Design and SAR of Novel Potassium Channel Openers Targeted for Urge Urinary Incontinence. 1. *N*-Cyanoguanidine Bioisosteres Possessing in Vivo Bladder Selectivity

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A structurally novel series of adenosine 5′-triphosphate-sensitive potassium (K_{ATP}) channel openers is described. As part of our efforts directed toward identifying novel, bladder-selective potassium channel openers (KCOs) targeted for urge urinary incontinence (UUI), we found that bioisosteric replacement of the *N*-cyanoguanidine moiety of pinacidil (**1**, Figure 1) with a diaminocyclobutenedione template afforded squaric acid analogue **2**, the prototype of a novel series of K_{ATP} channel openers with unique selectivity for bladder smooth muscle in vivo. Further modification of the heterocyclic ring to give substituted aryl derivatives (**3**) afforded potent KCOs that possessed the desired detrusor selectivity when administered orally. The effects of these potassium channel agonists on bladder contractile function was studied in vitro using isolated rat detrusor strips. Potent relaxants were evaluated in vivo in a rat model of bladder instability. Lead compounds were evaluated concomitantly in normotensive rats for their effects on mean arterial blood pressure (MAP) and heart rate as a measure of in vivo bladder selectivity. (*R*)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-3-ethyl-benzonitrile (**79**) met our potency and selectivity criteria and represents an attractive development candidate for the treatment of UUI. Electrophysiological studies using isolated rat bladder detrusor myocytes have demonstrated that compound **79** produces significant hyperpolarization which is glyburide-reversed, thus consistent with the activation of K_{ATP}. The design, synthesis, structure−activity relationships (SAR), and pharmacological activity associated with this series of novel KCOs will be discussed.

Introduction

Urinary incontinence (UI), officially defined as involuntary loss of urine, can become a devastating disorder to the patient due to its significant social and hygienic impact and negative effect on quality of life.[1] UI often proceeds undiagnosed and untreated in up to 50% of patients due to embarrassment or their unwillingness to discuss the symptoms with their primary care physician. UI affects over 13 million people in the United States alone and about 10−20% of the world population.[2] In the United States, this amounts to over $16 billion in health care costs.

The pharmacology of incontinence and current treatment strategies have been thoroughly reviewed.[1,3−10] Briefly, UI can be categorized into three major types: urge incontinence (bladder instability), stress incontinence, and overflow incontinence. Urge UI, also known as hyperreflexive bladder, is characterized by abnormal spontaneous detrusor contractions which are unrelated to bladder urine volume and accounts for 35−65% of all UI cases depending on the age of the patient.[2,11] The persistent instability causes chronic sensory urgency and involuntary urine loss. The etiology of urge UI is multivariant and can be associated with spinal chord injury, bladder hypertrophy secondary to outlet obstruction, or urinary tract infections. Antimuscarinic agents are widely prescribed for the treatment of urge UI although their usefulness is limited by antimuscarinic side effects resulting in low patient compliance (<30%). Stress UI is characterized by involuntary urine loss during physical exertion and occurs when intravesical pressure exceeds the maximum urethral pressure in the absence of detrusor overactivity. Sympathomimetic agents are often prescribed for the treatment of stress UI because the bladder sphincter is mostly under sympathetic control. Postmenopausal women may be successfully treated with hormone replacement therapy because bladder and urethral tone is largely under estrogenic control. Overflow incontinence is defined as involuntary urine loss associated with overdistension of the bladder. Other than agents used to treat the symptoms of urge or stress UI, few treatments are available specifically for this type of incontinence.

Potassium channels play a crucial role in controlling physiological function of excitable cells; thus modulation of these channels provides novel mechanistic approaches to treat cell dysfunction. The structure and function of

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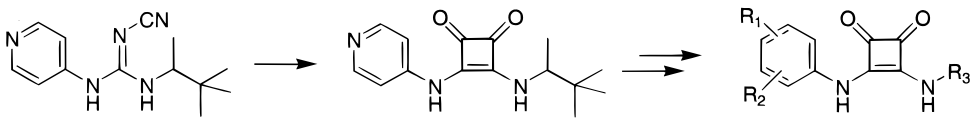
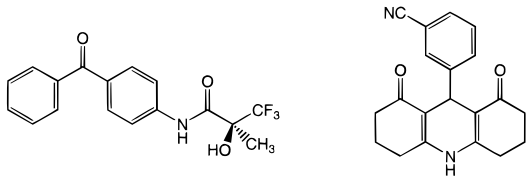


1 (pinacidil) **2** **3**

Figure 1. Bioisosteric replacement of the pinacidil (**1**) *N*-cyanoguanidine moiety with diaminocyclobutenedione affords a novel series of bladder selective KCOs (**3**).



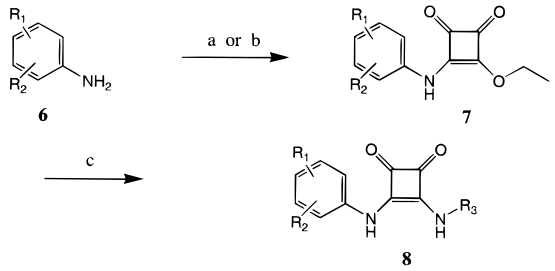
4 (ZD6169) **5** (ZM244085)

Figure 2. Bladder-selective KCOs reported by AstraZeneca.

physiologically important potassium channels and relevant SAR have been thoroughly reviewed.[12−25] By virtue of their ability to hyperpolarize cell membranes and prevent the influx of Ca^{2+} ions through voltage-gated Ca^{2+} channels, KCOs relax smooth muscle cells. Several potent K_{ATP} channel openers such as pinacidil (Chart 1) and cromakalim have been studied clinically as antihypertensive agents. It has been shown that the ATP-dependent potassium channel does exist in the bladder and that it can be activated by numerous antihypertensive KCOs.[26−29] The results of a small clinical study with cromakalim suggest that KCOs may show promise for the treatment of detrusor instability and hyperreflexia although full utility will only be realized with compounds that have minimal hemodynamic side effects (hypotension and tachycardia).[30,31] Workers at Zeneca Pharmaceuticals have recently described a structurally novel series of anilide tertiary carbinol KCOs (**4**)[32,33] and a series of dihydropyridine KCOs (**5**)[34] which are claimed to be bladder-selective smooth muscle relaxants in vivo. When administered orally, ZD6169 (**4**) (Figure 2) has been shown to activate the bladder K_{ATP} channel and to selectively increase bladder compliance in the rat and dog without significant hemodynamic effects.[35,36] This compound is currently undergoing Phase II clinical trials for the treatment of urge UI.

In this article, we report the results of our investigation of a novel series of diaminocyclobutenedione derivatives that were demonstrated to be potent and bladder-selective agonists of the K_{ATP} channel. A preliminary account of these efforts has been presented.[37] Our strategy was to utilize the 1,2-diaminocyclobutene-3,4-dione template as a putative bioisostere for the *N*-cyanoguanidine moiety seen in the pinacidil class of antihypertensive KCOs to generate a structurally novel series of KCOs. The compounds were evaluated for their ability to relax KCl precontracted isolated rat bladder smooth muscle strips in vitro. Electrophysiological studies on isolated bladder myocytes demonstrated that relaxation within this class of agents was mediated through activation of the ATP-sensitive potassium channel. In vivo performance of the active compounds was assessed in a rodent model of unstable bladder. Upon oral administration, lead compounds produced significant bladder relaxant effects at doses that caused minimal effects on hemodynamics, thus demonstrating a clear distinction between these selective agents and the antihypertensive KCOs.

Scheme 1. Synthesis of *N*-Aryl-1,2-diaminocyclobutenediones[a]



6 **7** **8**

[a] (a) THF or Et_2O, 3,4-diethoxy-3-cyclobutene-1,2-dione, reflux; (b) NaH, DMF, 3,4-diethoxy-3-cyclobutene-1,2-dione, 80 °C; (c) R_3NH_2, EtOH, reflux.

Chemistry

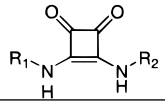
A series of 1,2-diaminocyclobutene-3,4-diones was generated using the synthetic route shown in Scheme 1. Treatment of 3,4-diethoxy-3-cyclobutene-1,2-dione with 1−1.3 equiv of the requisite substituted aniline or heteroarylamine in refluxing THF, ethanol, or diethyl ether (method A) afforded the intermediate amido-esters **7** in modest to excellent yields. For some deactivated anilines possessing bulky or electron-withdrawing groups, it was necessary to carry out the reaction in the presence of sodium hydride in DMF or THF to preform the more reactive sodium salt of the aniline (method B).

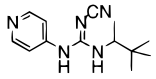
The di-*n*-butoxy and diisopropoxy analogues of 3,4-diethoxy-3-cyclobutene-1,2-dione could also be used as starting material. The amido-esters **7** were converted to 1,2-diaminocyclobutene-3,4-diones **8** by treatment with 1−5 equiv of the desired alkylamine in ethanol. Recrystallization from ethanol or acetonitrile typically afforded analytically pure compounds. Optically active (*R*)- and (*S*)-2-amino-3,3-dimethylbutane were prepared by the method of Manley and Quast from pinacolone using α-methylbenzylamine as a chiral auxiliary.[38] Chiral purities of enantiomers **42** and **43** were determined to be 99% ee and 97% ee, respectively (see Experimental Section). As it is unlikely for epimerization of the chiral center to occur during the displacement reactions, any derivative possessing the (*R*)-2-amino-3,3-dimethylbutane moiety is assumed to be ≈99% ee. Optically active (*R*) and (*S*) starting materials for the syntheses of compounds **60**, **61**, **63**, **64**, **67**−**69** were obtained commercially. Percent yields and physical properties for test compounds **2** and **9**−**87** are shown in Tables 1−3 and 5−6.

Pharmacology

Compounds were first evaluated in vitro for their ability to relax KCl precontracted rat detrusor muscle strips. Following stabilization, increasing concentrations

Table 1. In Vitro Effects of Pyridyl Analogues on Precontracted Rat Bladder Smooth Muscle Strips (IC_{50}, μM)[a]



Comp.	R_1	R_2	% Yield Step 1 (method)	% Yield Step 2	mp, °C	formula [b]	anal. [c]	IC_{50}[a] *In Vitro* rat bladder strip	n [d]
2	4-C_5H_4N	3,3-dimethyl-2-butyl	13 (A)	79	255-257	$C_{15}H_{19}N_3O_2$	C,H,N	1.36 ± 0.48	4
9	4-C_5H_4N	(*R*) 3,3-dimethyl-2-butyl	-- [e]	62	275-279 (dec)	$C_{15}H_{19}N_3O_2$	C,H,N	1.68 ± 0.10	2
10	3-C_5H_4N	3,3-dimethyl-2-butyl	49 (A)	66	243-245	$C_{15}H_{19}N_3O_2$	C,H,N	1.95 ± 0.78	2
11	3-C_5H_4N	(*R*) 3,3-dimethyl-2-butyl	-- [f]	68	283-285	$C_{15}H_{19}N_3O_2$	C,H,N	1.90 ± 0.32	2
12	2-C_5H_4N	(*R*) 3,3-dimethyl-2-butyl	21 (A)	57	279-281	$C_{15}H_{19}N_3O_2$	C,H,N	contracted	3
13	3-C_5H_4N	*t*-butyl	-- [f]	36	250-252	$C_{13}H_{15}N_3O_2$	C,H,N	2.80 ± 1.2	2
14	3-(2-Cl)C_5H_3N	*t*-butyl	25 (A)	73	213-216	$C_{13}H_{14}ClN_3O_2$	C,H,N	1.77 ± 0.6	3
15	3-(2-OCH_3)C_5H_3N	*t*-butyl	11 (A)	44	192-195	$C_{14}H_{17}N_3O_3$	C[g],H,N	2.56 ± 0.73	3
16	3-(2-OH)C_5H_3N	(*R*) 3,3-dimethyl-2-butyl	11% for 2 steps		340-345 (dec)	$C_{15}H_{19}N_3O_3$	C[h],H.N	>30	3
17	3-(5-Br)C_5H_3N	3,3-dimethyl-2-butyl	62 (A)	83	266-268	$C_{15}H_{18}BrN_3O_2$	C,H,N	11.74 ± 4	6
18	3-C_5H_4N	2-propyl	-- [f]	71	258-260	$C_{12}H_{13}N_3O_2$	C[i],H.N	>30	2
19	3-C_5H_4N	H	-- [f]	93	297 (dec)	$C_9H_7N_3O_2$	C,H,N	>30	2
1		(±) pinacidil	--	--	--	--	--	0.63 ± 0.11	8

[a] IC_{50}: drug concentration (μM) that relaxed KCl-induced contractions in rat detrusor strips by 50%. [b] Structures of compounds confirmed by ^{1}H NMR, IR, and MS. [c] Analytical results are within±0.4% of the theoretical value unless otherwise noted. [d] Number of experiments. [e] See compound **2**. [f] See compound **10**. [g] C: calcd, 61.08; found, 60.37. [h] C: calcd, 62.27; found, 61.40. [i] C: calcd, 62.32; found, 62.86.

of test compounds were superfused and isometric force was measured. Glyburide, a specific blocker of the K_{ATP} channel, was added at the end of each experiment and percent recovery of contractile activity was recorded. As a measure of intrinsic selectivity, some compounds were screened in similar tissue baths using rat aortic rings.

Selected compounds were evaluated for in vivo efficacy in a rat pathophysiological model of bladder instability previously described by Malmgren and coworkers.[39,40] Compounds that were efficacious in reducing the frequency of spontaneous bladder contractions in vivo were selected for hemodynamic assessment in a separate group of animals. Effects on mean arterial pressure and heart rate were recorded after oral administration of drug.

Finally, to confirm the mechanism of action of our biological lead compounds, we studied their effects on membrane currents in isolated rat detrusor smooth muscle cells using voltage clamp and current clamp recording techniques.

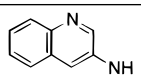
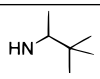
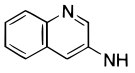
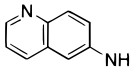
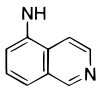
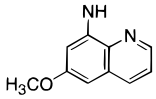
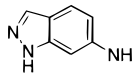
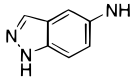
Result and Discussion

The in vitro bladder relaxant effects of pinacidil (**1**) and pyridyl cyclobutenedione analogues **2** and **9**−**19** are shown in Table 1. Compound **2**, the direct cyclobutenedione analogue of (±)pinacidil, was found to be about 2-fold less potent than pinacidil as a bladder smooth muscle relaxant (IC_{50}s = 1.36 μM and 0.63 μM, respectively). The corresponding 3-pyridyl analogue (**10**) was only slightly less potent than **2**, while the 2-pyridyl derivative (**12**) was found to possess bladder smooth muscle contracting properties. This SAR is consistent with that reported for the pinacidil-type *N*-cyanoguanidine antihypertensive series where the order of potency was reported as 4-pyridyl ≅ 3-pyridyl ≫ 2-pyridyl.[41] The *R*-configuration of the 3,3-dimethyl-2-butyl alkyl moiety retains the activity of the racemate in both the 4- (**9** vs **2**) and 3-pyridyl (**11** vs **10**) series. It has been shown in a series of pinacidil-type KCOs that when the alkylamine moiety contains an α-methyl group in addition to a $C(CH_3)$ group, only the *R*-configuration can be readily accommodated at the receptor.[38]

Due to the similar potencies of 4- and 3-pyridyl analogues **9** and **11** and to the consistently higher yields of the arylamine condensation reaction in the 3-pyridyl series over the 4-pyridyl series, the SAR of the alkylamine moiety (R_2) and pyridyl ring substituents were explored within the 3-pyridyl series. Exchange of a *tert*-butyl group for the 3,3-dimethyl-2-butyl moiety results in slightly lower activity (**13** vs **10**), while reducing the steric bulk further to *i*-propyl (**18**) or H (**19**) results in complete loss of activity. This requirement for a bulky lipophilic group on the alkylamine side is consistent with the SAR reported for the pinacidil-type *N*-cyanoguanidine antihypertensives.[41] Substitution is tolerated at the 2-position of the pyridine ring as seen with compounds **14** (2-Cl) and **15** (2-OCH_3). Interestingly, while a 2-OCH_3 group is tolerated, a 2-OH substituent (**16**) results in complete loss of activity most likely due to existence of the pyridinone tautomer. Substitution of a Br atom onto the 5-position of the 3-pyridyl ring results in a 6-fold loss in activity (**17** vs **10**).

In an effort to expand the SAR to bicyclic heteroaryl derivatives, compounds **20** −**27** were prepared and the results are shown in Table 2. Replacement of the aminopyridine ring with 3-, 6-, or 8-amino-quinoline resulted in loss of activity. The 5-amino-isoquinoline derivative **24** was slightly more potent than the corresponding pyridyl analogue **13**. Interestingly, replacing the 3-pyridyl group of compound **11** with 5-amino-

Table 2. In Vitro Effects of Bicyclic Heteroaryl Analogues on Precontracted Rat Bladder Smooth Muscle Strips (IC_{50}, μM)[a]

Comp.	X	Y	% Yield Step 1 (method)	% Yield Step 2	mp, °C	formula [b]	anal. [c]	IC_{50}[a] *In Vitro* rat bladder strip	n [d]
20			80 (A)	83	242-246	$C_{19}H_{21}N_3O_2$	C,H,N	>30	6
21			-- [e]	85	257-260	$C_{17}H_{17}N_3O_2$ 0.75 H_2O	C,H,N	>30	4
22			84 (A)	31	234-236	$C_{17}H_{17}N_3O_2$ 0.25H_2O	C,H,N	>30	8
23			70 (A)	81	244-245	$C_{19}H_{21}N_3O_2$	C,H,N	8.1± 3.4	4
24			29 (A)	39	268-270 (dec)	$C_{17}H_{17}N_3O_2$ 0.14H_2O	C,H,N	1.84 ± 0.3	4
25			29 (A)	71	243-245	$C_{20}H_{23}N_3O_3$	C,H,N	>30	4
26			53 (A)	43	264-267 (dec)	$C_{17}H_{20}N_4O_2$ 0.1 CH_2Cl_2	C,H,N	4.6 ± 3.3	2
27			42 (A)	84	290-297 (dec)	$C_{17}H_{20}N_4O_2$ 0.05 CH_2Cl_2	C,H,N	0.74 ± 0.01	2

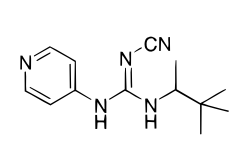
[a] IC_{50}: drug concentration (μM) that relaxed KCl-induced contractions in rat detrusor strips by 50%. [b] Structures of compounds confirmed by ^{1}H NMR, IR, and MS. The presence of hydrates and partial solvent adducts was confirmed by KF analysis and ^{1}H NMR. [c] Analytical results are within ±0.4% of the theoretical value unless otherwise noted. [d] Number of experiments. [e] See compound **20**.

indazolyl (**27**) resulted in a 2.5-fold increase in bladder relaxant activity while the corresponding 6-amino-indazolyl analogue (**26**) was 2.4-fold less active.

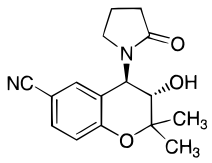
We next sought to examine *N*-(4-substituted)-aryl analogues as potential surrogates for the 4-pyridyl moiety. Keeping R_2 constant as 3,3-dimethyl-2-butyl (either the *R*-configuration or racemic), *N*-aryl analogues **28**−**41** were prepared, and the in vitro bladder relaxant effects are shown in Table 3. The unsubstituted phenyl analogue **28** was found to be 3.2-fold less potent than the corresponding 4-pyridyl analogue **9**. As seen with examples **29**−**40**, bladder relaxant activity is increased 10-fold when a cyano group is installed at the 4-position (**34**). A bromine atom (**29**) or a methoxy group (**30**) at position-4 is tolerated; however, replacement with any of the other groups shown in Table 3 caused a precipitous loss of bladder relaxant activity. Homologation of the cyano group in **34** with a methylene group to give phenyl-acetonitrile analogue **41** resulted in retention of potent bladder relaxant activity. The observed SAR for the 4-position is consistent with that reported for a series of aryl-*N*-cyanoguanidine KCOs .[42] Compound **89** (BMS-182264), a putative hybrid of pinacidil (**1**) and cromakalim (**88**), was shown to be the most potent aortic smooth muscle relaxant in a series of structurally novel KCOs (Chart 1).[42] In our study, it relaxed bladder smooth muscle strips with an IC_{50} = 0.19 μM.

Due to the potent bladder relaxant effects associated with cyanophenyl-aminocyclobutenedione compound **34**

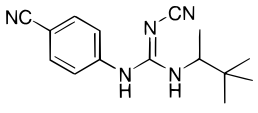
Chart 1



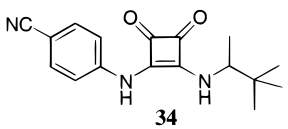
1 (pinacidil)



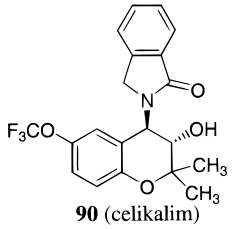
88 (cromakalim)



89 (BMS-182264)



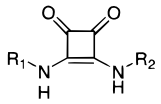
34

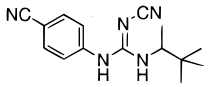


90 (celikalim)

(IC_{50} = 0.52 μM), we chose to evaluate its performance in a pathophysiological model of bladder instability. The in vivo effects of **34** and *N*-cyanoguanidine analogue **89** in the rat model of bladder instability and effects on MAP are shown in Table 4. When administered orally at 10 mg/kg, compound **34** reduced the frequency of spontaneous bladder contractions by 83% while, in a separate set of animals, no significant changes in MAP

Table 3. In Vitro Effects of Aryl Analogues on Precontracted Rat Bladder Smooth Muscle Strips—SAR at Position-4. (IC_{50}, μM)[a]



Comp.	R_1	R_2	% Yield Step 1 (method)	% Yield Step 2	mp, °C	formula [b]	anal. [c]	IC_{50}[a] *In Vitro* rat bladder strip	n [d]
28	C_6H_5	(*R*) 3,3-dimethyl-2-butyl	67 (A)	86	279-280	$C_{16}H_{20}N_2O_2$	C,H,N	5.4 ± 2.2	2
29	4-(Br)C_6H_4	3,3-dimethyl-2-butyl	47 (A)	77	308-309	$C_{16}H_{19}BrN_2O_2$	C[f],H,N	1.26 ± 0.72	3
30	4-(OCH_3)C_6H_4	3,3-dimethyl-2-butyl	75 (A)	85	254-255	$C_{17}H_{22}N_2O_3$	C,H,N	2.4 ± 1.3	4
31	4-(SCH_3)C_6H_4	3,3-dimethyl-2-butyl	80 (A)	89	285-286	$C_{17}H_{22}N_2O_2S$	C,H,N	>30	2
32	4-(OCF_3)C_6H_4	3,3-dimethyl-2-butyl	53 (A)	91	282-285	$C_{17}H_{19}F_3N_2O_3$	C,H,N	>30	3
33	4-(SO_2NH_2)C_6H_4	3,3-dimethyl-2-butyl	98 (A)	20	233-235	$C_{16}H_{21}N_3O_4S$ 0.5H_2O	C,H,N	>30	4
34	4-(CN)C_6H_4	3,3-dimethyl-2-butyl	81 (A)	71	241-243	$C_{17}H_{19}N_3O_2$	C,H,N	0.52 ± 0.03	2
35	4-($COCH_3$)C_6H_4	(*R*) 3,3-dimethyl-2-butyl	53 (A)	75	291 (dec)	$C_{18}H_{22}N_2O_3$	C[h],H,N	21.2 ± 3.2	2
36	4-(CO_2CH_3)C_6H_4	(*R*) 3,3-dimethyl-2-butyl	48 (A)	72	279-280	$C_{18}H_{22}N_2O_4$	C,H,N	>30	4
37 [e]	4-(SO_2CH_3)C_6H_4	3,3-dimethyl-2-butyl	-- [e]	-- [e]	307-310	$C_{17}H_{22}N_2O_4S$ 0.75H_2O	C,H[g],N	>30	2
38	4-(C_6H_5)C_6H_4	3,3-dimethyl-2-butyl	85 (A)	88	>300 (dec)	$C_{22}H_{24}N_2O_2$	C,H,N	>30	3
39	4-(1-imidazolyl)C_6H_4	3,3-dimethyl-2-butyl	92 (A)	82	269-271	$C_{19}H_{22}N_4O_2$	C,H,N	>30	3
40	4-(CF_3)C_6H_4	(*R*) 3,3-dimethyl-2-butyl	19 (A)	79	296-299	$C_{17}H_{19}F_3N_2O_2$	C,H,N	>30	2
41	4-(CH_2CN)C_6H_4	(*R*) 3,3-dimethyl-2-butyl	74 (A)	44	271-273 (dec)	$C_{18}H_{21}N_3O_2$	C[i],H,N	0.34 ± 0.02	2
89		(BMS-182264)	--	--	--	--	--	0.19 ± 0.03	2

[a] IC_{50}: drug concentration (μM) that relaxed KCl-induced contractions in rat detrusor strips by 50%. [b] Structures of compounds confirmed by ^{1}H NMR, IR, and MS. The presence of hydrates and partial solvent adducts was confirmed by KF analysis and ^{1}H NMR. [c] Analytical results are within ±0.4% of the theoretical value unless otherwise noted. [d] Number of experiments. [e] Prepared by oxidation of sulfide **31**; see Experimental Section. [f] C: calcd, 54.71; found, 54.08. [g] H: calcd, 6.51; found, 6.03. [h] C: calcd, 68.77; found, 68.20. [i] C: calcd, 69.43; found, 68.88

Table 4. In Vivo Effects of Compounds **34** and **89** on Frequency of Spontaneous Blader Contractions in the Rat Hypertrophic Bladder Model and Effects on MAP in Normotensive Rats (Percent Change from Pre-Drug Value, X ± SE)

	spontaneous bladder contractions			blood pressure[c]		
comp	dose (mg/kg) po	n^a	frequency (% change)[b]	dose (mg/kg) po	n^a	maximum % change
34	10	4	−83 ± 12	30	4	+8 ± 3
89	10	8	−27 ± 10	10	7	−19 ± 6

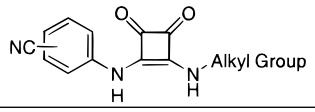
[a] Number of experiments. [b] Vehicle (PEG-200) effects: −2 ± 11. [c] Initial BP values ranged from 105 ± 2 to 116 ± 5 mmHg.

were observed at a dose of 30 mg/kg. In contrast, the direct *N*-cyanoguanidine analogue **89** only marginally reduced the frequency of bladder contractions at 10 mg/kg, while causing a 19% drop in MAP. Having demonstrated this remarkable in vivo bladder smooth muscle selectivity relative to hemodynamic effects associated with compound **34**, our SAR efforts turned to optimization of in vitro potency while maintaining in vivo selectivity.

We chose to vary the position of the nitrile on the arylamine and the structure of the alkylamine moiety. Compounds **42**−**74** were prepared, and the results are shown in Table 5. Consistent with prior trends, detrusor relaxant activity resides mostly in the (*R*)- antipode as demonstrated by enantiomers **42** (IC_{50} = 0.37 μM) and **43** (IC_{50} = 24.3 μM). Transposing the nitrile group to the meta (**44**, IC_{50} = 3.05 μM) and ortho (**45**, IC_{50} > 30 μM) positions results in progressive loss of activity. The *tert*-butyl group (**46**, IC_{50} = 0.56 μM) was found to be a suitable surrogate for the chiral 3,3-dimethyl-2-butyl group. Bladder relaxant activity was found to be remarkably sensitive to steric modifications on the lipophilic alkylamine side chain. The requirement of α-branching is demonstrated by the total loss of activity observed by simply removing the α-methyl group (**49**, IC_{50} > 30 μM). Modulation of branching and steric bulk at the position β to the nitrogen also reduces activity, but the effects are much less dramatic (**47**, IC_{50} = 1.9 μM; **48**, IC_{50} = 3.34 μM; and **50**; IC_{50} = 2.1 μM). Further reducing the overall lipophilicity and steric bulk to 2-propyl (**51**, IC_{50} = 13.7 μM) or methyl (**52**, IC_{50} > 30 μM), or installing a linear lipophilic group (**53**, IC_{50} > 30 μM), abolished activity. In a similar way, cyclic alkyl groups (i.e., **55**, **56**, **59**) are not tolerated. Interestingly, the endo-norbornyl analogue **57** retains some bladder relaxant activity, while the corresponding exo-analogue **58** is completely devoid of activity. This observation is consistent with SAR reported by workers at Green Cross for a series of related *N*-pyridyl-*N*′-bicycloalkyl-*N*″-cyanoguanidine derivatives shown to be potent antihypertensive KCOs.[43]

Compared to compound **34**, a remarkable 10-fold enhancement of in vitro potency is observed with the (*R*)-α-methylbenzylamine analogue, **60** (IC_{50} = 0.056 μM). Consistent with previously observed trends, the corresponding (*S*)-enantiomer (**61**, IC_{50} > 30 μM) was much less potent. In an attempt to further optimize in vitro activity associated with **60**, modifications in the α-methylbenzylamine moiety were studied with compounds **62**−**74**, and surprisingly, we found a very

Table 5. In Vitro Effects of Cyanophenyl Analogues on Precontracted Rat Bladder Smooth Muscle Strips—Alkyl SAR (IC_{50}, μM)[a]



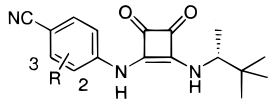
Comp.	CN Position	Alkyl Group	% Yield Step 1 (method)	% Yield Step 2	mp, °C	formula [b]	anal. [c]	IC_{50} [a] *In Vitro* rat bladder strip	n [d]
34	4	3,3-dimethyl-2-butyl	81 (A)	50	241-243	$C_{17}H_{19}N_3O_2$	C,H,N	0.52 ± 0.03	2
42	4	(*R*) 3,3-dimethyl-2-butyl	-- [e]	62	273-274	$C_{17}H_{19}N_3O_2$	C,H,N	0.37 ± 0.07	4
43	4	(*S*) 3,3-dimethyl-2-butyl	-- [e]	29	241-243	$C_{17}H_{19}N_3O_2$ 0.1 H_2O	C,H.N	24.3 ± 4.4	4
44	3	3,3-dimethyl-2-butyl	80 (A)	54	296-298 (dec)	$C_{17}H_{19}N_3O_2$	C,H,N	3.05 ± 1.44	4
45	2	(*R*) 3,3-dimethyl-2-butyl	22 (A)	80	258-259	$C_{17}H_{19}N_3O_2$ 0.2H_2O	C,H,N	>30	2
46	4	*t*-butyl	-- [e]	18	257-260 (dec)	$C_{15}H_{15}N_3O_2$ 0.05H_2O	C,H,N	0.56 ± 0.12	4
47	4	3-methyl-2-butyl	-- [e]	24	222-224	$C_{16}H_{17}N_3O_2$ 0.12H_2O	C,H,N	1.9 ± 0.93	7
48	4	3-pentyl	-- [e]	34	252-255 (dec)	$C_{16}H_{17}N_3O_2$ 0.25 H_2O	C,H,N	3.34 ± 0.57	12
49	4	2,2-dimethyl-1-propyl	-- [e]	83	272-275 (dec)	$C_{16}H_{17}N_3O_2$	C,H,N	>30	4
50	4	2-butyl	-- [e]	95	245-247	$C_{15}H_{15}N_3O_2$	C,H,N	2.1 ± 0.9	4
51	4	2-propyl	-- [e]	74	290-292 (dec)	$C_{14}H_{13}N_3O_2$ 0.12H_2O	C,H,N	13.7 ± 3.5	4
52	4	methyl	-- [e]	38	>300	$C_{12}H_9N_3O_2$ 0.03CH_2Cl_2	C,H,N	>30	2
53	4	1-heptyl	-- [e]	66	265-267 (dec)	$C_{18}H_{21}N_3O_2$	C,H,N	>30	3
54	4	*t*-amyl	-- [e]	50	266-267 (dec)	$C_{16}H_{17}N_3O_2$	C,H,N	>30	4
55	4	cyclopentyl	-- [e]	33	294-296 (dec)	$C_{16}H_{15}N_3O_2$	C,H,N	11.5 ± 4.4	6
56	4	cyclohexyl	-- [e]	82	>300	$C_{17}H_{17}N_3O_2$	C[k],H,N	>30	2
57	4	endo-norbornyl	-- [e]	68	251-252 (dec)	$C_{18}H_{17}N_3O_2$	C,H,N	3.35 ± 0.67	4
58	4	exo-norbornyl	-- [e]	79	288-290 (dec)	$C_{18}H_{17}N_3O_2$	C,H,N	>30	3
59	4	cycloheptyl	-- [e]	82	273-275 (dec)	$C_{18}H_{19}N_3O_2$	C,H,N	contracted [i]	4
60	4	(*R*) $CH(CH_3)C_6H_5$	-- [e]	28	273-274	$C_{19}H_{15}N_3O_2$	C,H,N	0.056 ± 0.01	6
61	4	(*S*) $CH(CH_3)C_6H_5$	-- [e]	26	269-270	$C_{19}H_{15}N_3O_2$ 0.1H_2O	C,H,N	>30	3
62	4	$CH_2C_6H_5$	-- [e]	62	288-290 (dec)	$C_{18}H_{13}N_3O_2$	C,H,N[f]	>30	4
63	4	(*R*) $CH(CH_3)C_6H_{11}$	-- [e]	84	275-280 (dec)	$C_{19}H_{21}N_3O_2$	C,H,N	2.6 ± 0.7	6
64	4	(*S*) $CH(CH_3)C_6H_{11}$	-- [e]	90	275-280 (dec)	$C_{19}H_{21}N_3O_2$ 0.13CH_2Cl_2	C,H,N	>30	3
65	4	$C(CH_3)_2C_6H_5$	-- [e]	37	>300 (dec)	$C_{20}H_{17}N_3O_2$ 1.0CH_3OH 0.05CH_2Cl_2	C,H,N	>30	3
66	4	$CH(CF_3)C_6H_5$	-- [e]	47	206-207	$C_{19}H_{12}F_3N_3O_2$	C,H,N	4.0 ± 2.3	2
67	4	(*S*) $CH(CH_2OH)C_6H_5$	-- [e]	80	247-250 (dec)	$C_{19}H_{15}N_3O_3$	C,H,N	14.3 ± 6.8	2
68	4	(*R*) $CH(CH_2CH_3)C_6H_5$	-- [e]	72	242-243 (dec)	$C_{20}H_{17}N_3O_2$	C,H,N	22.5 ± 4	3
69	4	(*S*) $CH(CH_2CH_3)C_6H_5$	-- [e]	66	241-243 (dec)	$C_{20}H_{17}N_3O_2$	C,H,N	>30	4
70	4	(*R*) $CH(CH_3)(4\text{-}CH_3\text{-}C_6H_4)$	-- [e]	75	>300	$C_{20}H_{17}N_3O_2$	C,H,N	>30	3
71	4	(*R*) $CH(CH_3)(4\text{-}OCH_3\text{-}C_6H_4)$	-- [e]	15	>300	$C_{20}H_{17}N_3O_3$ 0.03CH_2Cl_2	C,H,N	contracted [i]	2
72	4	(*R*) $CH(CH_3)(4\text{-}OCF_3\text{-}C_6H_4)$	-- [e]	45	281-284 (dec)	$C_{20}H_{14}F_3N_3O_3$	C,H,N	>30	2
73	4	(*R*) $CH(CH_3)(4\text{-}NO_2\text{-}C_6H_4)$	-- [e]	78	290-295 (dec)	$C_{19}H_{14}N_4O_4$	C[g],H,N[h]	NE [j]	4
74	4	(*R*) $CH(CH_3)(4\text{-}F\text{-}C_6H_4)$	-- [e]	65	284 (dec)	$C_{19}H_{14}FN_3O_2$	C,H,N	NE [j]	2

[a] IC_{50}: drug concentration (μM) that relaxed KCl-induced contractions in rat detrusor strips by 50%. [b] Structures of compounds confirmed by ^1H NMR, IR, and MS. The presence of hydrates and partial solvent adducts was confirmed by KF analysis and ^1H NMR. [c] Analytical results are within ±0.4% of the theoretical value unless otherwise noted. [d] Number of experiments. [e] See compound **34**. [f] N: calcd, 13.85; found, 12.89. [g] C: calcd, 62.98; found, 62.38. [h] N: calcd, 15.46; found, 14.95. [i] The compound caused a further contraction of the detrusor strip. [j] No effect. [k] C: calcd, 69.14; found 68.57.

narrow window of activity in this subset of compounds. Consistent with the (*R*)-3,3-dimethyl-2-butyl SAR, removal of the α-methyl group (**62**, IC_{50} > 30 μM) or introduction of a second methyl group (**65**, IC_{50} > 30 μM) is detrimental to activity. Exchange of the α-methyl group for α-trifluoromethyl (**66**), α-hydroxymethyl (**67**), or α-ethyl (**68**, **69**) all resulted in attenuation of activity. Likewise, smooth muscle relaxant properties were diminished with a variety of substituents at position-4 of the benzene ring (**70**−**74**). Replacement of the benzene ring with a cyclohexane ring (**63**, IC_{50} = 2.6 μM) resulted in a 50-fold drop in activity.

Our criteria for lead progression required that an in vivo bladder inhibitory ED_{50} dose in the rat should not cause greater than a 10% drop in mean arterial pressure. Potent in vitro lead **60** was evaluated in vivo (data

Table 6. In Vitro Effects of *N*-4-Cyanophenyl-*N′*-(*R*)-3,3-dimethyl-2-butyl Analogues on Precontracted Rat Bladder Smooth Muscle Strips (IC_{50}, μM)[a]



Comp.	R Position	R	% Yield Step 1 (method)	% Yield Step 2	mp, °C	formula [b]	anal. [c]	IC_{50}[a] In Vitro rat bladder strip	n [d]
34	-	H	81 (A)	50	241-243	$C_{17}H_{19}N_3O_2$	C,H,N	0.52 ± 0.03	2
75	3	Cl	20 (A)	88	>300	$C_{17}H_{18}ClN_3O_2$	C,H,N	4.3 ± 3.9	4
76	3	CH_3	45 (A)	52	288-289	$C_{18}H_{21}N_3O_2$	C,H,N	0.63 ± 0.13	2
77	3	C_2H_5	55 (A)	42	255-256	$C_{19}H_{23}N_3O_2$	C,H,N	17.6 ± 8.6	3
78	2	CH_3	24 (A)	70	226-228	$C_{18}H_{21}N_3O_2$ 0.2CH_2Cl_2	C,H,N	1.08 ± 0.97	4
79	2	C_2H_5	34 (A);59 (B)	52	236-237	$C_{19}H_{23}N_3O_2$	C,H,N	0.09 ± 0.021	5
80	2	C_3H_7	21 (A)	87	147-152	$C_{20}H_{25}N_3O_2$ 0.25H_2O	C,H,N	23.1 ± 0.66	2
81	2	OCH_3	24 (A);41(B)	67	280-282	$C_{18}H_{21}N_3O_3$	C,H,N	0.29 ± 0.12	4
82 [e]	2	OH	57 (A)	71	>200	$C_{17}H_{19}N_3O_3$ 0.25H_2O	C,H,N	6.05 ± 3.95	2
83 [e]	3	OH	50 (A)	37	180-187	$C_{17}H_{19}N_3O_3$ 0.5H_2O	C,H,N	NE [g]	4
84	2	Cl	21 (B)	61	220-222	$C_{17}H_{18}ClN_3O_2$ 0.2 H_2O	C,H,N	0.041 ± 0.009	3
85	2	Br	56 (B)	59	229-231	$C_{17}H_{18}BrN_3O_2$ 0.2H_2O	C,H,N[f]	0.075 ± 0.05	2
86	2	COC_6H_5	80 (B)	40	243-244	$C_{24}H_{23}N_3O_3$ 0.1H_2O	C,H,N	4.78 ± 1.46	4
87	2-3	fused cyclohexyl	29 (A)	38	259-264 (dec)	$C_{21}H_{25}N_3O_2$	C,H,N	0.67 ± 0.11	2

[a] IC_{50} drug concentration (μM) that relaxed KCl-induced contractions in rat detrusor strips by 50%. [b] Structures of compounds confirmed by ^{1}H NMR, IR, and MS. The presence of hydrates and partial solvent adducts has been confirmed by KF analysis and ^{1}H NMR. [c] Analytical results are within ±0.4% of the theoretical value unless otherwise noted. [d] Number of experiments. [e] Racemic compound. [f] N: calcd, 11.06; found, 10.37. [g] No effect.

not shown) and was found to not meet that criteria and thus was dropped from further consideration as a biological lead.

Revisiting *N*-4-cyanophenyl-*N′*-(*R*)-3,3-dimethyl-2-butyl analogue **34** as a starting point for a second iteration of SAR, we chose to focus on the introduction of additional substituents onto the aromatic ring, and the results are shown in Table 6. The 3-chloro analogue (**75**, IC_{50} = 4.3 μM) was somewhat less potent than **34**. The corresponding 3-methyl analogue (**76**, IC_{50} = 0.63 μM) retained most of the activity of the parent compound while homologation to the 3-ethyl species (**77**, IC_{50} = 17.6 μM) resulted in a 34-fold reduction in activity. The 3-hydroxy analogue (**83**) was totally devoid of activity.

Introduction of a 2-methyl substituent (**78**, IC_{50} = 1.08 μM) had little effect on potency. In contrast to the trend at position-3, homologation of methyl to ethyl (**79**, IC_{50} = 0.09 μM) at position-2 results in a 6-fold increase in bladder relaxant potency. However, further increase in lipophilic steric bulk from 2-ethyl to 2-propyl (**80**, IC_{50} = 23.1 μM) abolishes most of the bladder relaxant activity. Interestingly, some bladder relaxant activity is maintained with a 2-benzoyl substituent (**86**, IC_{50} = 4.78 μM), albeit at a level 9-fold lower than **34**. Other substituents at position-2 which were found to improve in vitro bladder relaxant activity include a methoxy group (**81**, IC_{50} = 0.29 μM), a chlorine atom (**84**, IC_{50} = 0.041 μM), and a bromine atom (**85**, IC_{50} = 0.075 μM).

As a result of the potent in vitro bladder relaxant properties associated with (*R*)-4-[3,4-dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-3-ethyl-benzonitrile (**79**, WAY-133537), we chose to fully evaluate the compound in our in vivo models for efficacy and bladder selectivity. The in vivo effects of **79** on inhibition of abnormal spontaneous contractions in the rat are shown in Table 7. Comparative data are presented for ZD-6169 (**4**), a compound which has been shown to activate the bladder K_{ATP} channel and to selectively increase bladder compliance in the rat and dog without significant hemodynamic effects, and for the antihypertensive K_{ATP} channel opener celikalim (**90**). A thorough pharmacological comparison of these three KCOs has been reported elsewhere.[44]

Table 7. Comparison of In Vitro and In Vivo Effects of Compounds **79**, ZD-6169 (**4**), and Celikalim (**90**)

	bladder effects		hemodynamic effects	selectivity
comp	in vitro IC_{50}[a] (μM)	in vivo ED_{50}[b] mg/kg	in vivo ED_{20}[c] mg/kg	ratio (MAP ED_{20}/ bladder ED_{50})
79	0.09	0.13	2.3	17.7
ZD-67169 (**4**)	0.93	2.4	6.96	2.9
Celikalim (**90**)	0.03	0.3	0.2	0.7

[a] IC_{50} drug concentration (μM) that relaxed KCl-induced contractions in rat detrusor strips by 50%. [b] ED_{50}: drug dose (po) that caused a 50% reduction in the frequency of spontaneous bladder contractions in the rat hypertrophied model of bladder instability. Vehicle (PEG-200) effects: −2 ± 11. [c] ED_{20}: drug dose (po) that caused a 20% drop in MAP in normotensive rats. Initial BP values ranged from 105 ± 2 to 116 ± 5 mmHg.

Oral administration of **79** at doses between 0.03 and 10 mg/kg produced a dose-dependent decrease in the frequency of spontaneous bladder contractions. The calculated ED_{50} value for the compound is 0.13 mg/kg (po). A representative cystometric recording before and after administration of **79** is shown in Figure 3. The upper portion shows the characteristic bladder instability and spontaneous spikes in bladder pressure that develop during the filling phase of the experiment. It is

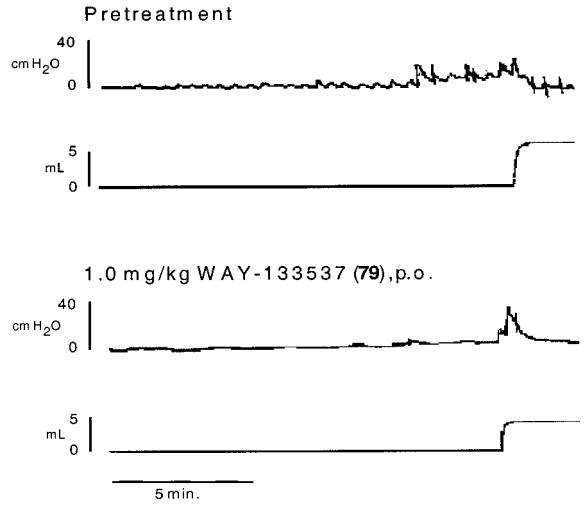


Figure 3. Rat hypertrophied bladder model: a representative cystometric tracing. Spontaneous spikes in bladder pressure are observed during the filling phase, indicating an increasing amount of instability prior to the micturition pressure (upper panel). After treatment with 1.0 mg/kg **79**, the frequency of spontaneous bladder contractions is significantly reduced and micturition pressure is unchanged (lower panel).

believed that the sense of urinary urgency that develops in patients suffering from this type of incontinence results from similar spontaneous bladder contractions. A sustained micturition contraction at the end of the cycle results in urine output. The lower portion of the chart shows bladder pressure and void volume after exposure to 1.0 mg/kg (po) **79**. Spontaneous bladder contractions are significantly reduced and, importantly, micturition pressure and void volume remain relatively unchanged. The maximal effect on reduction in the frequency of bladder contractions was observed at 1.0 h post-dose. As shown in Table 7, celikalim (ED_{50} = 0.3 mg/kg) possessed similar in vivo potency to **79**; however, in this model, ZD-6169 (ED_{50} = 2.4 mg/kg) was approximately 18-fold less potent than **79**.

As a measure of relative in vivo selectivity, we chose to compare the blood pressure lowering properties of **79**, ZD-6169, and celikalim, and the data are shown in Table 7. All three compounds dose-dependently lowered MAP in normotensive rats following oral administration. Celikalim (ED_{20} = 0.2 mg/kg), a KCO developed as an antihypertensive, was the most potent blood pressure lowering agent, followed **79** (ED_{20} = 2.3 mg/kg) and ZD-6169 (ED_{20} = 6.96 mg/kg). Although all three agents were effective at inhibiting spontaneous bladder contractions, compound **79**, with a selectivity ratio of 17.7, demonstrated bladder efficacy at doses which produced minimal hemodynamic changes. In our studies, ZD-6169 also exhibited some degree of bladder selectivity in vivo with a ratio of 2.9 in favor of bladder effects. The antihypertensive agent celikalim, with a ratio of 0.7, showed no selectivity toward the bladder over cardiovascular effects. A graphical representation of the observed bladder and hemodynamic effects of **79** and celikalim is shown in Figure 4. The top panel illustrates the remarkable in vivo bladder selectivity of **79** in contrast to the nonselective effects of celikalim shown in the lower panel.

To elucidate the underlying mechanism of action of this novel series of KCOs, several studies were under-

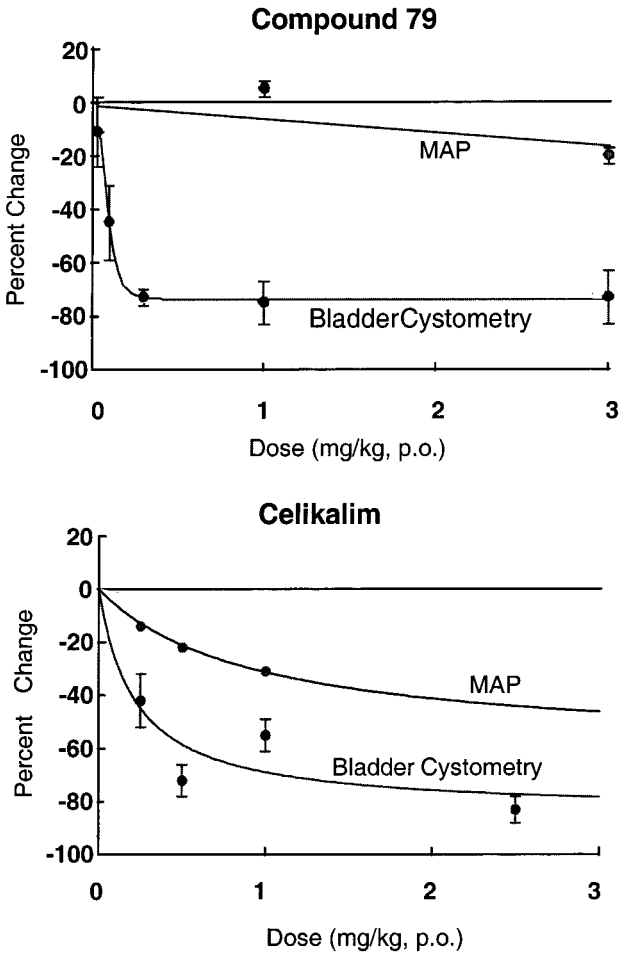


Figure 4. Comparison of in vivo bladder relaxant and hemodynamic effects of **79** and celikalim (**90**).

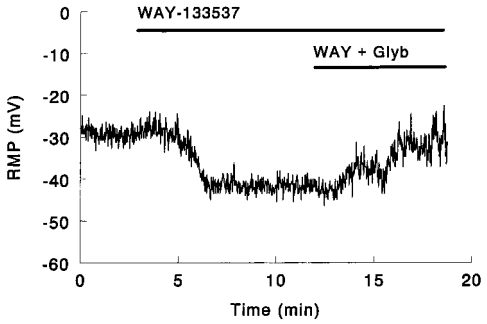


Figure 5. Representative current clamp tracing from a single rat detrusor myocyte. Control RMP was approximately −30 mV; after exposure to **79** (WAY-133537, 1 μM), the cell hyperpolarized by approximately 14 mV. This hyperpolarization was reversed with the addition of glyburide (5 μM) to the tissue bath.

taken. Contractile inhibition with all test compounds in the isolated bladder strip assay was reversed upon exposure to 6 μM glyburide; thus suggesting that the relaxant mechanism involves activation of the ATP-dependent potassium channel in the rat bladder. A full electrophysiological profile of **79** has been reported.[44] For the purpose of this manuscript, some cell electrophysiological data are shown in Figures 5 and 6. In current clamp studies on isolated rat detrusor myocytes, exposure to 1 μM **79** caused a hyperpolarization of approximately 14 mV which was reversed with the addition of 5 μM glyburide (Figure 5). In whole-cell

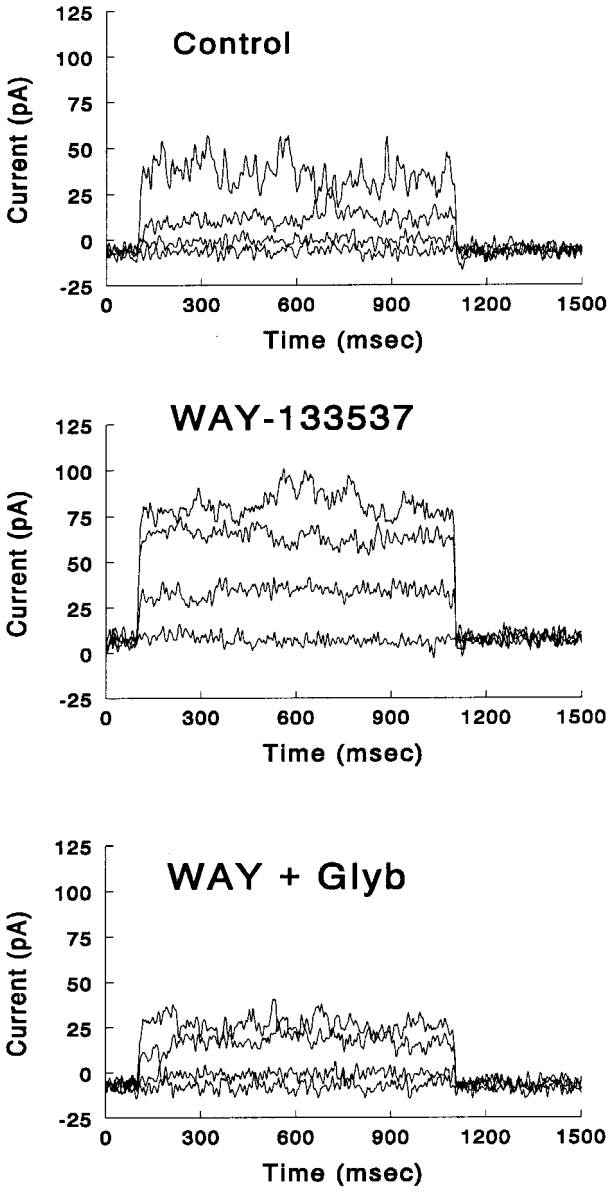


Figure 6. Whole-cell voltage clamp tracings from an isolated rat detrusor myocyte. Cells were held at −50 mV and stepped in +10 mV increments for 1000 ms (figure shows currents elicited at −50, −20, 10, and 40 mV). Compound **79** (WAY-133537) increased outward current at test potentials over the voltage range associated with activation of the ATP-dependent potassium channel. The increases in outward current were reversed in the presence of **79** (WAY-133537) with the subsequent addition of glyburide (5 μM) to the tissue bath.

voltage clamp studies on isolated rat detrusor myocytes, **79** increased outward current at all test potentials over the voltage range associated with activation of the ATP-dependent potassium channel (Figure 6). The increase in outward current was also reversed by exposure to glyburide. Results from both of these cell electrophysiological studies are consistent with activation of the K_{ATP} channel.

Although the mechanism of action studies are conclusive for activation of the K_{ATP} channel, the underlying mechanism of action for the in vivo bladder selectivity of this series of compounds and their differentiation from the vascular-selective antihypertensive KCOs is less clear. Intrinsically, neither compound **79** or ZD-6169 demonstrated in vitro selectivity for detrusor muscle over isolated aortic rings (data not shown). In general, in vitro IC_{50} values for relaxation of precontracted aortic tissues were 2−3 times lower than values observed for detrusor muscle. Favorable pharmacodynamics and/or pharmacokinetics may play a role in the observed in vivo selectivity associated with cyclobutenedione **79** and its analogues. Alternatively, it has been proposed[45] that bladder selectivity with agents such as ZD-6169 may be explained by a biphasic concentration−response for activation of the ATP-dependent potassium channel (i.e., observed activation of the ATP-dependent potassium channel at lower concentrations of ZD-6169, but inhibition of the channel at concentrations > 20 μM ZD-6169) as well as by possible effects on other potassium channels. Studies to further understand the observed in vivo bladder selectivity of **79** and its analogues are currently in progress.

Conclusions

We have demonstrated that structural modifications to prototypical KCOs in the *N*-cyanoguanidine class of agents originally developed as antihypertensives have provided novel compounds with potential utility in *noncardiovascular* therapeutic areas. Replacement of the *N*-cyanoguanidine template in pinacidil with a 1,2-diaminocyclobutene-3,4-dione moiety afforded a novel series of K_{ATP} channel openers possessing potent in vitro bladder relaxant activity. A systematic SAR study on both the aryl and alkylamine groups produced definitive trends which are summarized in Figure 7. Several agents were evaluated in vivo in a pathophysiological model of bladder instability in the rat and also for their effects on MAP and heart rate in normotensive rats. These studies culminated with the identification of (*R*)-4-[3,4-dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-3-ethyl-benzonitrile (**79**) as an agent which selectively inhibits spontaneous detrusor contractions when administered orally at doses that caused no significant changes in hemodynamic parameters. Mechanistic studies have demonstrated that the underlying mechanism of action for the compound involves activation of the K_{ATP} channel in the bladder.

Cyclobutenedione **79** was evaluated in a full battery of safety assessment and ancillary studies. Its oral bioavailability in the rat (calculated from drug plasma levels after oral vs iv administration) was determined to be 70%. It was found to be clean in a NOVA Screen receptor binding profile and was found to be AMES negative at all concentrations tested. A two week pilot toxicological study in the rat at doses up to 200 mg/kg resulted in no observed drug-related abnormalities. In our studies, compound **79** compared favorably in overall profile to ZD-6169, a compound currently undergoing Phase II clinical evaluation for the treatment of urge UI. Due to its potent bladder relaxant effects and its unique in vivo selectivity profile, compound **79** represents a true "second-generation" bladder-selective KCO with potential for development as a novel agent to treat urge urinary incontinence.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus or on an Electrothermal IA9300 and are uncorrected. ^{1}H NMR spectra were recorded on either a Varian XL-300 or on a Varian Unity Plus-400 spectrometer

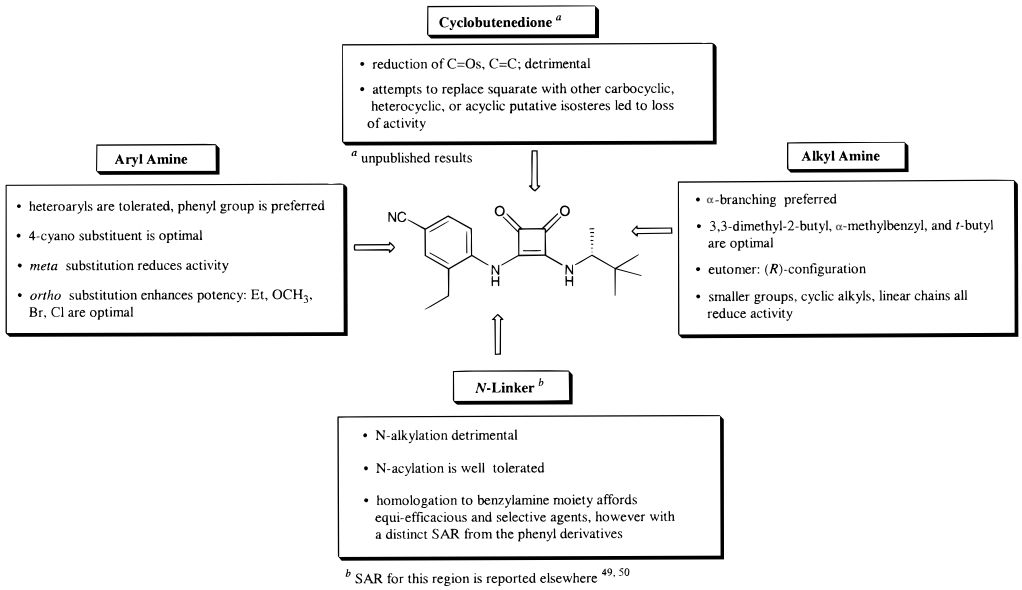


Figure 7. 1,2-Diaminocyclobutene-3,4-diones: trends in in vitro SAR.

using tetramethylsilane (TMS) as an internal standard. The chemical shifts are reported in parts per million (δ) downfield from TMS. Mass spectra were recorded on either a Finnigan MAT 8230 or a Finnigan MAT SSQ710C spectrometer. The infrared spectra were recorded on a Nicolet Avatar 360 spectrophotometer. C, H, N combustion analyses were determined on a Perkin-Elmer 2400 analyzer, and all compounds are within ±0.4% of the theoretical value unless otherwise indicated. Organic extracts were dried over magnesium sulfate or sodium sulfate and were concentrated in vacuo with rotary evaporators. When necessary, purification of compounds was accomplished by flash column chromatography using 230−400 mesh silica gel, by radial chromatography using a Chromatotron 7924 (Harrison Research) with precast silica gel rotors obtained from Analtech, Inc., or by HPLC using a Waters Prep 500 instrument with silica Prep-Pak cartridges. Thin-layer chromatography was performed on silica gel 60 F-254 (0.25 mm thickness) plates. Visualization was accomplished with UV light, I_2 vapor, or 10% phosphomolybdic acid in ethanol.

Structures of all tested compounds have been confirmed by ^{1}H NMR, IR, MS, and combustion analysis. Yields, melting points, and analytical results are tabulated in Tables 1−3 and 5−6. The syntheses and spectral data for representative compounds from each of the Tables 1−3 and 5−6 are shown below.

Representative Pyridyl Analogues in Table 1: (±)-3-(Pyridin-4-ylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (2). To a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (5.00 g, 29.4 mmol) in absolute ethanol (100 mL) was added a suspension of 4-aminopyridine (2.77 g, 29.4 mmol) in ethanol (50 mL). The reaction mixture was refluxed for 4 h and was then concentrated to give crude product. Flash chromatography (ethyl acetate) afforded 0.68 g (13%) of monoadduct as a white solid: mp 120−125 °C. To the compound (0.332 g, 1.52 mmol) in acetonitrile (30 mL) was added (±)-2-amino-3,3-dimethylbutane (0.200 mL, 1.52 mmol). A precipitate formed while stirring overnight. The crude reaction mixture was vacuum filtered, and the precipitate was dried in vacuo to yield 0.328 g (79%) of title compound as a pale yellow solid: mp 255−257 °C; ^{1}H NMR (DMSO-d_6) δ 9.80 (s, 1H), 8.42 (dd, 2H), 7.72 (d, 1H), 7.45 (dd, 2H), 3.96−4.00 (m, 1H), 1.18 (d, 3H), 0.91 (s, 9H); IR (KBr) 3200, 1800, 1675, 1600 cm^{-1}; MS (*m*/*z*) 274 (MH^+).

(±)-3-(Pyridin-3-ylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (10). To a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (5.00 g, 29.4 mmol) in absolute ethanol (100 mL) was added a suspension of 3-aminopyridine (2.77 g, 29.4 mmol) in ethanol (50 mL). The mixture was heated at reflux for 18 h, then concentrated. Flash chromatography (4:1 ethyl acetate/hexane) afforded 3.15 g (49%) of monoadduct as a white solid: mp 140−145 °C. To the compound (0.328 g, 1.50 mmol) in acetonitrile (30 mL) was added (±)-2-amino-3,3-dimethylbutane (0.200 mL, 1.52 mmol). A precipitate formed while stirring overnight. The crude reaction mixture was vacuum filtered, and the precipitate was dried in vacuo to yield 0.27 g (66%) of title compound as a white solid: mp 243−245 °C; ^{1}H NMR (DMSO-d_6) δ 9.67 (s, 1H), 8.58 (d, 1H),7.98 (d, 1H), 7.66 (d, 1H), 7.38 (m, 1H), 3.96−4.00 (m, 1H), 1.18 (d, 3H), 0.95 (s, 9H); IR (KBr) 3200, 1800, 1665, 1600 cm^{-1}; MS (*m*/*z*) 273 (M^+).

3-*tert*-Butylamino-4-(pyridin-3-ylamino)-cyclobut-3-ene-1,2-dione (13). Prepared in the manner described for compound **10**: mp 250−252 °C; ^{1}H NMR (DMSO-d_6) δ 8.57 (s, 1H), 8.23 (d, 1H), 7.96 (d, 1H), 7.37 (m, 1H) 1.43 (s, 9H); IR (KBr) 1790, 1685, 1600 cm^{-1}; MS (*m*/*z*) 245 (M^+).

3-(Isopropylamino)-4-(pyridin-3-ylamino)-cyclobut-3-ene-1,2-dione (18). Prepared in the manner described for compound **10**: mp 258−260 °C; ^{1}H NMR (DMSO-d_6) δ 9.62 (br s, 1H), 8.55 (s, 1H), 8.22 (d, 1H), 7.94 (br d, 1H), 7.73 (br s, 1H), 7.36 (dd, 1H), 4.20 (br m, 1H), 1.25 (d, 6H); IR (KBr) 3200, 1800, 1660, 1610 cm^{-1}; MS (*m*/*z*) 231(M^+).

3-Amino-4-(pyridin-3-ylamino)-cyclobut-3-ene-1,2-dione (19). Prepared in the manner described for compound **10**: mp 297 °C (dec); ^{1}H NMR (DMSO-d_6) δ 8.56 (s, 1H), 8.22 (d, 1H), 7.92 (d, 1H), 7.37 (m, 1H); IR (KBr) 3200, 1800, 1670, 1625 cm^{-1}; MS (*m*/*z*) 189 (M^+).

Representative Bicyclic Heteroaryl Analogues In Table 2: (±)-3-(Quinolin-3-ylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (20). To a solution of 3-aminoquinoline (2.12 g, 14.69 mmol) in ethanol (60 mL) was added 3,4-diethoxy-3-cyclobutene-1,2-dione (2.50 g, 14.69 mmol), and the resulting mixture was heated to reflux for 24 h. The mixture was cooled, and the precipitate was filtered, washed with diethyl ether, and dried in vacuo to give 3.14 g (80%) of monoadduct as a yellow solid which was used without purification: ^{1}H NMR (DMSO-d_6) δ 11.14 (s, 1H), 8.92 (dd, 1H), 8.20 (dd, 1H), 7.90 (m, 2H), 7.63 (m, 2H). To the compound (0.30 g, 1.12 mmol) in ethanol (8 mL) was added (±)-2-amino-3,3-dimethylbutane (0.18 mL, 1.34 mmol), and the resulting mixture was heated at 50 °C overnight. The precipitate was filtered, washed with diethyl ether, and dried in vacuo to afford 0.30 g (83%) of title compound as a light tan solid: mp 242−246 °C; ^{1}H NMR (DMSO-d_6) δ 9.93 (s, 1H), 8.90 (d, 1H), 8.38 (br s, 1H), 7.97 (d, 1H), 7.84 (dd, 1H), 7.75−7.55 (m, 2H), 4.00 (m, 1H), 1.20 (d, 3H), 0.94 (s, 9H); IR (KBr) 3160, 2960, 1795, 1650 cm^{-1}; MS (*m*/*z*) 324 (MH^+).

3-*tert*-Butylamino-4-(quinolin-3-ylamino)-cyclobut-3-ene-1,2-dione (21). Prepared in the manner described for

compound **20**: mp 257−260 °C; ^{1}H NMR (DMSO-d_6) δ 10.00 (br d, 1H), 8.9 (d, 1H), 8.4 (m, 1H), 8.02 (d, 1H), 7.96 (br d, 1H), 7.86 (dd, 1H), 7.66−7.54 (m, 2H), 1.45 (s, 9H); IR (KBr) 3400, 3200, 2980, 1785, 1680 cm^{-1}; MS (*m*/*z*) 296 (MH^+).

3-*tert*-Butylamino-4-(quinolin-6-ylamino)-cyclobut-3-ene-1,2-dione (22). To a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (5.00 g, 29.4 mmol) in absolute ethanol (100 mL) was added a suspension of 6-aminoquinoline (4.24 g, 29.4 mmol) in ethanol (50 mL). The mixture was heated at reflux for 18 h, cooled, and vacuum filtered to afford 6.64 g (84%) of monoadduct (mp: 185−187, dec) which was used without further purification. An aliquot (3.00 g, 11.2 mmol) was dissolved in *tert*-butylamine (50 mL). The solution was refluxed for 3 h, cooled, and concentrated. The residue was recrystallized twice from ethanol and then triturated with diethyl ether to give 1.05 g (31%) of the title compound as a pale yellow solid: mp 234−236 °C; ^{1}H NMR (DMSO-d_6) δ 9.92 (s, 1H), 8.76 (d, 1H), 8.24 (d, 1H), 8.01 (d, 1H), 7.98 (s, 1H), 7.97 (d, 1H), 7.88 (d, 1H), 7.48 (m, 1H), 1.45 (s, 9H). IR (KBr) 1780, 1665, 1610 cm^{-1}; MS (*m*/*z*) 295 (M^+).

(±)-3-(Quinolin-8-ylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (23). To a solution of 8-aminoquinoline (1.00 g, 6.94 mmol) in ethanol (20 mL) was added 3,4-diethoxy-3-cyclobutene-1,2-dione (1.03 mL, 6.94 mmol), and the resulting mixture was heated to reflux for 24 h. The mixture was cooled, diluted with ethanol, and filtered. The crude product was triturated with chloroform/hexanes and then purified by flash chromatography (ethyl acetate/hexane) to give 1.31 g (70%) of monoadduct: ^{1}H NMR (DMSO-d_6) δ 9.75 (br m, 1 H), 8.86 (dd, 1 H), 8.26 (br m, 1H), 8.20 (dd, 1H), 7.57 (m, 2H), 7.53 (dd, 1H), 4.95 (q, 2H), 1.59 (t, 3H). To the compound (0.300 g, 1.12 mmol) in ethanol (5 mL) was added (±)-2-amino-3,3-dimethylbutane (0.18 mL, 1.34 mmol), and the resulting mixture was heated at 45 °C overnight, diluted with hexanes, and filtered to give 0.294 g (81%) of title compound as a yellow solid: mp 244−245; ^{1}H NMR (DMSO-d_6) δ 10.45 (s, 1H), 8.97 (dd, 1H), 8.62 (d, 1H), 8.41 (dd, 1H), 8.29 (dd, 1H), 7.6 (m, 3H), 4.10 (m, 1H), 1.21 (d, 3H), 0.94 (s, 9H); IR (KBr) 3280, 2960, 1790, 1670 cm^{-1}; MS (*m*/*z*) 323 (MH^+).

3-*tert*-Butylamino-4-(isoquinolin-5-ylamino)-cyclobut-3-ene-1,2-dione (24). To a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (5.00 g, 29.4 mmol) in absolute ethanol (100 mL) was added a suspension of 5-aminoisoquinoline (4.24 g, 29.4 mmol) in ethanol (50 mL). The mixture was heated to reflux overnight and then filtered to yield 2.30 g (29%) of monoadduct as a solid: mp 182 (dec) °C. The compound (0.300 g, 1.12 mmol) was dissolved in *tert*-butylamine (50 mL) and refluxed for 3 h. The mixture was cooled, concentrated, and triturated with diethyl ether to afford 0.120 g (39%) of the title compound as a white solid, one-eighth hydrate: mp 268−270 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.75 (s, 1H), 9.35 (s, 1H), 8.62 (d, 1H), 8.19 (s, 1H), 8.01 (d, 1H), 7.88 (d, 1H), 7.80 (d, 1H), 7.68 (t, 1H), 1.47 (s, 9H); IR (KBr) 3200, 1785, 1670, 1600 cm^{-1}; MS (*m*/*z*) 295 (M^+).

(±)-3-(6-Methoxy-quinolin-8-ylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (25). Prepared in the manner described for compound **23** utilizing 8-amino-6-methoxyquinoline: mp 243−245 °C; ^{1}H NMR (DMSO-d_6) δ 10.43 (br s, 1H), 8.78 (dd, 1H), 8.64 (d, 1H), 8.28 (dd, 1H), 8.13 (d, 1H), 7.56 (dd, 1H), 7.02 (d, 1H), 4.11 (m, 1H), 3.88 (s, 3H), 1.20 (d, 3H), 0.94 (s, 9H); IR (KBr) 3400, 3220, 2950, 1780 cm^{-1}; MS (*m*/*z*) 353 (M^+).

Representative Aryl Analogues in Table 3: (+)-(*R*)-3-Phenylamino-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (28). Prepared in the manner described for compound **34**: mp 279−280 °C; $[\alpha]^{25}_D$ = +6.96; ^{1}H NMR (DMSO-d_6) δ 9.55 (s, 1H), 7.57 (d, 1H), 7.40 (d, 2H), 7.33 (m, 2H), 7.01 (m, 1H), 3.99 (m, 1H), 1.17 (d, 3H), 0.91 (s, 9H); MS (*m*/*z*) 273 (M + H)$^+$.

(±)-3-(4-Methylsulfanyl-phenylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (31). 4-(Methylthio)-aniline (0.722 mL, 5.80 mmol) was added to a stirring solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (1.29 mL, 8.70 mmol) in absolute ethanol (29 mL). The mixture was stirred overnight at room temperature, diluted with diethyl ether, and filtered to give 1.22 g (80%) of monoadduct of sufficient purity for the second step. The compound (0.50 g, 1.90 mmol) was added to a solution of (±)-2-amino-3,3-dimethylbutane (0.51 mL, 3.80 mmol) in ethanol (10 mL) and was stirred at room temperature overnight. Filtration afforded 0.536 g (89%) of title compound as a yellow solid: mp 285−286 °C; ^{1}H NMR (DMSO-d_6) δ 9.55 (s, 1H), 7.57 (d, 1H), 7.41 (d, 2H), 7.25 (d, 2H), 3.95 (m, 1H), 2.44 (s, 3H), 1.17 (d, 3H), 0.91 (s, 9H); IR (KBr) 3400, 3200, 2920, 1800, 1660, 1575, 1525, 1450 cm^{-1}; MS (*m*/*z*) 318 (M^+).

(±)-3-(4-Trifluoromethoxy-phenylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (32). Prepared in the manner described for compound **34**: mp 282−285 °C; ^{1}H NMR (DMSO-d_6) δ 9.66 (s, 1H), 7.60 (d, 1H), 7.53 (d, 2H), 7.33 (d, 2H), 3.99 (m, 1H), 1.17 (d, 3H), 0.91 (s, 9H); MS (*m*/*z*) 357 (M + H)$^+$.

(±)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (34). 4-Aminobenzonitrile (17.58 g, 149 mmol) was added to a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (25.31 g, 149 mmol) in absolute ethanol (450 mL). The mixture was heated at reflux overnight and the resulting suspension was filtered hot to remove a small amount of bis-adduct. The filtrate was gradually concentrated to afford several crops of 4-(3,4-dioxo-2-ethoxy-cyclobut-1-enylamino)-benzonitrile, as a bright yellow solid, which were collected by filtration and combined: yield 29.11 g (81%); ^{1}H NMR (DMSO-d_6) δ 11.07 (s, 1H), 7.81 (d, 2H), 7.56 (d, 2H), 4.79 (q, 2H), 1.46 (t, 3H). To this product (13.00 g, 53.7 mmol) in ethanol (360 mL) was added (±)-2-amino-3,3-dimethylbutane (7.2 mL, 54 mmol). The mixture was heated at reflux overnight. Gradual concentration of the reaction solution afforded two crops of 4-[3,4-dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzonitrile, as a yellow precipitate, which were collected by filtration and combined: yield 11.34 g (71%); mp 241−243 °C; ^{1}H NMR (DMSO-d_6) δ 9.89 (s, 1H), 7.78 (d, 2H), 7.72 (d, 1H), 7.60 (d, 2H), 3.96 (m, 1H), 1.18 (d, 3H), 0.91 (s, 9H).

(+)-(*R*)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzoic Acid Methyl Ester (36). Prepared in the manner described for compound **34**: mp 279−280 °C; $[\alpha]^{25}_D$ = +7.54° (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.87 (s, 1H), 7.93 (d, 2H), 7.68 (d, 1H), 7.56 (d, 2H), 4.99 (m, 1H), 3.82 (s, 3H), 1.18 (d, 3H), 0.92 (s, 9H); MS (*m*/*z*) 331 (M + H)$^+$.

(±)-3-(4-Methanesulfonyl-phenylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (37). To a suspension of 3-(4-methylsulfanyl-phenylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (**31**, 0.100 g, 0.314 mmol) in ethanol (3 mL) was added potassium peroxymonosulfate (OXONE, 0.77 g, 1.26 mmol) as a solution in water (2 mL). The mixture was stirred overnight at room temperature and filtered, and the solid was washed with water and dried in vacuo. The solid was triturated with methanol/ethyl acetate to afford 0.05 g (45%) of sulfone as an off-white solid: mp 307−310 °C; ^{1}H NMR (DMSO-d_6) δ 9.91 (s, 1H), 7.85 (d, 2H), 7.71 (d, 1H), 7.65 (d, 2H), 3.99 (m, 1H), 3.17, (s, 3H), 1.20 (d, 3H), 0.91 (s, 9H); IR (KBr) 3400, 3150, 2925, 1800, 1680, 1575, 1530, 1450 cm^{-1}; MS (*m*/*z*) 351 (M + H)$^+$.

(+)-(*R*)-3-(4-Trifluoromethyl-phenylamino)-4-(1,2,2-trimethyl-propylamino)-cyclobut-3-ene-1,2-dione (40). Prepared in the manner described for compound **34**: mp 296−299 °C; $[\alpha]^{25}_D$ = +4.20° (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.81 (s, 1H), 7.63 (m, 5H), 4.00 (m, 1H), 1.17 (d, 3H), 0.91 (s, 9H); MS (*m*/*z*) 341 (M + H)$^+$.

Representative Benzonitrile Analogues in Table 5: (+)-(*R*)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (42). Prepared in the manner described for compound **34**: mp 273−274 °C; $[\alpha]^{25}_D$ = +5.88° (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.88 (s, 1H), 7.79 (d, 2H), 7.71 (d, 1H), 7.61 (d, 2H), 3.99 (m, 1H), 1.17 (d, 3H), 0.91 (s, 9H); MS (*m*/*z*) 298 (M + H)$^+$. The chiral purity of **42** was determined using a Chiralpak AS column with an 80:20 isocratic hexane:ethanol mobile phase at a flow rate of 1.0 mL/min. Under these conditions, compound **42** possessed a retention time of 9.40 min (>99% ee).

(−)-(*S*)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (43). To a solution of (*S*)(−)-2-amino-3,3-dimethyl-butane (0.50 g, 4.90 mmol) in ethanol (100 mL) was added 4-(3,4-dioxo-2-ethoxy-cyclobut-1-enylamino)-benzonitrile (1.00 g, 4.13 mmol). The mixture was stirred for 2 days at room temperature and vacuum filtered. The solid was dissolved in methanol and filtered. The filtrate was concentrated in vacuo and the residue was triturated with acetone to afford 350 mg (29%) of title compound as a pale yellow solid: mp 241−243 °C; $[\alpha]^{25}_D = -7.7°$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.89 (s, 1H), 7.78 (d, 2H), 7.72 (d, 1H), 7.60 (d, 2H), 3.96−3.98 (m, 1H), 1.17 (d, 3H), 0.91 (s, 9H); IR (KBr) 3210, 2250, 1800, 1685 cm^{-1}; MS (*m*/*z*) 297 (M^+). The chiral purity of **43** was determined using a Chiralpak AS column with an 80:20 isocratic hexane: ethanol mobile phase at a flow rate of 1.0 mL/min. Under these conditions, compound **43** possessed a retention time of 7.52 min (97% ee).

(±)-3-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (44). Prepared in the manner described for compound **34**: mp 296−298 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.79 (s, 1H), 7.94 (m, 1H), 7.63−7.69 (m, 2H), 7.51−7.55 (m, 1H), 7.44−7.46 (m, 1H), 3.93−4.03 (m, 1H), 1.17 (d, 3H), 0.91 (s, 9H); IR (KBr) 3200, 2200, 1800, 1675 cm^{-1}; MS (*m*/*z*) 297 (M^+).

(+)-(*R*)-2-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (45). Prepared in the manner described for compound **34**: mp 258−259 °C; $[\alpha]^{25}_D = +45.29°$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.69 (s, 1H), 8.02 (br d, 1H), 7.79 (dd, 1H), 7.62 (m, 1H), 7.57 (d, 1H), 7.22 (m, 1H), 4.03 (m, 1H), 1.19 (d, 3H), 0.92 (s, 9H); MS (*m*/*z*) 298 ($M + H)^+$.

4-(2-*tert*-Butylamino-3,4-dioxo-cyclobut-1-enylamino)-benzonitrile (46). Prepared in the manner described for compound **34**: mp 257−260 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.97 (s, 1H), 8.02 (s, 1H), 7.78 (d, 2H), 7.61 (d, 2H), 1.42 (s, 9H); IR (KBr) 3220, 2240, 1800, 1680, 1620 cm^{-1}; MS (*m*/*z*) 269 (M^+).

4-(2-Isopropylamino-3,4-dioxo-cyclobut-1-enylamino)-benzonitrile (51). Prepared in the manner described for compound **34**: mp 290−292 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.89 (s, 1H), 7.79 (d, 1H), 7.76 (d, 2H), 7.57 (d, 2H), 4.19−4.20 (m, 1H), 1.25 (d, 6H); IR (KBr) 3220, 2230, 1800, 1620 cm^{-1}; MS (*m*/*z*) 255 (M^+).

(±)-(Exo)-4-[2-(bicyclo[2.2.1]hept-2-ylamino)-3,4-dioxo-cyclobut-1-enylamino]-benzonitrile (58). Prepared in the manner described for compound **34**: mp 288−290 °C; ^{1}H NMR (DMSO-d_6) δ 9.79 (s, 1H), 7.78 (d, 2H), 7.76 (m, 1H), 7.58 (d, 2H), 3.90−3.98 (m, 1H), 2.22−2.34 (m, 2H), 1.76−1.86 (m, 1H), 1.08−1.56 (m, 7H); IR (KBr) 2220, 1790, 1650, 1600 cm^{-1}; MS (*m*/*z*) 307 (M^+).

4-(2-Cycloheptylamino-3,4-dioxo-cyclobut-1-enylamino)-benzonitrile (59). Prepared in the manner described for compound **34**: mp 273−275 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.89 (s, 1H), 7.86 (d, 1H), 7.77 (d, 2H), 7.58 (d, 2H), 4.07−4.11 (m, 1H), 1.42−1.98 (m, 12H); IR (KBr) 3250, 2200, 1800, 1675 cm^{-1}; MS (*m*/*z*) 309 (M^+).

(−)-4-[3,4-Dioxo-2-((*R*)-1-phenyl-ethylamino)-cyclobut-1-enylamino]-benzonitrile (60). Prepared in the manner described for compound **34**: mp 273−274 °C; $[\alpha]^{25}_D = -53.20$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.91 (s, 1H), 8.21 (d, 1H), 7.72 (d, 1H), 7.79−7.31 (m, 9H), 5.29 (m, 1H), 1.59 (d, 3H); IR (KBr) 3200, 2230, 1790, 1670, 1600 cm^{-1}; MS (*m*/*z*) 317 (M^+).

(+)-4-[3,4-Dioxo-2-((*S*)-1-phenyl-ethylamino)-cyclobut-1-enylamino]-benzonitrile (61). Prepared in the manner described for compound **34**: mp 269−270 °C; $[\alpha]^{25}_D = +46.47$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.91 (s, 1H), 8.21 (d, 1H), 7.72 (d, 1H), 7.79−7.31 (m, 9H), 5.29 (m, 1H), 1.59 (d, 3H); IR (KBr) 3200, 2230, 1790, 1670, 1600 cm^{-1}; MS (*m*/*z*) 317 (M^+).

4-[3,4-Dioxo-2-(benzylamino)-cyclobut-1-enylamino]-benzonitrile (62). Prepared in the manner described for compound **34**: mp 288−290 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.91 (s, 1H), 8.10 (m, 1H), 7.79 (d, 2H), 7.75 (d, 2H), 7.55 (d, 2H), 7.91−7.78 (m, 5H), 4.82 (d, 2H); IR (KBr) 3190, 2220, 1790, 1660, 1575 cm^{-1}; MS (*m*/*z*) 303 (M^+).

4-[3,4-Dioxo-2-(1-methyl-1-phenyl-ethylamino)-cyclobut-1-enylamino]-benzonitrile (65). Prepared in the manner described for compound **34**: mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 10.08 (s, 1H), 8.38 (s, 1H), 7.79 (d, 2H), 7.61 (d, 2H), 7.48−7.27 (m, 5H), 1.78 (s, 6H); IR (KBr) 3200, 2230, 1790, 1675, 1600 cm^{-1}; MS (*m*/*z*) 331 (M^+).

(±)-4-[3,4-Dioxo-2-(2,2,2-trifluoro-1-phenyl-ethylamino)-cyclobut-1-enylamino]-benzonitrile (66). To a solution of *N*-2,2,2-trifluoro-1-phenylethyl-*N*-1′-(phenyl)ethylamine[46] (1.65 g, 6.22 mmol) and ammonium formate (1.17 g, 18.6 mmol) in methanol (150 mL) was added 10% palladium on activated carbon. The suspension was refluxed for 4 h, filtered through Celite, and concentrated to a volume of approximately 10 mL. 4-(3,4-Dioxo-2-ethoxy-cyclobut-1-enylamino)-benzonitrile (1.00 g, 4.13 mmol) and ethanol (20 mL) were added, and the mixture was heated at reflux for 18 h, cooled slightly, and vacuum filtered to remove a small amount of solid. The filtrate was chromatographed (methanol/dichloromethane), and the resulting yellow solid was crystallized from chloroform and ether to afford 0.72 g (47%) of product as a pale yellow solid: mp 206−207 °C; ^{1}H NMR (DMSO-d_6) δ 9.99 (s, 1H), 8.88 (d, 1H), 7.82 (d, 2H), 7.60−7.46 (m, 9H), 5.98 (m, 1H), 3.73 (s, 3H); IR (KBr) 3200, 2200, 1800, 1690, 1570 cm^{-1}; MS (*m*/*z*) 371 (M^+).

(−)-4-[3,4-Dioxo-2-((*R*)-1-phenyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (68). Prepared in the manner described for compound **34**: mp 242−243 °C; $[\alpha]^{25}_D = -52.73$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.84 (s, 1H), 8.12 (br d, 1H), 7.76 (d, 2H), 7.56 (d, 2H), 7.42−7.27 (m, 5H), 5.06 (m, 1H), 1.94 (m, 2H), 0.90 (t, 3H); IR (KBr) 3200, 2220, 1790, 1670, 1600 cm^{-1}; MS (*m*/*z*) 331 (M^+).

(+)-4-[3,4-Dioxo-2-((*S*)-1-phenyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (69). Prepared in the manner described for compound **34**: mp 241−243 °C; $[\alpha]^{25}_D = +52.33$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.84 (s, 1H), 8.21 (br d, 1H), 7.76 (d, 2H), 7.56 (d, 2H), 7.42−7.27 (m, 5H), 5.06 (m, 1H), 1.94 (m, 2H), 0.90 (t, 3H); IR (KBr) 3200, 2220, 1790, 1670, 1600 cm^{-1}; MS (*m*/*z*) 331 (M^+).

(−)-(*R*)-4-{2-[1-(4-Methyl-phenyl)-ethylamino]-3,4-dioxo-cyclobut-1-enylamino}-benzonitrile (70). Prepared in the manner described for compound **34**: mp >300 °C; $[\alpha]^{25}_D = -56.01$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.81 (s, 1H), 8.10 (m, 1H), 7.76 (d, 2H), 7.55 (d, 2H), 7.29 (d, 2H), 7.19 (d, 2H), 5.26 (m, 1H), 1.57 (d, 3H); IR (KBr) 3200, 2220, 1790, 1670, 1600 cm^{-1}; MS (*m*/*z*) 331 (M^+).

(−)-(*R*)-4-{2-[1-(4-Methoxy-phenyl)-ethylamino]-3,4-dioxo-cyclobut-1-enylamino}-benzonitrile (71). To a solution of (1*R*, 1′*R*)-*N*-(1′-phenylethyl)-1-(4″-methoxyphenyl)ethylamine (1.37 g, 5.36 mmol; prepared from 4′-methoxyacetophenone utilizing the procedure described by Manley and Quast[38]) and ammonium formate (1.01 g, 16.0 mmol) in methanol (125 mL) was added 10% palladium on activated carbon. The suspension was refluxed for 2 h, filtered through Celite, and concentrated. 4-(3,4-Dioxo-2-ethoxy-cyclobut-1-enylamino)-benzonitrile (1.00 g, 4.13 mmol) was added to a solution of the resulting residue in ethanol (30 mL). The mixture was heated at reflux for 18 h, cooled slightly, and vacuum filtered. The precipitate was chromatographed (methanol/dichloromethane) and recrystallized (methanol/dichloromethane) to afford 0.21 g (15%) of product as a yellow solid: mp >300 °C; $[\alpha]^{25}_D = -46.95$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.89 (s, 1H), 8.13 (d, 1H), 7.77 (d, 2H), 7.56 (d, 2H), 7.34 (d, 2H), 6.95 (d, 2H), 5.23 (m, 1H), 3.73 (s, 3H), 1.57 (d, 3H); IR (KBr) 3200, 2200, 1800, 1670, 1575 cm^{-1}; MS (*m*/*z*) 347 (M^+).

(−)-(*R*)-4-{3,4-Dioxo-2-[1-(4-trifluoromethoxy-phenyl)-ethylamino]-cyclobut-1-enylamino}-benzonitrile (72). To a solution of (1*R*,1′*R*)-*N*-(1′-phenylethyl)-1-(4″-trifluoromethoxyphenyl)-ethylamine (1.92 g, 6.21 mmol; prepared from 4′-trifluoromethoxyacetophenone utilizing the procedure described by Manley and Quast[38]) and ammonium formate (1.17 g, 18.6 mmol) in methanol (150 mL) was added 10% palladium on activated carbon. The suspension was refluxed for 2 h, filtered through Celite, and concentrated. 4-(3,4-Dioxo-2-ethoxy-cyclobut-1-enylamino)-benzonitrile (1.00 g, 4.13 mmol)

was added to a solution of the resulting residue in ethanol (35 mL). The mixture was heated at reflux for 18 h, cooled slightly, and vacuum filtered. The precipitate was combined with a second crop of solid obtained from the cooled filtrate, chromatographed (methanol/dichloromethane), and recrystallized (methanol/dichloromethane) to afford 0.74 g (45%) of product as a white solid: mp 281−284 °C (dec); $[\alpha]^{25}_D = -55.94$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.94 (s, 1H), 8.22 (d, 1H), 7.78 (d, 2H), 7.60−7.51 (m, 4H), 7.40 (d, 2H), 5.33 (m, 1H), 1.60 (d, 3H); IR (KBr) 3200, 2200, 1800, 1670, 1560 cm^{-1}; MS (*m*/*z*) 401 (M^+).

(−)-(*R*)-4-{2-[1-(4-Nitro-phenyl)-ethylamino]-3,4-dioxo-cyclobut-1-enylamino}benzonitrile (73). To 4-(3,4-dioxo-2-ethoxy-cyclobut-1-enylamino)-benzonitrile (0.598 g, 2.47 mmol) in ethanol (50 mL) were added (*R*)-α-methyl-4-nitrobenzylamine hydrochloride (0.50 g, 2.5 mmol) and *N*,*N*-diisopropylethylamine (0.43 g, 2.5 mmol). The mixture was heated at reflux for 16 h. After cooling, the precipitate was filtered off to afford 0.70 g (78%) of product as an orange solid: mp 290−295 °C; $[\alpha]^{25}_D = -100.52$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.98 (s, 1H), 8.32 (d, 1H), 8.25 (d, 2H), 7.79 (d, 2H), 7.68 (d, 2H), 7.57 (d, 2H), 5.42 (m, 1H), 1.61 (d, 3H); IR (KBr) 3200, 2220, 1790, 1670, 1600 cm^{-1}; MS (*m*/*z*) 362 (M^+).

Representative *N*-4-Cyanophenyl-*N*′-(*R*)-3,3-dimethyl-2-butyl Analogues in Table 6: (+)-(*R*)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-3-ethyl-benzonitrile (79). Method A. 4-Amino-3-ethylbenzonitrile (0.86 g, 5.88 mmol) and 3,4-diethoxy-3-cyclobutene-1,2-dione (1.0 g, 5.88 mmol) were refluxed in acetonitrile (2 mL) for 21 h. Additional 3,4-diethoxy-3-cyclobutene-1,2-dione (0.5 g, 2.9 mmol) was added to the reaction mixture. After 48 h the reaction mixture was cooled to room temperature and then diluted with ethyl acetate, and a solid was filtered. The filtrate was concentrated, and the resulting solid was taken up in ethyl acetate (5 mL) and sonicated. Filtration gave 0.54 g (34%) of monoadduct as a light brown solid: ^{1}H NMR (DMSO-d_6) δ 10.56 (br s, 1H), 7.70 (br s overlapping a doublet at d 7.69, 2H), 7.30 (d, 1H), 4.71 (q, 2H), 2.74 (q, 2H), 1.37 (t, 3H), 1.15 (t, 3H). 4-(2-Ethoxy-3,4-dioxo-cyclobut-1-enylamino)-3-ethylbenzonitrile (1.26 g, 4.66 mmol) and a solution of (*R*)-2-amino-3,3-dimethylbutane (9.3 mmol) in ethanol (58 mL) were stirred at room temperature for 24 h. The resulting yellow solution was concentrated to a yellow oil, which was dissolved in acetonitrile (20 mL) and stirred at room temperature. The resulting yellow solid was filtered and rinsed with ethyl acetate to yield 0.79 g (52%) of title compound as an off-white solid: mp 236−237 °C; $[\alpha]^{25}_D = +68.20$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 8.04 (br s, 1H), 8.04 (d, 1H), 7.69−7.57 (m, 3H), 4.05 (m, 1H), 2.71 (q, 2H), 1.27−1.16 (overlapping doublet and triplet, 6H), 0.91 (s, 9H); IR (KBr) 3278, 2959, 2222, 1793, 1674, 1598, 1576, 1522 cm^{-1}; MS (*m*/*z*) 325 (M^+).

(+)-(*R*)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-3-ethyl-benzonitrile (79). Method B. To a solution of 4-amino-3-ethylbenzonitrile (10 g, 68.5 mmol) in DMF (150 mL) at 0 °C was added, portion-wise, NaH (2.26, 80%, 75.3 mmol). The mixture was heated to 60 °C for 1 h and was then cooled to room temperature. The 3,4-diethoxy-3-cyclobutene-1,2-dione (13.17 mL, 89.04 mmol) was added, and the resulting mixture was heated to 80 °C for 4 h. The reaction was cooled, diluted with brine (150 mL), and extracted with dichloromethane. The organic phase was washed with 1.0 N HCl and brine. It was dried and concentrated under heated vacuum to afford an oil. Trituration with diethyl ether/hexanes afforded 10.93 g (59%) of 4-(2-ethoxy-3,4-dioxo-cyclobut-1-enylamino)-3-ethyl-benzonitrile as an off-white solid. The intermediate (7.8 g, 28.88 mmol) was added to a solution of (*R*)-2-amino-3,3-dimethylbutane in ethanol (209 mL of a 0.166 N solution; 34.7 mmol) and was stirred for 48 h at room temperature. The reaction mixture was concentrated, and the residue was triturated with 5:1 petroleum ether/diethyl ether. Filtration afforded 4.86 g (52%) of analytically pure title compound. An additional crop of 1.95 g (21%) was obtained from the mother liquor. The compound was identical in all respects with the sample obtained from method A. The chiral purity of **79** was determined using a Pirkle covalent (*S*,*S*) Whelk O1 column with a mobile phase consisting of either 100% ethanol or 1:1 hexane:ethanol at a flow rate of 0.5 mL/min. Under these conditions, the (*R*)-(+)-enantiomer (eutomer, 98.2%) possessed a retention time of 9.51 min and the (*S*)-(−)-enantiomer (distomer, 1.6%) possessed a retention time of 11.49 min. Chiral purity was determined to be 96.6% ee.

(+)-(*R*)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-3-methoxy-benzonitrile (81). Method A. To a cooled solution of 3-methoxybenzonitrile (10.00 g, 75 mmol) in acetic anhydride (150 mL) was added 70% nitric acid (60 mL) at a rate that kept the reaction mixture below 50 °C. After 1 h the reaction mixture was poured onto ice (400 g) and extracted with ethyl acetate. The ethyl acetate layer was washed with water and dried (Na_2SO_4). Concentration under reduced pressure and flash chromatography with ethyl acetate/hexanes gave 2.69 g (20%) of 3-methoxy-4-nitrobenzonitrile: ^{1}H NMR (DMSO-d_6) δ 8.04 (d, 1H), 7.93 (d, 1H), 7.61 (dd, 1H), 3.97 (s, 3H). 3-Methoxy-4-nitrobenzonitrile (2.6 g, 14.6 mmol) was suspended in ethanol (100 mL) and added to a mixture of 5% platinum on carbon (250 mg) in ethanol (150 mL). The reaction mixture was stirred under a hydrogen gas atmosphere. After 24 h the reaction was filtered, concentrated, and chromatographed (ethyl acetate/hexanes, 25/75) to yield 1.3 g (60%) of 4-amino-3-methoxybenzonitrile: ^{1}H NMR (DMSO-d_6) δ 7.1 (m, 2H), 6.65 (d, 1H), 5.77 (br s, 2H), 3.79 (s, 3H). 4-Amino-3-methoxybenzonitrile (1.3 g, 8.77 mmol) and 3,4-diethoxy-3-cyclobutene-1,2-dione (1.5 g, 8.82 mmol) in ethanol (50 mL) were heated in an oil bath at 110−115 °C for 3 days. The reaction mixture was filtered hot, and the filtrate was reduced to half its volume. Filtration gave 0.58 g (24%) of monoadduct as a yellow solid: mp 159−161 °C; ^{1}H NMR (DMSO-d_6) δ 10.42 (br s, 1H), 7.54 (d, 1H), 7.44 (dd, 1H), 7.39 (d, 1H), 4.70 (q, 2H), 3.86 (s, 3H), 1.36 (t, 3H); IR (KBr) 3413, 3026, 2650, 2576, 2439, 1719, 1630, 1548 cm^{-1}; MS (*m*/*z*) 272 (M^+). 4-(2-Ethoxy-3,4-dioxo-cyclobut-1-enylamino)-3-methoxybenzonitrile (0.58 g, 2.1 mmol) and a solution of (*R*)-2-amino-3,3-dimethylbutane (4.2 mmol) in ethanol (21 mL) were stirred at room temperature for 24 h. The reaction mixture was filtered, and the filtrate was concentrated to a foam, which was dissolved in acetonitrile (10 mL). Upon standing at room temperature fluffy pale yellow needles formed. This was filtered and rinsed sparingly with acetonitrile to yield 0.46 g (67%) of title compound as pale yellow needles: mp 280−282 °C (dec); $[\alpha]^{25}_D = +62.86$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.49 (br s, 1H), 8.31 (d, 1H), 7.99 (d, 1H), 7.50 (d, 1H), 7.43 (dd, 1H), 4.03 (m, 1H), 3.97 (s, 3H), 1.18 (d, 3H), 0.90 (s, 9H); MS (*m*/*z*) 327 (M^+).

(+)-(*R*)-4-[3,4-Dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-3-methoxy-benzonitrile (81). Method B. 4-Amino-3-methoxybenzonitrile (1.32 g, 8.90 mmol) was dissolved in DMF (20 mL) and purged with nitrogen. NaH (0.263 g, 80%, 8.9 mmol) was added, and the resulting mixture was stirred for 30 min at 70 °C. The mixture was cooled to room temperature and added via syringe to a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (2.27 g, 13.35 mmol) in DMF (10 mL). The mixture was stirred for 48 h at room temperature and diluted with brine (50 mL) and extracted with dichloromethane. The organic phase was washed with 1.0 N HCl and brine. It was dried and concentrated under heated vacuum to afford an oil. Trituration with diethyl ether/hexanes afforded 0.97 g (41%) of 4-(2-ethoxy-3,4-dioxo-cyclobut-1-enylamino)-3-methoxy-benzonitrile as a light yellow solid. This was converted to the title compound by treatment with (*R*)-2-amino-3,3-dimethylbutane as described above in method A. The compound was identical in all respects with the sample obtained from method A.

(+)-(*R*)-3-Chloro-4-[3,4-dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (84). To 3,4-diethoxy-3-cyclobutene-1,2-dione (2.0 g, 11.75 mmol) and 4-amino-3-chlorobenzonitrile (1.8 g, 11.80 mmol) in DMF (10 mL) was added 60% NaH (0.94 g, 23.5 mmol). The mixture was stirred at room temperature overnight and then parti-

tioned between dichloromethane and 10% citric acid. The organic phase was washed with brine, dried, and concentrated. The residue was purified by flash column chromatography (hexanes/ethyl acetate) to afford 0.70 g (21%) of 4-(2-ethoxy-3,4-dioxo-cyclobut-1-enylamino)-3-chloro-benzonitrile. The compound (0.3 g, 1.08 mmol) was added to an ethanolic solution of (*R*)-2-amino-3,3-dimethylbutane (5.4 mL of 0.2 N solution; 1.08 mmol). The reaction was stirred overnight at room temperature and then concentrated. The residue was triturated with acetonitrile, sonicated, and filtered to afford 0.22 g (61%) of title compound: 220−222 °C; $[\alpha]^{25}_{D} = +61.39$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.42 (s, 1H), 8.31 (d, 1H), 8.06 (d, 1H), 7.81 (m, 2H), 4.05 (m, 1H), 1.19 (d, 3H), 0.91 (s, 9H); MS (*m*/*z*) 332 $(M + H)^{+\cdot}$.

(+)-(*R*)-3-Bromo-4-[3,4-dioxo-2-(1,2,2-trimethyl-propylamino)-cyclobut-1-enylamino]-benzonitrile (85). To 60% NaH (0.5 g, 12.5 mmol) in THF (50 mL) was added a solution of 4-amino-3-bromobenzonitrile (1.2 g, 6.09 mmol) and 3,4-diethoxy-3-cyclobutene-1,2-dione (1.40 mL, 9.5 mmol) in THF (10 mL). The mixture was heated for 2 h under argon at 80 °C. The reaction was cooled, quenched with 2 N HCl, and stirred for 30 min at room temperature. The water layer was removed by pipet, and the organic layer was diluted with dichloromethane, dried, and concentrated to afford crude monoadduct which was purified by flash column (dichloromethane) to give 1.09 g (56%) of 4-(2-ethoxy-3,4-dioxo-cyclobut-1-enylamino)-3-bromo-benzonitrile. The compound (0.4 g, 1.25 mmol) was added to an ethanolic solution of (*R*)-2-amino-3,3-dimethylbutane (8.1 mL of 0.2 N solution; 1.62 mmol). The reaction was stirred overnight at room temperature and then concentrated. The residue was triturated with acetonitrile, sonicated, and filtered to afford 0.28 g (59%) of title compound: 229−231 °C; $[\alpha]^{25}_{D} = +51.85$ (DMSO); ^{1}H NMR (DMSO-d_6) δ 9.30 (s, 1H), 8.29 (d, 1H), 8.21 (d, 1H), 7.87 (dd, 1H), 7.65 (d, 1H), 4.05 (m, 1H), 1.20 (d, 3H), 0.93 (s, 9H); MS (*m*/*z*) 376/378 $(M + H)^{+\cdot}$.

Pharmacological Methods. All animal studies were approved by the Wyeth-Ayerst Institutional Animal Care and Use Committee and were performed in accordance with the guidelines of the Animal Welfare Act and the American Association for Accreditation of Laboratory Animal Care.

In Vitro Studies: A. Isolation of Rat Detrusor Cells. Rat detrusor cells were isolated in a manner previously described for guinea-pig detrusor.[47] Male Sprague−Dawley rats (Charles River, Wilmington, MA; 200−400 g) were euthanized by CO_2 inhalation and exsanguination. The urinary bladder was rapidly removed and placed in 37 °C physiological salt solution (PSS) with the following composition (mM): Na glutamate (80.0), NaCl (54.7), KCl (5.0), $NaHCO_3$ (25.0), $MgCl_2 \cdot 2H_2O$ (2.5), D-glucose (11.8), and $CaCl_2$ (0.2) gassed with O_2-CO_2, 95%/5%, for a final pH of 7.4. The dome of the bladder was isolated from the trigone region, and the mucosa was removed. The detrusor was then cut into 2−3 mm wide strips and placed into fresh buffer for 1 h. Tissues were then transferred into 10 mL of PSS isolation buffer plus collagenase type VIII (1.0 mg/mL) and pronase (0.25 mg/mL). After 10 min the isolation buffer was replaced with fresh buffer for an additional 10 min. The tissue was then washed 3 times in fresh collagenase and pronase free PSS and stored at room temperature until studied. Cells for study were prepared by triturating one to two pieces of detrusor tissue in 5 mL of fresh isolation buffer for 5 min with a polished Pasteur pipet (tip diameter ~1.5 mm) attached to a modified Harvard Respirator pump (Harvard Apparatus, Southnatic, MA) at a rate of 20×/min with an approximate volume of 5 mL. Cells were studied in a temperature regulated tissue bath at 32.5° C and continually superfused with PSS.

B. Cell Electrophysiology. Single-cell recordings were performed with a List-Medical EPC-7 patch clamp amplifier (Adams & List Assoc., Westbury, NY). Pipet electrodes had tip resistances of 2−4 MΩ and were filled with the following composition (mM): KCl (126.0), $MgCl_2 \cdot 6H_2O$ (4.5), ATP Mg salt (4.0), GTP tris salt (0.3), creatine PO_4 (14.0), D-glucose (9.0), EGTA (9.0), HEPES (9.0). The pH was adjusted to 7.4 with KOH. Signals were acquired (3 kHz high-frequency cutoff, 12 bit resolution) and analyzed using a 586-based personal computer.

Cell resting membrane potential (RMP) was assessed by adding nystatin to the pipet solution (100 μg/mL) and utilizing the perforated patch technique.[48] After stable intracellular access was achieved, RMP was recorded for a 5 min control period. Next, compound (0.3 and 1.0 μM) was superfused until changes in voltage amplitude reached steady state (approximately 3−5 min). After this time, the effects of glyburide (5 μM) on RMP was evaluated.

Whole-cell voltage clamp recordings were made without nystatin using broken patch access. Currents were evoked using the voltage steps described in the figure legends. After stability was achieved, control currents were recorded. Next, compound (0.3 and 1 μM) was added to the superfusate. Currents were recorded for 5 to 10 min or until compound effects reached steady state. This was followed either by washout or addition of glyburide (5 μM) to the superfusate.

C. Isolated Bladder Strip Contraction Studies. Female Sprague−Dawley rats (Charles River, Wilmington, MA; 250 to 350 g) were rendered unconscious via inhalation of CO_2 and exsanguinated. The entire bladder was removed and placed into room temperature PSS of the following composition (mM): NaCl (118.4), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $NaHCO_3$ (24.9), and D-glucose (11.1) gassed with O_2-CO_2, 95%/5%, to achieve a pH of 7.4. The dome of the bladder was isolated from the trigone region, and the mucosa was removed. The detrusor was then cut into strips 4−5 mm wide by 10 mm long. One end was secured to the bottom of a 10 mL tissue bath and the other to a Grass isometric force transducer (Grass Instruments, Quincy, MA). Tissues were pretensioned (0.25 to 0.5 g) and allowed to equilibrate for 30 min. Strips were then contracted with an additional 15 mM KCl and again allowed to equilibrate for approximately 90 min. Compounds were administered directly into the tissue baths as cumulative concentrations, and responses were allowed to reach steady-state. Signals were digitized (486 based personal computer, 12 bit resolution, 1 s sampling interval, custom software) for online analysis. Since isolated bladder strips contract with irregular frequency and amplitude, a 5 min area-under-the-contraction curve was used to assess contractility after achieving steady state for each concentration.

In Vivo Studies: A. Rat Hypertrophied Bladder Model. The method for producing hypertrophied, unstable bladders was modified from that reported by Malmgren et al.[39] Briefly, female Sprague−Dawley rats (Charles River, Wilmington, MA; 190−210 g) were anesthetized with isoflurane, and the bladder and urethra were exposed through a 2 cm midline incision. A 4−0 silk ligature was tied around the proximal urethra in the presence of a stainless steel rod (1 mm diameter). The rod was then removed thus resulting in a partial premeasured occlusion. The abdominal musculature was closed using 3−0 silk, and the skin was closed with surgical staples. Each rat received 150 000 units (im) of bicilin C-R (Wyeth Laboratories, Philadelphia, PA). During the following 6 week period, the increased urethral outlet resistance from the partial occlusion caused the bladders to hypertrophy and become unstable.

After 6 weeks, the ligature was removed under isoflurane anesthesia, and a flared catheter (PE60) was placed in the dome of the bladder and secured with a purse-string suture. The catheter was exteriorized under the skin and through an opening in the back of the neck. The abdominal incision was sutured and the free end of the catheter sealed. Following surgery, animals were given bicilin C-R (150 000 units/rat, im).

Two days after catheter implantation, the animals were used for cystometric evaluation. The night before testing, the animals were placed into metabolic cages. The catheter was connected to a Harvard infusion pump, and bladders were perfused overnight with saline at a rate of 2 mL/h. The next morning a Statham pressure transducer (model P23Db) was positioned in line with the Harvard infusion pump (using a "T" connector) to record bladder pressure. A plastic beaker attached to a force displacement transducer (Grass FTO3) was

placed under the metabolic cage to collect and record urine volume. The cystometric evaluation of bladder function was started by infusing saline (20 mL/h), and after the first micturition the infusion was maintained for 20 min. Two hours after the first cystometry period, the rats were dosed orally with the test compound, and a second cystometry was approximately 1 h after administration of test compound. Vehicle (poly(ethylene glycol) 200) was similarly administered to groups of rats that served as controls.

B. Hemodynamic Assessment. Male Sprague−Dawley rats (Charles River, Wilmington, MA; 315−410 g) were anesthetized with isofluorane. A femoral artery and vein were cannulated with polyethylene tubing (PE50). The rats were placed in Bollman cages, and the tail along with two cannulas was extended through a hole at one end of the cage. The animal was further immobilized by securely taping the tail to the benchtop. Arterial blood pressure (BP) was obtained from the cannulated femoral artery by means of a Statham pressure transducer (model P23Db). Transducer signals were recorded on a Grass (model 7) polygraph. Heart rate (HR) was calculated manually from the BP traces.

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