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl)-2-phenylethenesulfonamide (342 mg, 68%). ^{1}H-NMR (DMSO-d_6) δ: 1.29 (9H, s), 3.10—3.28 (2H, m), 3.82 (3H, s), 4.37 (2H, t, J=7.5 Hz), 6.70—6.86 (2H, m), 7.00—7.12 (2H, m), 7.36—8.04 (8H, m), 8.58 (1H, d, J=5.5 Hz), 9.08 (2H, d, J=6.0 Hz), 10.85 (1H, s), 11.56 (1H, s). FAB-MS m/z: 700 (M^++1). To an ice-cooled solution of (*E*)-*N*-(6-{2-[(*N'-tert*-butoxycarbonylsulfamoyl)amino]ethoxy}-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl)-2-phenylethenesulfonamide (320 mg, 0.457 mmol) in CH_2Cl_2 (2 ml) was added trifluoroacetic acid (TFA) (2 ml), and the mixture was stirred for 3 h at room temperature. The mixture was concentrated *in vacuo.* The residue was chromatographed over silica gel using 20 : 1 $CHCl_3$–MeOH to give an oil. The oil was crystallized from Et_2O to give **5g** (140 mg, 51%), mp 192—194 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.04—3.14 (2H, m), 3.83 (3H, s), 4.36—4.50 (2H, m), 6.62 (2H, s), 6.72—6.90 (2H, m), 7.00—7.12 (2H, m), 7.40—7.50 (3H, m), 7.66—7.78 (3H, m), 7.88 (1H, d, J=20 Hz), 7.98 (1H, d, J=20 Hz), 9.08 (2H, d, J=4.0 Hz), 11.43 (1H, s). FAB-MS m/z: 600 (M^++1). *Anal.* Calcd for $C_{25}H_{25}N_7O_7S \cdot 0.25H_2O$: C, 49.70; H, 4.25; N, 16.23; S, 10.62. Found: C, 49.55; H, 4.14; N, 16.24; S, 10.52.

(*E*)-*N*-{6-[2-(3-Isopropylureido)ethoxy]-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl}-2-phenylethenesulfonamide (5h) To a solution of **5e** (400 mg, 0.768 mmol) in DMF (6 ml) was added 2-propylisocyanate (78 mg, 0.922 mmol) in DMF, and the mixture was stirred for 3 h at room temperature. The mixture was concentrated *in vacuo.* The residue was chromatographed over silica gel using 20 : 1 $CHCl_3$–MeOH to give an oil. The oil was crystallized from Et_2O–EtOH to give **5h** (21 mg, 45%), mp 177—179 °C. ^{1}H-NMR (DMSO-d_6) δ: 0.98 (6H, d, J=8.5 Hz), 3.04—3.22 (2H, m), 3.52—3.68 (1H, m), 3.82 (3H, s), 4.20—4.40 (2H, m), 5.58—5.82 (2H, m), 6.76—6.92 (2H, m), 7.00—7.16 (2H, m), 7.36—7.54 (3H, m), 7.62—7.80 (3H, m), 7.84 (1H, d, J=20 Hz), 7.96 (1H, d, J=20 Hz), 9.08 (2H, d, J=4.0 Hz), 11.38 (1H, s). FAB-MS m/z: 606 (M^++1). *Anal.* Calcd for $C_{29}H_{31}N_7O_6S \cdot 0.25H_2O$: C, 57.08; H, 5.20; N, 16.07; S, 5.26. Found: C, 57.19; H, 5.07; N, 16.19; S, 5.34.

Ethyl (*E*)-({5-(2-Methoxyphenoxy)-6-[(2-phenylethenesulfonyl)amino]-2-(pyrimidin-2-yl)pyrimidin-4-yl}oxy)acetate (5i) To an ice-cooled solution of ethyl 2-hydroxyacetate (2.10 g, 20.2 mmol) and **4** (2.00 g, 4.03 mmol) in DMF (50 ml) was added 60% sodium hydride in mineral oil (1.14 g, 28.5 mmol), and the mixture was stirred for 2 h at room temperature and for 30 min at 50 °C. It was poured into ice-water and 1 N HCl, and the resulting precipitate was collected by filtration. The solid was chromatographed over silica gel using 20 : 1 $CHCl_3$–MeOH to give an oil **5i** (960 mg, 48%). ^{1}H-NMR (DMSO-d_6) δ: 1.11 (3H, t, J=6.5 Hz), 3.84 (3H, s), 4.08 (2H, q, J=6.5 Hz), 4.96 (2H, s), 6.72 (1H, d, J=8.0 Hz), 6.82 (1H, t, J=7.5 Hz), 7.04 (1H, t, J=7.5 Hz), 7.10 (1H, d , J=8.0 Hz), 7.40—7.48 (3H, m), 7.62—7.78 (3H, m), 7.83 (1H, d, J=15.5 Hz), 7.93 (1H, d, J=15.5 Hz), 9.05 (2H, d, J=5.0 Hz), 11.53 (1H, s). FAB-MS m/z: 564 (M^++1). *Anal.* Calcd for $C_{27}H_{25}N_5O_7S \cdot 0.5H_2O \cdot 0.4DMF$: C, 56.28; H, 4.82; N, 12.57; S, 5.33. Found: C, 56.44; H, 4.68; N, 12.70; S, 5.14.

(*E*)-*N*-[6-(1*H*-Imidazol-4-ylmethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (5k) To a solution of [1-(triphenylmethyl)imidazol-4-yl]methanol (673 mg, 1.98 mmol) in DMF (15 ml) was added 60% sodium hydride in mineral oil (130 mg, 3.26 mmol). **4** (490 mg, 0.988 mmol) was added to the mixture and the mixture was stirred for 3.5 h at 60 °C. It was poured into ice-water and acidified with 1 N HCl (pH=4). The mixture was then extracted with EtOAc. The organic layer was washed with brine, dried, and concentrated *in vacuo.* The residue was chromatographed over silica gel using 40 : 1 $CHCl_3$–MeOH to give an oil. The oil was crystallized from Et_2O–EtOH to give (*E*)-*N*-{5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)-6-[1-(triphenylmethyl)imidazol-4-yl-methoxy]-pyrimidin-4-yl}-2-phenylethenesulfonamide **5j** (320 mg, 20%). ^{1}H-NMR (DMSO-d_6) δ: 3.75 (3H, s), 5.29 (2H, s), 6.45 (1H, d, J=7.5 Hz), 6.63 (1H, t, J=7.5 Hz), 6.88—7.04 (7H, m), 7.06—7.34 (10H, m), 7.36—7.40 (6H, m), 7.72—8.08 (4H, m), 8.81 (2H, d, J=4.5 Hz). FAB-MS m/z: 800 (M^++1). A solution of **5j** in TFA (10 ml) was stirred at room temperature for 30 min. The mixture was concentrated *in vacuo.* To the residue was added sat.$NaHCO_3$ and the resulting precipitate was collected by filtration. The solid was chromatographed over silica gel using 20 : 1 $CHCl_3$–MeOH to give a solid. The solid was washed with Et_2O to give **5k** (225 mg, 42%), mp 147—149 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.79 (3H, s), 5.34 (2H, s), 6.59 (1H, d, J=8.0 Hz), 6.75 (1H, t, J=8.0 Hz), 6.98 (1H, t, J=8.0 Hz), 7.04 (1H, d, J=8.0 Hz), 7.28 (1H, s), 7.38—7.50 (3H, m), 7.63 (1H, d, J=15.5 Hz), 7.64—7.80 (3H, m), 7.96—8.08 (2H, m), 9.15 (2H, d, J=5.0 Hz). FAB-MS m/z: 558 (M^++1). *Anal.* Calcd for $C_{27}H_{23}N_7O_5S \cdot 1.5H_2O$: C, 55.47; H, 4.48; N, 16.77; S, 5.49. Found: C, 55.35; H, 4.24; N, 16.76; S, 5.44.

(*E*)-*N*-[6-(2-Fluoroethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (5n) To an ice-cooled solution of 2-fluoroethanol (258 mg, 4.03 mmol) in DMF (20 ml) was added 60% sodium hydride in mineral oil (194 mg, 484 mmol) and the mixture was stirred at room temperature for 30 min. **4** (400 mg, 0.807 mmol) was added to the solution and stirred at room temperature for 3 h. It was poured into ice-water and 1 N HCl. The mixture was then extracted with $CHCl_3$. The organic layer was concentrated *in vacuo.* The residue was chromatographed

over silica gel using 40 : 1 $CHCl_3$–MeOH to give an oil. The oil was crystallized from Et_2O–EtOH to give **5n** (240 mg, 57%). mp 142—143 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.82 (3H, s), 4.40—4.68 (4H, m), 6.78 (1H, d, J=8.0 Hz), 6.83 (1H, t, J=7.5 Hz), 7.05 (1H, t, J=7.5 Hz), 7.09 (1H, d, J=8.0 Hz), 7.40—7.52 (3H, m), 7.62—7.80 (3H, m), 7.84 (1H, d, J=16.0 Hz), 7.98 (1H, d, J=16.0 Hz), 9.08 (2H, d, J=4.0 Hz), 11.47 (1H, s). FAB-MS m/z: 524 (M^++1). *Anal.* Calcd for $C_{25}H_{22}N_5O_5SF$: C, 57.35; H, 4.24; N, 13.38; S, 6.12; F, 3.63. Found: C, 57.21; H, 4.34; N, 13.44; S, 6.09; F, 3.60.

In the same manner, compounds **5l**, **5m**, **5o**, and **5p** were synthesized.

(*E*)-*N*-{6-[2-(Methanesulfonylamino)ethoxy]-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl}-2-phenylethenesulfonamide (5l) Yellow amorphous (420 mg, 69%). ^{1}H-NMR (DMSO-d_6) δ: 2.83 (3H, s), 3.14—3.26 (2H, m), 3.83 (3H, s), 4.34—4.50 (2H, m), 6.74—6.98 (2H, m), 7.00—7.16 (2H, m), 7.27 (1H, t, J=6.0 Hz), 7.38—7.52 (3H, m), 7.62—7.80 (3H, m), 7.85 (1H, d, J=15.0 Hz), 7.97 (1H, d, J=15.0 Hz), 9.08 (2H, d, J=4.0 Hz), 11.44 (1H, s). FAB-MS m/z: 599 (M^++1). *Anal.* Calcd for $C_{26}H_{26}N_6O_7S_2\cdot 0.5H_2O\cdot 1.7CHCl_3$: C, 50.06; H, 4.36; N, 13.38; S, 10.21; Cl, 2.88. Found: C, 50.20; H, 4.37; N, 13.55; S, 9.92; Cl, 2.62.

(*E*)-*N*-[6-(2-Methoxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (5m) Crystallized from Et_2O (240 mg, 56%), mp 129—130 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.08 (3H, s), 3.36—3.48 (2H, m), 3.82 (3H, s), 4.42—4.56 (2H, m), 6.76—6.90 (2H, m), 7.00—7.14 (2H, m), 7.40—7.54 (3H, m), 7.62—7.80 (3H, m), 7.82 (1H, d, J=16.0 Hz), 7.97 (1H, d, J=16.0 Hz), 9.00—9.14 (2H, m), 11.41 (1H, s). FAB-MS m/z: 536 (M^++1). *Anal.* Calcd for $C_{26}H_{25}N_5O_6S$: C, 58.31; H, 4.70; N, 13.08; S, 5.99. Found: C, 58.26; H, 4.83; N, 13.10; S, 5.96.

(*E*)-*N*-[6-(2,2-Difluoroethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (5o) Crystallized from Et_2O (246 mg, 58%). mp 190—191 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.81 (3H, s), 4.66 (2H, t, J=15.0 Hz), 6.15 (1H, t, J=55 Hz), 6.78—6.85 (2H, m), 7.04—7.10 (2H, m), 7.45—7.46 (3H, m), 7.70—7.74 (3H, m), 7.86 (1H, d, J=15.0 Hz), 7.98 (1H, d, J=15.0 Hz), 9.10 (2H, d, J=5.0 Hz), 11.57 (1H, s). FAB-MS m/z: 542 (M^++1). *Anal.* Calcd for $C_{25}H_{21}N_5O_5SF_2$: C, 55.45; H, 3.91; N, 12.93; S, 5.92; F, 7.02. Found: C, 55.46; H, 3.82; N, 12.95; S, 5.95; F, 6.98.

(*E*)-*N*-[5-(2-Methoxyphenoxy)-2-(pyrimidin-2-yl)-6-(2,2,2-trifluoroethoxy)pyrimidin-4-yl]-2-phenylethenesulfonamide (5p) Crystallized from Et_2O (325 mg, 72%), mp 204—205 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.79 (3H, s), 5.03 (2H, q, J=8.5 Hz), 6.76—6.88 (2H, m), 7.02—7.14 (2H, m), 7.42—7.52 (3H, m), 7.66—7.80 (3H, m), 7.87 (1H, d, J=15.0 Hz), 7.96 (1H, d, J=15.0 Hz), 9.09 (2H, d, J=4.5 Hz), 11.67 (1H, s). AB-MS m/z: 560 (M^++1). *Anal.* Calcd for $C_{25}H_{20}N_5O_5SF_3$: C, 53.67; H, 3.60; N, 12.52; S, 5.73; F, 10.19. Found: C, 53.53; H, 3.60; N, 12.43; S, 5.68; F, 10.28.

(*E*)-({5-(2-Methoxyphenoxy)-6-[(2-phenylethenesulfonyl)amino]-2-(pyrimidin-2-yl)pyrimidin-4-yl}oxy)acetic Acid (5q) To a solution of **5i** (490 mg, 0.869 mmol) in THF (10 ml) was added 1 N sodium hydroxide in water (3.48 ml, 3.28 mmol) and the mixture was stirred for 12 h at room temperature. The mixture was concentrated *in vacuo.* To the residue 1 N HCl in water was added and the resulting precipitate was collected by filtration. The solid was washed with water and EtOH to give **5q** (294 mg, 63%), mp 206—207 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.84 (3H, s), 4.92 (2H, s), 6.74 (1H, d, J=8.0 Hz), 6.79—6.81 (1H, m), 7.01—7.04 (1H, m), 7.09 (1H, d, J=8.0 Hz), 7.40—7.52 (3H, m), 7.64—7.78 (3H, m), 7.82 (1H, d, J=15.0 Hz), 7.95 (1H, d, J=15.0 Hz), 9.06 (2H, d, J=4.5 Hz), 11.47 (1H, s). AB-MS m/z: 536 (M^++1). *Anal.* Calcd for $C_{25}H_{21}N_5O_7S\cdot 0.25H_2O$: C, 55.60; H, 4.01; N, 12.97; S, 5.94. Found: C, 55.54; H, 4.13; N, 12.71; S, 5.83.

(*E*)-*N*-Isopropyl-2-({5-(2-methoxyphenoxy)-6-[(2-phenylethenesulfonyl)amino]-2-(pyrimidin-2-yl)pyrimidin-4-yl}oxy)acetamide (5r) Compound **5i** (300 mg, 0.532 mmol) and isopropylamine (3 ml) were heated under reflux for 5.5 h. The mixture was concentrated *in vacuo.* The residue was chromatographed over silica gel using 20 : 1 $CHCl_3$–MeOH to give an amorphous solid. The amorphous solid was crystallized from Et_2O to give **5r** (140 mg, 46%), mp 149—150 °C. ^{1}H-NMR (DMSO-d_6) δ: 0.96 (6H, d, J=8.5 Hz), 3.72—3.84 (1H, m), 3.84 (3H, s), 4.76 (2H, s), 6.76—6.90 (2H, m), 6.98—7.16 (2H, m), 7.24—7.36 (1H, m), 7.40—7.54 (3H, m), 7.62—7.78 (3H, m), 7.83 (1H, d, J=20 Hz), 7.94 (1H, d, J=20 Hz), 9.06 (2H, d, J=5.5 Hz), 11.48 (1H, s). FAB-MS m/z: 577 (M^++1). *Anal.* Calcd for $C_{28}H_{28}N_6O_6S\cdot H_2O$: C, 56.56; H, 5.08; N, 14.13; S, 5.39. Found: C, 56.67; H, 4.96; N, 14.17; S, 5.56.

Potassium (*E*)-*N*-[6-(3-formylpropoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl]-2-phenylethenesulfonamidate (5s) To a solution of (*E*)-*N*-[6-(4-hydroxybutoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl]-2-phenylethenesulfonamide (free acid of **5c**) (1.55 g, 2.81 mmol) in dichloromethane was added pyridinium dichromate (PDC) (2.70 g, 7.17 mmol), and the mixture was stirred at room temperature overnight. To the mixture was added PDC (1.21 g, 3.21 mmol). After the mixture was stirred for 6 h, the mixture was filtered. The filtrate was concentrated *in vacuo.* The residue was chromatographed over silica gel using 30 : 1 $CHCl_3$–MeOH to give 905 mg (59%) of (*E*)-*N*-[6-(3-formylpropoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl]-2-phenylethenesulfonamide as an amorphous solid. MS m/z: 548 (M^++1). (*E*)-*N*-[6-(3-formylpropoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]-2-phenylethenesulfonamide (104 mg, 0.200 mmol) was added to a 0.1 N KOH solution in EtOH (1.75 ml, 0.175 mmol) and stirred at room temperature for 30 min. The mixture was concentrated *in vacuo.* The residue was crystallized from Et_2O to give **5s** (88 mg, 72%). mp 140—145 °C. ^{1}H-NMR (DMSO-d_6) δ: 1.30—1.48 (1H, m), 1.50—1.60 (1H, m), 1.70—1.82 (1H, m), 2.22—2.32 (1H, m), 3.80 (3H, s), 4.18—4.30 (2H, m), 6.39—6.52 (1H, m), 6.69—6.78 (1H, m), 6.80—6.94 (1H, m), 6.98—7.06 (1H, m), 7.08—7.20 (1H, m), 7.28—7.36 (1H, m), 7.38—7.46 (2H, m), 7.56—7.66 (3H, m), 8.24 (1H, d, J=15 Hz), 9.02 (2H, d, J=5.0 Hz), 9.51 (1H, s). FAB-MS m/z: 548 (M^++1). *Anal.* Calcd for $C_{27}H_{24}N_5O_6SK\cdot H_2O$: C, 53.72; H, 4.34; N, 11.60; S, 5.31; K, 6.48. Found: C, 53.61; H, 4.47; N, 11.41; S, 5.25.

Binding Assay For competition studies, [^{125}I]ET-1 (200 pM) was added to each membrane preparation, which was incubated with various concentrations of compounds in 250 μl of assay buffer containing 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 0.01% BSA. Binding reactions were initiated by the addition of the membrane preparations. After the incubation period (180 min, room temperature), the reaction was terminated by the addition of 3 ml of ice-cold Tris buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 0.01% BSA) followed by rapid filtration through Whatman GF/C filters. The filters were rinsed twice, and the radioactivity retained on the filters was counted using a gamma counter at 60% efficiency. Each assay was performed in duplicate, and nonspecific binding was assessed in the presence of 100 nM unlabeled ET-1. The IC_{50} values were calculated by a non-linear regression analysis.

Vessel Contraction (Inhibition of Big ET-1 Induced Contraction of Ring Preparation Sample of Rat Aorta) Male Wistar rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and the thoracic aorta was quickly removed and placed in a Krebs–Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11.1 mM glucose). The endothelium was removed by gentle rubbing of the intimal surface using a small cotton ball and each ring was suspended in a 10 ml isolated organ chamber (siliconized) containing gassed (95% O_2–5% CO_2) and warmed (37 °C) Krebs–Henseleit solution. Vessel segments were attached to an isometric force transducer linked to a physiographic recorder for monitoring tension change. Baseline tension was set at 1.0 g, and the tissues were allowed to equilibrate for 60 min. The tissues were contracted with phenylephrine (1 μM) followed by challenge with acetylcholine (1 μM). A negative relaxant response to acetylcholine confirmed the absence of endothelium. The rings were stimulated to contract with 60 mM KCl repeatedly until the contractile response to KCl became stable before starting the experiments. Cumulative concentration–response curves to ET-1 were performed in the presence or absence of test compounds after a 30-min pretreatment period. Contractile responses were expressed as a percentage of the response elicited by 60 mM KCl. The effective concentration of ET-1 causing 50% maximum response (EC_{50}) in the presence or absence of test compounds was determined by regression analysis. Dose ratios were determined and the results analyzed for competitiveness. The pA_2 value was estimated by plotting the log of (dose ratio−1) as a function of the negative log of concentration of test compounds.

Functional Assay *in Vivo* (Inhibition of Pressor Response to Big ET-1): Pithed Rats *In vivo* antagonistic activity in pithed rats was evaluated according to the method of Clozel *et al.* described previously (Clozel *et al.*, 1994). Briefly, male Wistar rats were pithed under sodium pentobarbital anesthesia and artificially ventilated with room air. The right common carotid artery and the left femoral vein were cannulated for blood pressure measurements and i.v. injection of drugs, respectively. After stabilization of blood pressure, various doses of (1 ml/kg) test compounds or vehicle (distilled water) were injected. Five minutes later, the first dose of big ET-1 was injected intravenously. In another series of experiments, the oral activities of test compounds were assessed. Varying doses of (5 ml/kg) the test compounds or vehicle (0.5% methyl cellulose) were administered by gastric gavage with a cannula. About 20 minutes later, the rats were anesthetized with sodium pentobarbital, and 30 minutes later, pithed and ventilated. After stabilization of the blood pressure, the first dose of big ET-1 was injected intra-

venously. In this study, the DR_2 value was defined as the dose of test compounds which was required to produce a 2-fold rightward shift of the dose–response curves of big ET-1 in diastolic blood pressure (DBP).

Functional Assay *in Vivo* (Inhibition of Pressor Response to Big ET-1): Conscious Normotensive Rats Male Wistar rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.). The right common carotid artery and the left jugular vein were cannulated with a polyethylene tube for determination of blood pressure and heart rate, and for i.v. administration of big ET-1 (0.5 nmol/kg). The animals were allowed to recover for 2 to 3 d after the operation, during which time they were housed in individual cages with free access to rat chow and water. After an appropriate equilibration period, bolus i.v. doses of big ET-1 were administered to determine control responses and patency of the catheters. Each rat was treated with a single *p.o.* dose of antagonist or vehicle (0.5% methyl cellulose) and any changes in blood pressure were noted. The percentage of the pressor response to big ET-1 challenges during the subsequent 6.5 h and at 24 h were used as a measure of big ET-1 inhibition.

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References and Notes

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