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Article

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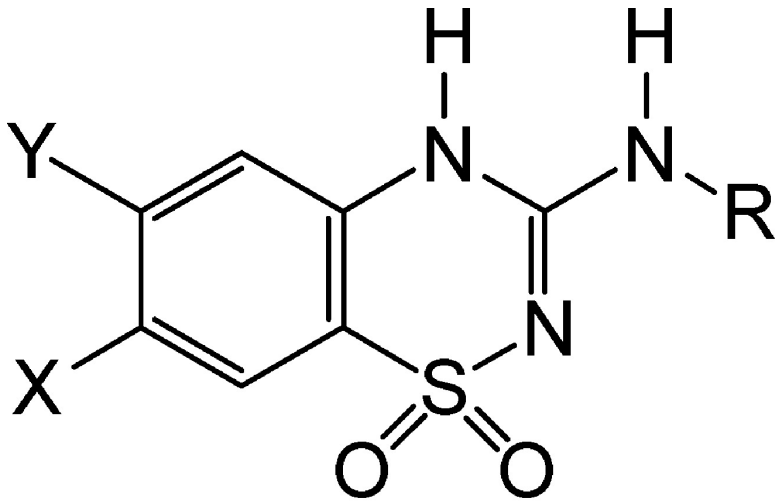

X = Cl, Br, F, OCH_3

Y = Cl, F, NH-alkyl

R = alkyl, cycloalkyl

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3-Alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-Dioxides as ATP-Sensitive Potassium Channel Openers: Effect of 6,7-Disubstitution on Potency and Tissue Selectivity

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A series of 6,7-disubstituted 4*H*-1,2,4-benzothiadiazine 1,1-dioxides bearing a short alkylamino side chain in the 3-position were synthesized. These compounds were tested on rat pancreatic islets and on rat aorta rings. In vitro data indicated that in most cases substitution in the 6 and the 7 positions increased their activity as inhibitors of insulin secretion, while the myorelaxant potency of the drugs was maintained or enhanced according to the nature of the substituent in the 7-position. The presence of either chlorine or bromine atoms in the 6 and 7 positions did not improve the apparent selectivity of the drugs for the pancreatic tissue. By contrast, the introduction of one or two fluorine atoms, as well as the presence of a methoxy group in the 7-position, generated potent and selective inhibitors of insulin release. Radioisotopic and fluorimetric experiments performed with the most potent compound inhibiting insulin release (**34**, BPDZ 259, 6-chloro-7-fluoro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide) confirmed that the drug activated K_{ATP} channels. **34** was found to be one of the most potent and selective pancreatic potassium channel openers yet described.

Introduction

ATP-sensitive potassium channels (K_{ATP} channels), which regulate the flow of potassium ions through the cell membrane, have been identified in a wide range of cell types and have been found to link the metabolic state to the electric state of the cell.[1−8] K_{ATP} channels are composed of two different protein subunits in a 4 + 4 stoichiometry.[9] The K_{ATP} channel pore belongs to the inwardly rectifying potassium channel family and is named Kir6.*x*.[10] The second subunit, the SUR (for sulfonylurea receptor) subunit, contains the regulatory sites for most drugs.[10] Four variants of SUR have been reported (SUR1, SUR2A, SUR2B, and SUR2C).[11] According to their tissue localization, K_{ATP} channels are composed of different subunits. For example, SUR1 combined with Kir6.2 forms the pancreatic K_{ATP} channel.[12] The combination of SUR2A and Kir6.2 subunits is found in cardiac and skeletal muscle, while the smooth muscle K_{ATP} channel is composed of SUR2B and Kir6.1 or Kir6.2 subunits.[13] Although pancreatic K_{ATP} channels are well-known to be involved in the insulin-releasing process[14,15] and smooth muscle K_{ATP} channels in the control of muscle tone,[16,17] the physiological roles of the different channel subtypes have not yet been thoroughly assessed.[18,19]

Several drugs, named PCOs (potassium channel openers), have been found to activate K_{ATP} channels,[20,21] leading to plasma membrane hyperpolarization and reduction in cell excitability. This may, in turn, provoke the relaxation of smooth muscles and/or the inhibition of endocrine releases.[22,23] Because of their broad therapeutic potential, a large variety of K_{ATP} channel agonists has been developed.[24,25] These drugs include chromane derivatives such as cromakalim,[26] cyanoguanidine compounds such as pinacidil,[27] and 1,2,4-benzothiadiazine derivatives such as diazoxide[28] (Figure 1). Selective activation of pancreatic K_{ATP} channels has been demonstrated to be of clinical value in the treatment of several metabolic disorders, including type I and type II diabetes, obesity, and hyperinsulinemia.[29−32] Until recently, diazoxide was the only compound reported to activate pancreatic K_{ATP} channels. Unfortunately, as a consequence of its lack of tissue selectivity, diazoxide induces many side effects such as hypertrichosis, edema, headache, and hypotension.[33]

In the search for new pancreatic selective PCOs, several years ago we developed a series of 3-alkylamino-4*H*-pyrido- and -1,2,4-benzothiadiazine 1,1-dioxides.[34−40] Among the drugs of this original series, BPDZ 44[37] (**1**, Figure 1), BPDZ 73[41] (7-chloro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide) (**2**, Figure 1), BPDZ 138 (7-fluoro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide)[40] (**3**, Figure 1), and BPDZ 216 (3-isopropylamino-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-dioxide)[42] (**4**, Figure 1) were identified as the first potent and selective pancreatic K_{ATP} channel openers. Usually, the benzenic derivatives appeared to exhibit a higher potency on vascular and/or pancreatic tissue in comparison with their pyridinic counterparts. More recently, 3-alkylamino-6-chloro-4*H*-thieno[3,2-*e*]-1,2,4-thiadiazine 1,1-dioxides, such as NN414 (**5**, Figure 1), have also

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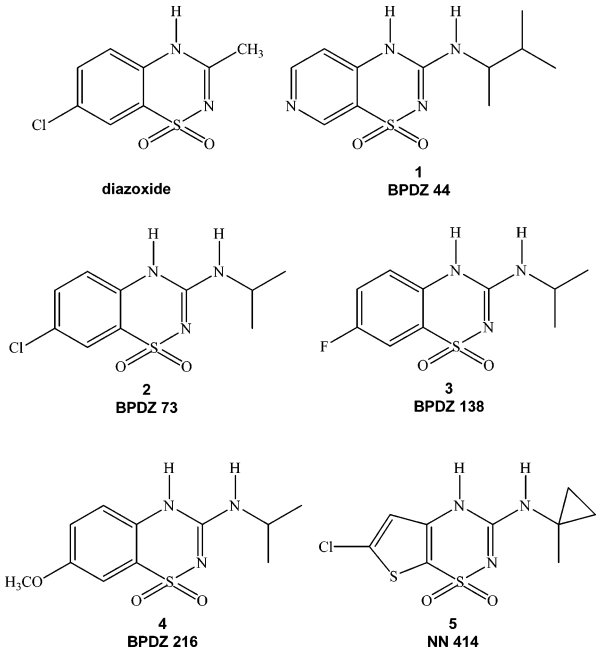


Figure 1. Chemical structure of diazoxide and several analogues described as potassium channels openers.

been proposed for the treatment of metabolic disorders linked to insulin secretion.[43]

Thirty years ago, Topliss and Yudis[44] demonstrated that the introduction of a small group in the 6-position of diazoxide improved its vasorelaxant activity, while Wales et al.[45] showed that the 6,7-dichloro analogue of diazoxide maintained the hyperglycemic properties of the parent compound. On this basis, it might be expected that the disubstitution of 3-alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxides in the 6 and 7 positions would enhance the K_{ATP} channel agonistic activity of diazoxide analogues. In line with this proposition and according to the structure−activity relationships (SAR) deduced from our previous work,[34,36,40] we synthesized in the present study a series of 6- and 7-substituted 4*H*-1,2,4-benzothiadiazine 1,1-dioxides bearing short and branched alkylamino side chains in the 3-position. These newly synthesized compounds were tested as putative K_{ATP} channel openers on a vascular and a pancreatic pharmacological model in order to evaluate the impact of the 6- and 7-disubstitution on both their potency and tissue selectivity. Moreover, further biological investigations were conducted with a selected drug to confirm the mechanism of action of these original compounds.

Chemistry

The different synthetic pathways used to prepare the 6,7-disubstituted 3-alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxides described in this manuscript are illustrated in Schemes 1−3. In the 6,7-dichloro, 7-bromo-6-chloro, 6-chloro-7-fluoro, and 6,7-difluoro series (Scheme 1), a chlorosulfonation step was used to give access to the *o*-aminobenzenesulfonamides key intermediates (**14**−**17**). Starting from the appropriate aniline (**6**−**9**), the treatment with chlorosulfonic acid led to the corresponding sulfonyl chloride (**10**−**13**), which reacted without further purification with diluted ammonia. Ring closure of *o*-aminobenzenesulfonamides, using 1,1′-thiocarbonyldiimidazole as previously described,[46] led to compounds **18**−**21**. The 3-imidazolyl group of the reactive intermediates was displaced by selected branched alkylamines to give the expected 3-alkylamino-substituted derivatives (**22**−**35**). Such a nucleophilic substitution occurred without any difficulties in the cases of 6,7-dichloro (**14**), 7-bromo-6-chloro (**15**), and 6-chloro-7-fluoro (**16**) intermediates. However, the fluorine atom in the 6-position of 6,7-difluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**17**) was substituted by the alkylamine, together with the imidazole group. Thus, application of this synthetic pathway to the 6,7-difluoro intermediate (**17**) led to compounds bearing two alkylamino side chains: the first one, as expected, in the 3-position and the second one, more surprisingly, in the 6-position (compounds **36**−**38**). So to obtain the 6,7-difluoro-substituted compounds (**42**−**44**), we decided to follow the route described by Flemming et al. for obtaining 3-alkylamino-substituted thienothiadiazine dioxides.[43] The action of different alkyl isothiocyanates on 2-amino-4,5-difluorobenzenesulfonamide (**17**) generated the corresponding sulfonylthioureas (**39**−**41**). Ring closure was then achieved at low temperature using phosgene and led to the expected drugs (Scheme 2, **42**−**44**).

The 6-chloro-7-methoxy derivatives (**49**−**51**) were prepared according to Scheme 3, starting from 3-chloro-4-methoxyaniline (**45**). Reaction with chlorosulfonyl isocyanate led to the 3-oxo intermediate (**46**). Subsequent conversion of the 3-oxo function into a 3-thioxo one, (**47**) followed by S-methylation, gave access to the expected reactive 3-methylsulfanyl-substituted intermediate (**48**). The nucleophilic substitution of the methylsulfanyl group was conducted with the appropriate alkylamine under reflux at normal pressure or in a sealed vessel (compounds **49**−**51**).

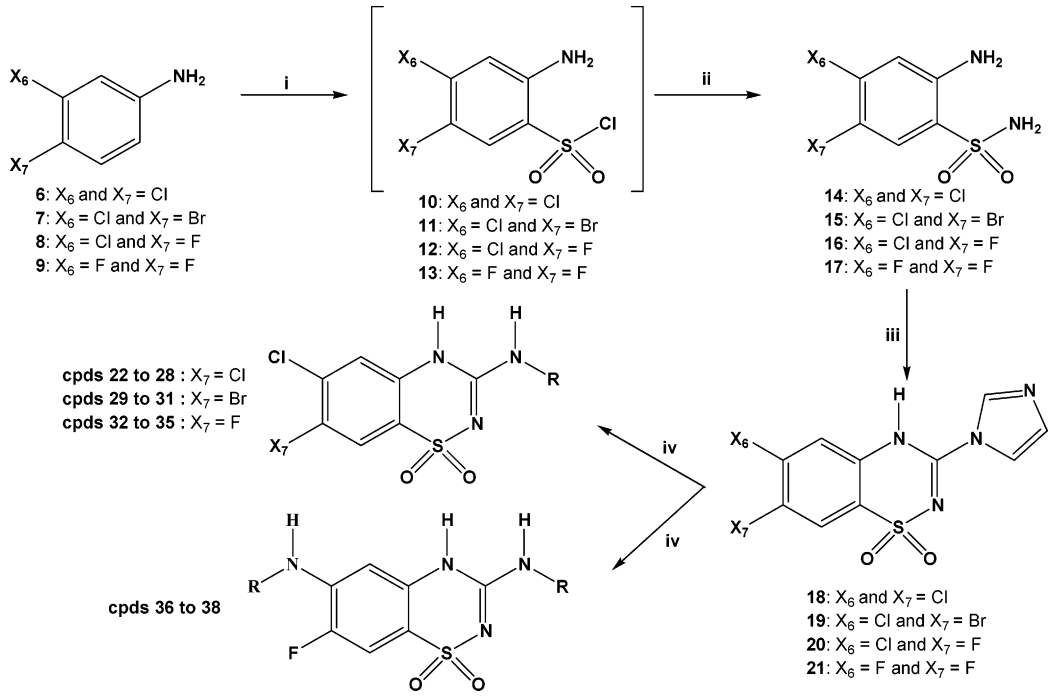
Results and Discussion

Twenty-four new 6,7-disubstituted 4*H*-1,2,4-benzothiadiazine 1,1-dioxides bearing, in most cases, a short alkylamino side chain in the 3-position were prepared. To determine their putative activity on K_{ATP} channels and also to compare their relative tissue selectivity, a pancreatic and a vascular in vitro model were used as pharmacological screening tools.

The ability of such compounds to inhibit glucose-induced insulin secretion was evaluated on isolated rat pancreatic islets and compared to that of diazoxide and the corresponding 7-monosubsituted analogues (Tables 1 and 2). Except for compounds exhibiting a large alkylamino side chain in the 3-position (*n*-hexyl substituent, **28**) and compounds substituted by an alkylamino chain in the 6-position (**36**−**38**) that were found to be inactive at 50 μM, all drugs markedly inhibited insulin release at 10 μM. At a lower concentration (1 μM), and whatever the nature of the 6 and 7 substituent, the 3-ethylamino and the 3-isopropylamino side chains appeared to be the more accurate choice to elicit a marked inhibitory activity on the insulin secreting rate (see compounds **22**, **24**, **29**, **30**, **32**, **34**, **42**, **43**, and **50**).

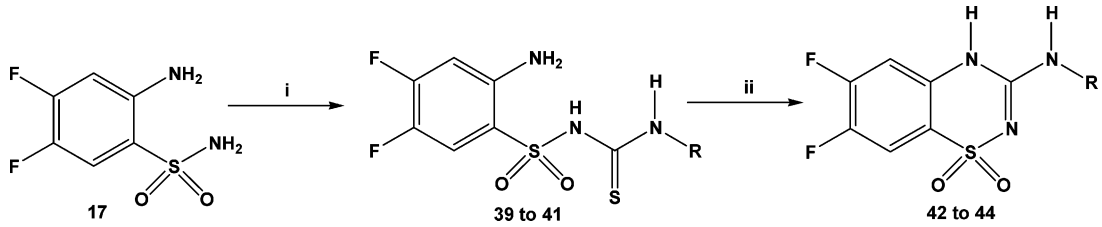
Another interesting hydrocarbon chain on the nitrogen atom in the 3-position appeared to be cyclobutyl (**27**, **31**, **35**, **51**). These results are in accordance with the SAR related to the nature of the alkylamino chain in

Scheme 1[a]



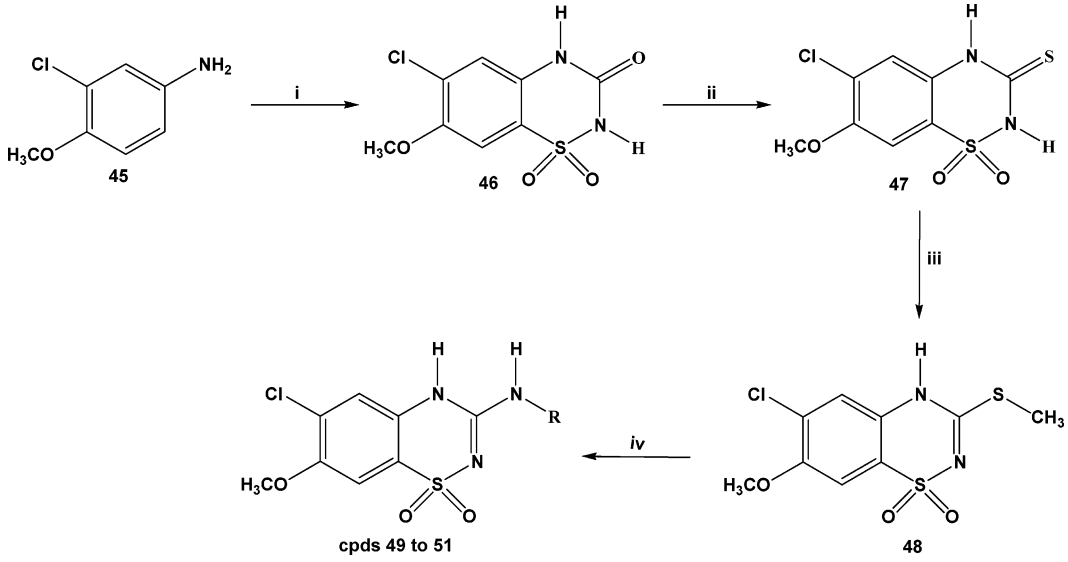
[a] Reagents: (i) $ClSO_3H$; (ii) NH_4OH; (iii) 1,1-thiocarbonyldiimidazole; (iv) RNH_2.

Scheme 2[a]



[a] Reagents: (i) K_2CO_3, S=C=N−R, dry acetone; (ii) TEA, 1.93 M $COCl_2$ in toluene, THF, 0 °C.

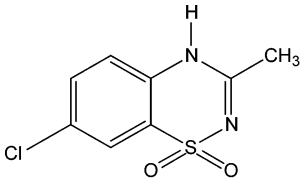
Scheme 3[a]



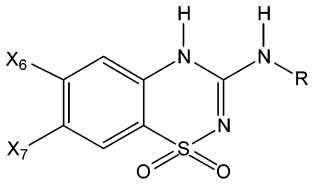
[a] Reagents: (i) $MeNO_2$, $ClSO_2NCO$, $AlCl_3$; (ii) P_2S_5, pyridine; (iii) K_2CO_3, CH_3I; (iv) RNH_2.

the 3-position and the SAR deduced from previous studies on 7-substituted 3-alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxides.[40,47] Moreover, on looking at the calculated IC_{50} values (Tables 1 and 2), it is noted that these disubstituted compounds appear to be more potent than their respective 7-monosubstituted analogues (**24** vs **2**, **34**, **43** vs **3**, and **50** vs **4**) and up to 150 times more potent than diazoxide. So in general the introduction of a halogen atom in the 6-position appeared to favor activity on the pancreatic tissue. The

Table 1. Effects of 6,7-Dichloro-Substituted or 6-Chloro-7-bromo-Substituted 3-Alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-Dioxides on 16.7 mM Glucose-Induced Insulin Release from Rat Pancreatic Islets and on Contractile Activity of K^+-Depolarized Rat Aorta Rings



Diazoxide

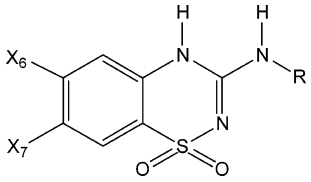


22 to 31

compd				% of residual insulin release (mean ± SEM (*n*)) 10 μM	1 μM	0.1 μM	IC_{50} (pancreas),[b] μM	EC_{50} (aorta),[c] μM	EC_{50}/IC_{50} [d]
22	Cl	Cl	CH_2CH_3	9.1 ± 1.7 (18)	8.5 ± 0.6 (29)	83.4 ± 3.8 (24)	0.25	1.0 ± 0.1 (8)	4
23	Cl	Cl	$CH_2CH_2CH_3$	9.9 ± 0.9 (14)	60.2 ± 4.1 (15)		1.37	4.7 ± 0.5 (4)	3.4
24	Cl	Cl	$CH(CH_3)_2$	6.3 ± 0.7 (12)[a]	13.2 ± 1.0 (26)[a]	84.9 ± 4.5 (21)[a]	0.28[a]	2.3 ± 0.2 (8)	8.2
25	Cl	Cl	$CH_2CH{=}CH_2$	13.5 ± 0.8 (11)	68.5 ± 4.1 (16)		1.89	1.2 ± 0.2 (4)	0.63
26	Cl	Cl	$CH(CH_2)_2$[e]	17.6 ± 2.0 (18)	92.5 ± 3.3 (14)		3.34	>30 (4)	>9
27	Cl	Cl	$CH(CH_2)_3$[f]	8.4 ± 1.7 (18)	58.1 ± 5.4 (16)		1.25	12.6 ± 2.0 (5)	10.1
28	Cl	Cl	$(CH_2)_5CH_3$	95.2 ± 4.7 (16)	ND[g]		ND[g]	>300 (4)	ND[g]
29	Cl	Br	CH_2CH_3	7.9 ± 0.7 (13)	19.7 ± 1.9 (13)	79.5 ± 3.8 (36)	0.27	3.3 ± 0.6 (6)	12.2
30	Cl	Br	$CH(CH_3)_2$	7.5 ± 0.7 (14)	25.7 ± 2.0 (15)	73.3 ± 4.2 (23)	0.26	5.6 ± 0.7 (6)	21.5
31	Cl	Br	$CH(CH_2)_3$[f]	9.5 ± 0.6 (14)	57.2 ± 5.6 (16)		1.21	6.6 ± 0.5 (6)	5.5
diazoxide	H	Cl	-	73.9 ± 4.4 (16)[a]	87.5 ± 5.0 (15)[a]		22.6[a]	22.4 ± 2.1 (11)[a]	1.0[a]
2	H	Cl	$CH(CH_3)_2$	4.9 ± 0.4 (32) [a]	36.2 ± 2.4 (31)[a]	90.4 ± 3.5 (23)[a]	0.73	36.3 ± 2.2 (6)[a]	49.7
52	H	Br	$CH(CH_3)_2$	8.1 ± 0.8 (12)[a]	34.9 ± 2.8 (12)[a]	91.0 ± 5.3 (13)[a]	0.47	4.8 ± 0.7 (5)[a]	10.2

[a] Published results (refs 32 and 40). [b] Drug concentration giving 50% inhibition of insulin release (estimated value). [c] Drug concentration giving 50% relaxation of the 30 mM KCl induced contraction of rat aorta rings (mean ± SEM (*n*)). [d] Estimated selectivity ratio. [e] Cyclopropyl. [f] Cyclobutyl. [g] ND: not determined.

Table 2. Effects of 6-Chloro-7-fluoro-Substituted, 6-Alkylamino-7-fluoro-Substituted, 6,7-Difluoro-Substituted, and 6-Chloro-7-methoxy-Substituted 3-Alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-Dioxides on 16.7 mM Glucose-Induced Insulin Release from Rat Pancreatic Islets and on Contractile Activity of K^+-Depolarized Rat Aorta Rings



32 to 51

compd				% of residual insulin release (mean ± SEM (*n*)) 10 μM	1 μM	0.1 μM	IC_{50} (pancreas),[b] μM	EC_{50} (aorta),[c] μM	EC_{50}/IC_{50} [d]
32	Cl	F	CH_2CH_3	12.7 ± 0.9 (14)	7.3 ± 0.6 (14)	73.1 ± 3.0 (24)	0.20	42.4 ± 2.8 (6)	212
33	Cl	F	$CH_2CH_2CH_3$	7.5 ± 0.6 (12)	37.6 ± 2.3 (13)	92.0 ± 4.7 (23)	0.52	45.3 ± 2.5 (5)	87.1
34	Cl	F	$CH(CH_3)_2$	7.0 ± 0.6 (14)	5.7 ± 0.4 (13)	64.4 ± 3.3 (31)	0.16	41.5 ± 1.4 (6)	259.4
35	Cl	F	$CH(CH_2)_3$[e]	12.8 ± 0.8 (13)	15.3 ± 1.4 (15)	74.5 ± 4.2 (23)	0.23	39.9 ± 1.3 (6)	173.5
36	$NH{-}CH(CH_3)_2$	F	$CH(CH_3)_2$	[70.1 ± 4.1 (20) at 50 μM]				>200 (4)	ND[f]
37	$NH{-}CH_2CH_2CH_3$	F	$CH_2CH_2CH_3$	[104.1 ± 4.1 (16) at 50 μM]				ND[f]	ND[f]
38	NH− $CH(CH_2)_3$[e]	F	$CH(CH_2)_3$[e]	[88.6 ± 3.8 (14) at 50 μM]				ND[f]	ND[f]
42	F	F	CH_2CH_3	10.5 ± 1.2 (15)	22.0 ± 0.9 (15)	94.5 ± 5.1 (16)	0.37	66.7 ± 8.5 (4)	180.3
43	F	F	$CH(CH_3)_2$	6.1 ± 0.5 (11)	22.9 ± 2.3 (12)	81.6 ± 3.4 (16)	0.30	102.4 ± 15.2 (4)	341.3
44	F	F	$CH_2CH(CH_3)_2$	42.3 ± 2.5 (15)	86.9 ± 3.2(16)		5.68	>30 (4)	>5.3
49	Cl	OCH_3	CH_2CH_3	7.2 ± 0.6 (15)	38.0 ± 4.1 (14)	85.3 ± 4.7 (21)	0.48	8.1 ± 0.9 (5)	16.9
50	Cl	OCH_3	$CH(CH_3)_2$	7.7 ± 0.7 (15)	20.6 ± 1.9 (13)	73.8 ± 4.1 (19)	0.24	37.0 ± 2.7 (6)	154.2
51	Cl	OCH_3	$CH(CH_2)_3$[e]	9.2 ± 0.8 (14)	38.1 ± 3.0 (14)	90.0 ± 4.6 (16)	0.51	23.4 ± 1.4 (5)	45.9
3	H	F	$CH(CH_3)_2$	3.7 ± 0.6 (13)[a]	47.3 ± 3.7 (23)[a]	96.9 ± 4.9 (16)[a]	0.76	43.1 ± 10.7 (5)[a]	56.7
4	H	OCH_3	$CH(CH_3)_2$	8.5 ± 0.9 (24)	67.6 ± 4.3 (20)		1.75	274.0 ± 19.0 (5)	156.6

[a] Published results (ref 40). [b] Drug concentration giving 50% inhibition of insulin release (estimated value). [c] Drug concentration giving 50% relaxation of the 30 mM KCl induced contraction of rat aorta rings (mean ± SEM (*n*)). [d] Estimated selectivity ratio. [e] Cyclobutyl. [f] ND: not determined.

replacement of the chlorine atom in the 7-position by a fluorine, a bromine atom, or a methoxy group had little impact on the effect of the drugs. Among these newly described drugs, compounds belonging to the 6-chloro-7-fluoro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide series (**32**−**35**), and more specifically the 3-ethyl (**32**), the 3-isopropyl (**34**), and the cyclobutyl (**35**) derivatives, can be considered as among the most potent inhibitors of insulin release reported to date.

The myorelaxant effect of these compounds was evaluated on the contractile activity of KCl-depolarized rat aorta rings. Results are presented in Tables 1 and 2 as the EC_{50} values, together with the EC_{50}/IC_{50} ratio, which reflects the apparent tissue selectivity (vascular versus pancreatic) of the drugs. As previously noted with 3-alkyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxides,[44] the introduction of two chlorine atoms in the 6 and 7 positions (compounds **22**−**27**) led to a sizable increase in myore-

laxant activity, in comparison with their previously reported 7-chloro-subtituted analogues,[40] except for the hindered 3-hexylamino-substituted compound (**28**). A marked enhancement in vasorelaxant activity was also noticed in the 6-chloro-7-methoxy series (**49**−**51**) (compare **50** to its 7-methoxy counterpart **4**). By contrast, in the 6-chloro-7-fluoro and in the 6-chloro-7-bromo series, the introduction of a chlorine atom in the 6-position failed to exert any obvious impact (**30** vs its 7-bromo counterpart **52** and **34** vs **3**). As reported previously with the 3-alkylamino-7-fluoro-4*H*-1,2,4-benzothiadiazine 1,1-dioxides,[40] the presence of two fluorine atoms on the benzene ring (compounds **42**−**44**) was deleterious for the myorelaxant activity of the drugs. The presence of a hindered group in the 3-position, such as *n*-hexyl (compound **28**), was also clearly unfavorable to myorelaxant activity.

Comparison of these in vitro results by means of the EC_{50}/IC_{50} ratio allows us to assess the apparent tissue selectivity (vascular versus pancreatic) of the compounds. Drugs of the 6,7-dichloro series (**22**−**27**), and more specifically the 3-ethylamino and the 3-isopropylamino derivatives (**22** and **24**), highly potent on pancreatic B-cells, were characterized by a decrease in tissue selectivity in comparison with compound **2**. The 7-bromo derivatives (**29**−**31**) expressed a moderate selectivity for the pancreatic tissue. As previously noted with monosubstituted 7-fluoro- and 7-methoxy-3-alkylaminobenzothiadiazine 1,1-dioxides,[40,42] the presence of at least one fluorine atom or one methoxy group in the 7-position of the benzothiadiazine ring led to drugs more selective for the pancreatic tissue. 6-Chloro-7-fluoro-3-ethylamino (**32**), 6-chloro-7-fluoro-3-isopropylamino (**34**), 6-chloro-7-fluoro-3-cyclobutylamino (**35**), and 6,7-difluoro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxides (**43**) were identified as the most promising compounds in terms of insulin inhibitory activity ($IC_{50} \leq 0.3\ \mu M$) and pancreatic selectivity (EC_{50}/IC_{50} ratio up to 340).

Taken as a whole, such in vitro results indicate that a halo substitution in the 6-position of the 7-substituted 3-alkylamino-1,2,4-benzothiadiazine ring yielded an increase in inhibitory activity on the insulin-releasing process, while the myorelaxant potency of the drugs was maintained or enhanced according to the nature of the substituent group in the 7-position.

Compound **34** (6-chloro-7-fluoro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide), namely, BPDZ 259, was found to be the most potent compound acting on pancreatic B-cells. Therefore, further radioisotopic and fluorimetric experiments were performed in order to characterize its mechanism of action. The drug (10 μM) provoked a rapid, sustained, and marked increase in ^{86}Rb outflow (^{42}K substitute) from prelabeled and perifused rat pancreatic islets (Figure 2). The stimulatory effect of **34** on ^{86}Rb FOR (fractional outflow rate) was reversible and totally abolished by the presence of glibenclamide (10 μM), a hypoglycemic sulfonylurea known to block K_{ATP} channels[48,49] (Figure 2). Such data indicate that BPDZ 259 induces an increase in membrane K^+ permeability through the activation of ATP-sensitive K^+ channels.[22,41,50]

Activation of K_{ATP} channels might be expected to hyperpolarize the plasma membrane and to restrict

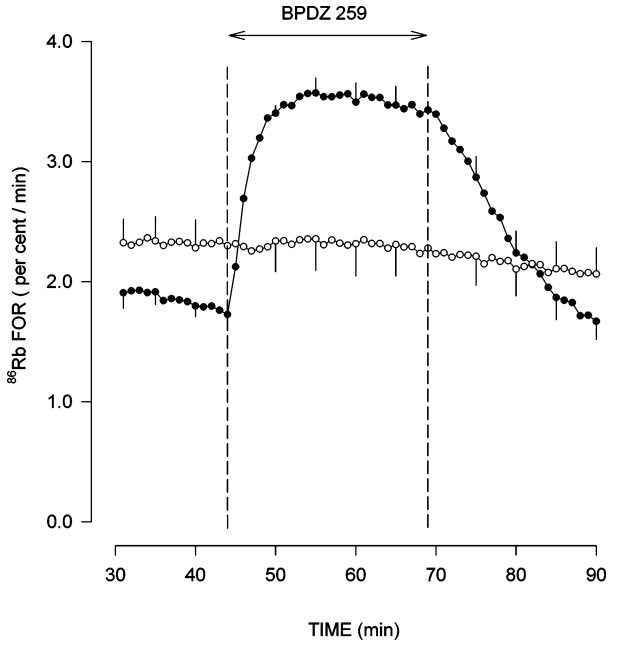


Figure 2. Effect of 10 μM BPDZ 259 (**34**) on ^{86}Rb outflow from rat pancreatic islets perifused throughout in the absence (●) or presence (○) of 10 μM glibenclamide. Basal media contained 5.6 mM glucose and extracellular Ca^{2+}. Mean values (±SEM) are from six individual experiments.

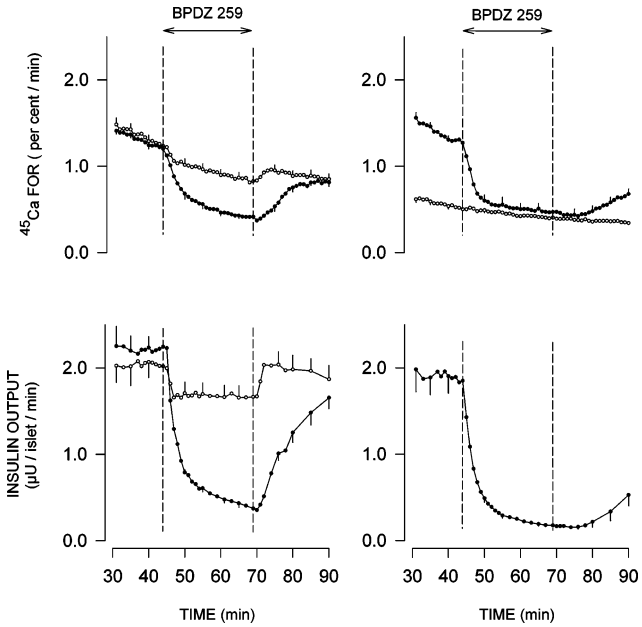


Figure 3. (Left panels) Effect of 100 nM (○) and 1 μM (●) BPDZ 259 (**34**) on ^{45}Ca outflow (upper) and insulin release (lower) from pancreatic islets perifused throughout in the presence of 16.7 mM glucose. Basal media contained extracellular Ca^{2+}. (Right panels) Effect of 10 μM BPDZ 259 (**34**) on ^{45}Ca outflow (upper) and insulin release (lower) from pancreatic islets perifused throughout in the presence of 16.7 mM glucose. Basal media contained extracellular Ca^{2+} (●) or were deprived of Ca^{2+} and enriched with EGTA (○). Mean values are from four to six individual experiments.

Ca^{2+} inflow through voltage-dependent Ca^{2+} channels. In agreement with this, Figure 3 (upper panels) clearly documents an inhibitory effect of **34** on ^{45}Ca outflow from rat pancreatic islets exposed to 16.7 mM glucose and extracellular Ca^{2+}. Under such experimental conditions, a decrease in ^{45}Ca FOR is known to result from a

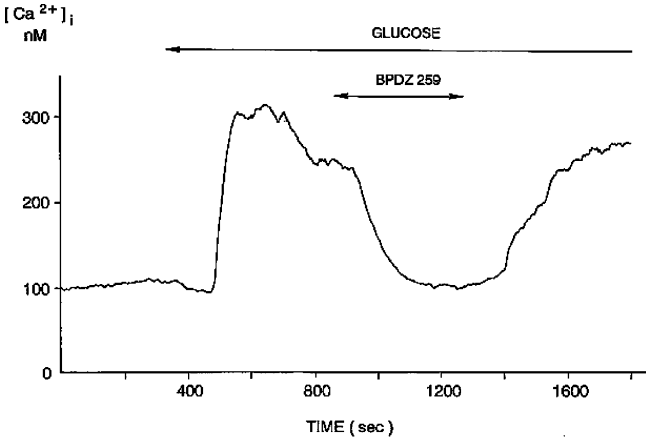


Figure 4. Effect of 1 μM BPDZ 259 (**34**) on glucose (20 mM) induced increase in $[Ca^{2+}]_i$. Basal media contained 2.8 mM glucose and extracellular Ca^{2+}. Each graph is a representative experiment conducted on a single cell.

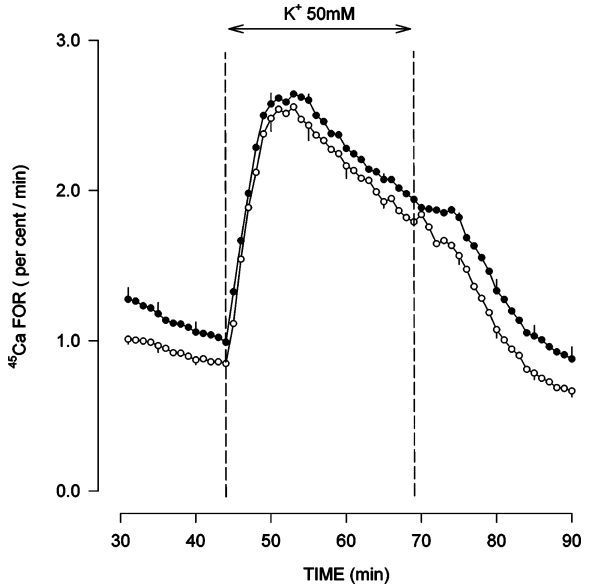


Figure 5. Effect of a rise in the extracellular K^+ concentration from 5 to 50 mM on ^{45}Ca outflow from rat pancreatic islets perifused throughout in the absence (○) or presence (●) of 10 μM BPDZ 259 (**34**). Basal media contained 2.8 mM glucose and extracellular Ca^{2+}. Mean values (±SEM) are from four individual experiments.

reduction in ^{40}Ca entry through voltage-sensitive Ca^{2+} channels.[50] This proposal is further confirmed by the lack of effect of **34** on ^{45}Ca outflow from pancreatic islets perifused in the absence of extracellular Ca^{2+} (Figure 3, right upper panel).

The inhibitory effect of **34** on ^{45}Ca FOR was sustained, reversible, and concentration-dependent. The paired difference in ^{45}Ca FOR before (40−44 min) and during (60−68 min) exposure to 100 nM, 1 μM, and 10 μM **34** averaged 0.40 ± 0.02, 0.78 ± 0.04, and 0.80 ± 0.03 %/min, respectively.

Moreover, the effect of **34** on ^{45}Ca outflow was accompanied by a concentration-dependent reduction in insulin output. The effect of **34** on the insulin-releasing process can be viewed as the result of the decrease in $^{40}Ca^{2+}$ entry because the dynamic measurement of the insulin secretory rate displayed a time course parallel to that of the ^{45}Ca FOR response.

Incidentally, the cationic and secretory responses to **34** were clearly reversible, indicating that the drug did not damage the pancreatic B-cells.

A decrease in Ca^{2+} entry as mediated by **34** should provoke a reduction in the cytosolic Ca^{2+} concentration. Such an effect of **34** is attested by calcium fluorimetry experiments, indicating that the drug was able to counteract the rise in cytosolic Ca^{2+} provoked by an insulinotropic glucose concentration (Figure 4).

Altogether, these radioisotopic and fluorimetric data suggest that in insulin-secreting cells compound **34** activates K_{ATP} channels. This in turn reduces Ca^{2+} entry, decreases the cytosolic Ca^{2+} concentration, and ultimately inhibits insulin output. Such a view is further supported by the failure of **34** to affect the increase in ^{45}Ca outflow from pancreatic islets exposed to 50 mM extracellular K^+ (Figure 5). Indeed, the ^{45}Ca response to high K^+ is known to be sensitive to Ca^{2+} channel blockers but resistant to potassium channel openers.[22,41,51]

Conclusions

Having previously explored the 7-monosubstituted 3-alkylamino-1,2,4-benzothiadiazine 1,1-dioxides as putative K_{ATP} channel openers, we synthesized and examined several 3-alkylamino derivatives substituted in the 6 and 7 positions by different halogen atoms or by a methoxy group. The effects of these compounds were characterized in two different models: rat insulin-secreting cells and rat aorta rings. The in vitro data indicate that halo-substitution in the 6-position of the 7-substituted 3-alkylamino-4*H*-1,2,4-benzothiadiazine ring enhanced the inhibitory effect on insulin release, while the myorelaxant properties of the drugs were unaffected or increased according to the nature of the substituent in the 7-position. A fluorine atom and a methoxy group were found to be substituents leading to marked tissue selectivity (pancreatic vs aortic).

Radioisotopic and fluorimetric experiments performed with the most potent drug inhibiting the insulin secretory rate, compound **34** (6-chloro-7-fluoro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide), also named BPDZ 259, confirmed that the drug activated the K_{ATP} channels. Thus, BPDZ 259, a potent and selective pancreatic K_{ATP} channel opener, might be considered as a valuable substitute for diazoxide in the treatment of glucose homeostasis disorders.

Materials and Methods

Chemistry. Melting points were determined on a Büchi-Tottoli capillary apparatus and are uncorrected. IR spectra were recorded as KBr pellets on a Perkin-Elmer 1000 FT spectrophotometer (Perkin-Elmer). The ^1H NMR spectra were taken on a Bruker AW-80 instrument, on a Bruker AM-400, and on a Bruker AM-500 (Bruker Belgium, Brussel, Belgium) in DMSO-d_6 with HMDS (hexamethyldisiloxane) or TMS (tetramethylsilane) as internal standard. Chemical shifts are reported in δ units (ppm) relative to internal HMDS. In the data presentation, s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, b = broad are used. Elemental analyses (C, H, N, S) were realized on a Carlo-Erba EA 1108-elemental analyzer and were within ±0.4% of the theoretical values. All reactions were routinely checked by TLC on Merck 60F 254 silica gel (Merck, Darmstad, Germany).

2-Amino-4,5-dichlorobenzenesulfonamide (14).[52] 3,4-Dichloroaniline (**6**, 20 g, 0.0123 mol) was added portionwise

to chlorosulfonic acid (70 mL) cooled in an ice/water bath. The suspension was then refluxed and supplemented after 1 h with thionyl chloride (20 mL). The reflux was maintained for another 30 min. After cooling, the mixture was poured on ice and extracted with AcOEt. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The crude residue of 2-amino-4,5-dichlorobenzenesulfonyl chloride (**10**) was dissolved in dioxane (50 mL) and added dropwise to a 10% w/v aqueous solution of ammonia (100 mL). After 10 min of being stirred, the solution was concentrated under reduced pressure to a small volume. The yellow-brown precipitate obtained was collected by filtration and washed with water. The crude product was suspended in water, and this suspension was adjusted to pH 12 with 2.5 M NaOH. The resulting solution was treated with charcoal and filtered. The filtrate was adjusted to pH 3−4 with concentrated HCl. The precipitate that appeared was collected by filtration, washed with water, dried, and crystallized in MeOH/water (44%). Mp 175−178 °C (lit., 175−178 °C[52]).

2-Amino-5-bromo-4-chlorobenzenesulfonamide (15).[53] 4-Bromo-3-chloroaniline (**7**, 2 g, 0.0097 mol) was added portionwise to chlorosulfonic acid (8 mL) cooled in an ice/water bath. The suspension was then heated at 150 °C for 1 h. After cooling, the mixture was poured onto ice and extracted with AcOEt. The organic layer was dried over anhydrous MgSO4, and the solvent was removed under reduced pressure. The crude residue of 2-amino-5-bromo-4-chlorobenzenesulfonyl chloride (**11**) was dissolved in dioxane (10 mL) and added dropwise to a 10% w/v aqueous solution of ammonia (50 mL). After 30 min of being stirred, the solution was treated with charcoal, filtered, and concentrated under reduced pressure to a small volume. The yellow-brown precipitate that appeared was collected by filtration, washed with water, and dried (42%). Mp 177−180 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_6H_6BrClN_2O_2S$) C, H, N, S.

2-Amino-4-chloro-5-fluorobenzenesulfonamide (16). 3-Chloro-4-fluoroaniline (**8**, 2 g, 0.0137 mol) was added portionwise to chlorosulfonic acid (6 mL) cooled on an ice/water bath. The suspension was heated at 150 °C and supplemented after 1 h with thionyl chloride (1 mL). The reflux was maintained for another 30 min. After cooling, the mixture was poured onto ice and the precipitate was filtered off. The crude residue of 2-amino-4-chloro-5-fluorobenzenesulfonyl chloride (**12**) was dissolved in dioxane (10 mL) and added dropwise to a 10% w/v aqueous solution of ammoniac (50 mL). After the mixture was stirred for 30 min, concentration under reduced pressure to a small volume gave rise to a precipitate of the title compound (45%). Mp 178−180 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_6H_6ClFN_2O_2S$) C, H, N, S.

2-Amino-4,5-difluorobenzenesulfonamide (17). 3,4-Difluoroaniline (**9**, 8.7 g, 0.067 mol) was added portionwise to chlorosulfonic acid (26 mL) cooled on an ice/water bath. The mixture was refluxed for 90 min. After cooling, the mixture was supplemented with thionyl chloride (10 mL) and refluxed for another 90 min. The mixture was poured onto ice and extracted with AcOEt. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The crude residue of 2-amino-4,5-difluorobenzenesulfonyl chloride (**13**) was dissolved in dioxane (30 mL) and added dropwise to a 10% w/v aqueous solution of ammonia (100 mL). After the mixture was stirred for 30 min, concentration under reduced pressure to a small volume gave rise to a precipitate of the title compound (57%). Mp 129−130 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_6H_6F_2N_2O_2S$) C, H, N, S.

6,7-Dichloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (18). The title compound was obtained as described in the literature,[46] starting from 2-amino-4,5-dichlorobenzenesulfonamide (**14**).

7-Bromo-6-chloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (19). 2-Amino-5-bromo-4-chlorobenzenesulfonamide (**15**, 0.2 g, 0.7 mmol) and 1,1′-thiocarbonyldiimidazole (0.45 g, 0.0025 mol) were dissolved in dioxane (3 mL). The mixture was heated under reflux for 2 h. The solvent was removed under reduced pressure, and the residue was triturated with water. The resulting solution was adjusted to pH 12 with 2.5 M NaOH, treated with charcoal, and filtered. The filtrate was adjusted to pH 3−4 with concentrated HCl. The precipitate that appeared was collected by filtration, washed with water, and crystallized in acetone/*n*-hexane (59%). Mp 261−264 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{10}H_6BrClN_4O_2S$) C, H, N, S.

6-Chloro-7-fluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide Monohydrate (20). 2-Amino-4-chloro-5-fluorobenzenesulfonamide (**16**, 4 g, 0.0178 mol) and 1,1′-thiocarbonyldiimidazole (8.84 g, 0.05 mol) were dissolved in dioxane (60 mL). The mixture was heated under reflux for 3 h. The solvent was removed under reduced pressure, and the residue was triturated with water. The resulting solution was adjusted to pH 12 with 2.5 M NaOH, treated with charcoal, and filtered. The filtrate was adjusted to pH 3−4 with concentrated HCl. The precipitate that appeared was collected by filtration, washed with water, and dried (48%). Mp 271−275 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{10}H_6ClFN_4O_2S\cdot H_2O$) C, H, N, S.

6,7-Difluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (21). 2-Amino-4,5-difluorobenzenesulfonamide (**17**, 5.5 g, 0.026 mol) and 1,1′-thiocarbonyldiimidazole (15 g, 0.084 mol) were dissolved in dioxane (50 mL). The mixture was heated under reflux for 4 h. The solvent was removed under reduced pressure, and the residue was triturated with water. The resulting solution was adjusted to pH 3−4 with 12 M HCl, and the precipitate that appeared was collected by filtration, washed with water, dried, and crystallized in acetone/diethyl ether (35%). Mp 264−266 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{10}H_6F_2N4O_2S$) C, H, N, S.

6,7-Dichloro-3-(ethylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (22). A mixture of 6,7-dichloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**18**, 0.5 g, 0.001 57 mol) and a 70% w/v aqueous solution of ethylamine (5 mL) was heated in a sealed vessel for 5 h at 150 °C. After the mixture was cooled, the excess amine was eliminated by distillation under reduced pressure. The residue was suspended in water and stirred for 1 h. The precipitate was collected by filtration, washed with water, dried, and crystallized in MeOH/water (60%). Mp 308−310 °C (lit., 302−304 °C[53]); IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_9H_9Cl_2N_3O_2S$) C, H, N, S.

6,7-Dichloro-3-(propylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide Monohydrate (23). The title compound was obtained as described for **22**, starting from 6,7-dichloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**18**, 0.5 g, 0.001 57 mol) and propylamine (5 mL). The final product was crystallized in MeOH/water (65%). Mp >300 °C (lit., 314−315 °C[53]); IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz) Anal. ($C_{10}H_{11}Cl_2N_3O_2S\cdot H_2O$) C, H, N, S.

6,7-Dichloro-3-(isopropylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide Monohydrate (24). The title compound was obtained as described for **22** starting from 6,7-dichloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**18**, 0.5 g, 0.001 57 mol) and isopropylamine (5 mL). The final product was crystallized from hot MeOH (58%). Mp 296−298 °C (lit., 289−291 °C[53]); IR (KBr); ^{1}H NMR (DMSO-d_6, 80 MHz). Anal. ($C_{10}H_{11}Cl_2N_3O_2S\cdot H_2O$) C, H, N, S.

3-Allylamino-6,7-dichloro-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (25). A mixture of 6,7-dichloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**18**, 0.5 g, 0.00157 mol) and allylamine (5 mL) was heated in a sealed vessel for 3 h at 140 °C. The excess amine was eliminated by distillation under reduced pressure. The residue was suspended in water, and the pH was adjusted to 3−4 with concentrated HCl. The precipitate was collected by filtration, washed with water, dried, and crystallized in MeOH/water (29%). Mp 298−301 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{10}H_9Cl_2N_3O_2S$) C, H, N, S.

3-Cyclopropylamino-6,7-dichloro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (26). A mixture of 6,7-dichloro-3-(1*H*-

imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**18**, 0.5 g, 0.001 57 mol) and cyclopropylamine (5 mL) was heated in a sealed vessel for 5 h at 150 °C. The excess amine was eliminated by distillation under reduced pressure. The residue was suspended in water, and the pH was adjusted to 12 with 2.5 M NaOH. The solution was treated with charcoal and filtered, and the pH of the filtrate was adjusted to pH 3−4 with concentrated HCl. The precipitate that appeared was collected by filtration, washed with water, dried, and crystallized in hot MeOH (62%). Mp 295−297 °C (lit., 282−285 °C[53]); IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{10}H_9Cl_2N_3O_2S$) C, H, N, S.

3-Cyclobutylamino-6,7-dichloro-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (27). 6,7-Dichloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**18**, 0.5 g, 0.001 57 mol) was dissolved in dioxane (4 mL) and supplemented with cyclobutylamine (1 mL). The mixture was heated in a sealed vessel for 8 h at 140 °C. The excess amine was eliminated by distillation under reduced pressure. The residue was suspended in water (20 mL), and the pH was adjusted to 12 with 2.5 M NaOH. The solution was treated with charcoal and filtered, and the pH was adjusted to pH 3−4 with concentrated HCl. The precipitate was collected by filtration, washed with water, dried, and crystallized in hot MeOH (60%). Mp 320−326 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{11}H_{11}Cl_2N_3O_2S$) C, H, N, S.

6,7-Dichloro-3-(hexylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (28). A mixture of 6,7-dichloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**18**, 0.5 g, 0.001 57 mol) and *n*-hexylamine (5 mL) was refluxed for 4 h. The excess amine was eliminated by distillation under reduced pressure. The residue was suspended in water and stirred for 1 h. The precipitate was collected by filtration, washed with water, dried, and crystallized in hot MeOH (64%). Mp 282−286 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{13}H_{17}Cl_2N_3O_2S$) C, H, N, S.

7-Bromo-6-chloro-3-(ethylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (29). The title compound was obtained as described for **22**, starting from 7-bromo-6-chloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**19**, 0.5 g, 0.001 38 mol) and an aqueous solution of ethylamine (70%, 5 mL). The final product was crystallized from hot MeOH (60%). Mp >290 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 80 MHz). Anal. ($C_9H_9BrClN_3O_2S$) C, H, N, S.

7-Bromo-6-chloro-3-(isopropylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (30). The title compound was obtained as described for **22**, starting from 7-bromo-6-chloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**19**, 0.5 g, 0.001 38 mol) and isopropylamine (5 mL) (62%). IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Mp >290 °C. Anal. ($C_{10}H_{11}BrClN_3O_2S$) C, H, N, S.

7-Bromo-6-chloro-3-(cyclobutylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (31). The title compound was obtained as described for **27**, starting from 7-bromo-6-chloro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**19**, 0.5 g, 0.001 38 mol) and cyclobutylamine (1 mL) in dioxane (5 mL) (58%). Mp >290 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{11}H_{11}BrClN_3O_2S$) C, H, N, S.

6-Chloro-3-(ethylamino)-7-fluoro-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (32). The title compound was obtained as described for **22**, starting from 6-chloro-7-fluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**20**, 0.5 g, 0.001 67 mol) and a 70% w/v aqueous solution of ethylamine (5 mL) (65%). Mp >290 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_9H_9ClFN_3O_2S$) C, H, N, S.

6-Chloro-7-fluoro-3-(propylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (33). The title compound was obtained as described for **22**, starting from 6-chloro-7-fluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**20**, 0.5 g, 0.001 67 mol) and propylamine (5 mL) (59%). Mp 287−289 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{10}H_{11}ClFN_3O_2S$) C, H, N, S.

6-Chloro-7-fluoro-3-(isopropylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (34). The title compound was obtained as described for **22**, starting from 6-chloro-7-fluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**20**, 0.5 g, 0.001 67 mol) and isopropylamine (5 mL) (59%). Mp 228−232 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 80 MHz). Anal. ($C_{10}H_{11}ClFN_3O_2S$) C, H, N, S.

6-Chloro-3-(cyclobutylamino)-7-fluoro-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (35). The title compound was obtained as described for **27**, starting from 6-chloro-7-fluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**20**, 0.5 g, 0.001 67 mol) and cyclobutylamine (1 mL) in dioxane (5 mL) (55%). Mp >290 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{11}H_{11}ClFN_3O_2S$) C, H, N, S.

7-Fluoro-3,6-di(isopropylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (36). The title compound was obtained as described for **22**, starting from 6,7-difluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**21**, 0.5 g, 0.001 76 mol) and isopropylamine (5 mL) (65%). Mp 234−237 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 80 MHz). Anal. ($C_{13}H_{19}FN_4O_2S$) C, H, N, S.

7-Fluoro-3,6-di(propylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (37). The title compound was obtained as described for **22**, starting from 6,7-difluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**21**, 0.5 g, 0.001 76 mol) and *n*-propylamine (5 mL) (62%). Mp 236 °C, then 256 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{13}H_{19}FN_4O_2S$) C, H, N, S.

3,6-Di(cyclobutylamino)-7-fluoro-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (38). The title compound was obtained as described for **27**, starting from 6,7-difluoro-3-(1*H*-imidazol-1-yl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**21**, 0.5 g, 0.001 76 mol) and cyclobutylamine (1 mL) in dioxane (5 mL) (45%); IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Mp 258 °C, then 297 °C. Anal. ($C_{15}H_{19}FN_4O_2S$) C, H, N, S.

***N*-Ethyl-*N*′-(2-amino-4,5-difluorobenzenesulfonyl)thiourea (39).** 2-Amino-4,5-difluorobenzenesulfonamide (**17**, 0.4 g, 0.0019 mol) was dissolved in dry acetone (3 mL) and supplemented with K_2CO_3 (0.32 g) and ethyl isothiocyanate (0.3 mL). The mixture was heated at 60 °C for 4 h. The solvent was removed under reduced pressure, and the residue was suspended in water (25 mL). The solution was adjusted to pH 3−4 with concentrated HCl and stirred at room temperature for a few hours. The precipitate was collected by filtration, washed with water, and dried. The crude product was used without further purification (52%).

***N*-Isopropyl-*N*′-(2-amino-4,5-difluorobenzenesulfonyl)-thiourea (40).** The title compound was obtained as described for **39**, starting from 2-amino-4,5-difluorobenzenesulfonamide (**17**) and isopropyl isothiocyanate. The crude product was used without further purification (48%).

***N*-Isobutyl-*N*′-(2-amino-4,5-difluorobenzenesulfonyl)-thiourea (41).** The title compound was obtained as described for **39**, starting from 2-amino-4,5-difluorobenzenesulfonamide (**17**) and isobutyl isothiocyanate. The crude product was used without further purification (37%).

6,7-Difluoro-3-(ethylamino)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (42). *N*-Ethyl-*N*′-(2-amino-4,5-difluorobenzenesulfonyl)thiourea (**39**, 0.2 g, 0.68 mmol) and triethylamine (0.2 mL) were dissolved in dry THF (5 mL). The mixture was cooled in an ice/water bath and slowly supplemented with a 20% phosgene solution in toluene (0.5 mL). After 2 h at 0 °C, the solvent was removed under reduced pressure and the residue was triturated with water, adjusted to pH 12 with 2.5 M NaOH, treated with charcoal, and filtered. The filtrate was adjusted to pH 3−4 with concentrated HCl, and the precipitate that appeared was collected by filtration, washed with water, and dried (75%). Mp 246−252 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_9H_9F_2N_3O_2S$) C, H, N, S.

6,7-Difluoro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (43). The title compound was obtained as described for **42**, starting from 2*N*-isopropyl-*N*′-(2-amino-4,5-difluorobenzenesulfonyl)thiourea (**40**) (68%). Mp 242−244 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{10}H_{11}F_2N_3O_2S$) C, H, N, S.

6,7-Difluoro-3-isobutylamino-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (44). The title compound was obtained as described for **42**, starting from 2*N*-isobutyl-*N*′-(2-amino-4,5-difluorobenzenesulfonyl)thiourea (**41**) (65%). Mp >270 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{11}H_{13}F_2N_3O_2S$) C, H, N, S.

6-Chloro-7-methoxy-3-oxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (46). Chlorosulfonyl isocyanate (5 mL, 0.057 mol) and nitroethane (50 mL) were mixed together in a closed, dried vessel. The mixture was cooled at −40 °C (acetone and carbogene) and protected from moisture during the slow addition, under vigorous stirring, of 3-chloro-4-methoxyaniline (**45**, 4.8 g, 0.03 mol) dissolved in nitroethane (10 mL). At the end of the addition, anhydrous $AlCl_3$ (5 g, 0.0375 mol) was added to the resulting suspension and the mixture was heated at 110 °C for 20 min. The hot solution was poured onto ice (200 g), and after the mixture was stirred and after complete melting of the ice, the resulting precipitate was collected by filtration and dissolved in an aqueous solution of sodium hydrogenocarbonate (5 g/150 mL). The solution was treated with charcoal and filtered, and the filtrate was adjusted to pH 1 by means of 12 N HCl. The white precipitate that appeared was collected by filtration, washed with water, and dried (28%). Mp 277−279 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_8H_7ClN_2O_4S$) C, H, N, S.

6-Chloro-7-methoxy-3-thioxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (47). A mixture of 6-chloro-7-methoxy-3-oxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (**46**, 2.62 g, 0.01 mol) and phosphorus pentasulfide (4.5 g) in anhydrous pyridine (25 mL) was refluxed for 3 h. The solvent was removed under reduced pressure, and the residue was dissolved in an aqueous solution of NaOH (5 g/100 mL). The solution was treated with charcoal and filtered, and the filtrate was adjusted to pH 1 by means of 12 N HCl. The precipitate was collected by filtration, washed with water, and suspended in an aqueous solution of sodium hydrogenocarbonate (3 g/100 mL). The suspension was heated until most of the insoluble material dissolved and was then treated with charcoal and filtered. The filtrate was adjusted to pH 1 with 12 N HCl, and the precipitate was collected by filtration, washed with water, and dried. Mp 233−235 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_8H_7ClN_2O_3S_2$) C, H, N, S.

6-Chloro-7-methoxy-3-methylsulfanyl-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide Monohydrate (48). 6-Chloro-7-methoxy-3-thioxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (**47**, 0.47 g, 0.0017 mol) was dissolved in a hydromethanolic 1:1 solution of sodium hydrogenocarbonate (1 g/10 mL), and an excess of methyl iodide (1.8 mL) was added. After 30 min of being stirred, the resulting suspension was adjusted to pH 5−6 by means of 6 N HCl. The suspension was concentrated under reduced pressure to half of the volume, and the white precipitate was collected by filtration, washed with water, and dried. Mp 271−275 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_9H_9ClN_2O_3S_2\cdot H_2O$) C, H, N, S.

6-Chloro-3-(ethylamino)-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (49). A mixture of 6-chloro-7-methoxy-3-methylsulfanyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide monohydrate (**48**, 0.5 g,0.017 mol) and a 70% w/v aqueous solution of ethylamine (5 mL) was heated in a sealed vessel for 4 h at 120 °C. The excess amine was eliminated by distillation under reduced pressure, and the residue was dissolved in an aqueous 2% w/v solution of NaOH (20 mL). The alkaline solution was treated with charcoal and was filtered, and the filtrate was adjusted to pH 4−5 with concentrated HCl. The precipitate was collected by filtration, washed with water, and dried. The compound was recrystallized in methanol/water (75%). Mp >300 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{10}H_{12}ClN_3O_3S$) C, H, N, S.

6-Chloro-3-isopropylamino-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (50). The title compound was obtained as described for **49**, starting from 6-chloro-7-methoxy-3-methylsulfanyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide monohydrate (**48**) and isopropylamine (70%). Mp 281−283 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 80 MHz). Anal. ($C_{11}H_{14}ClN_3O_3S$) C, H, N, S.

6-Chloro-3-cyclobutylamino-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (51). The title compound was obtained as described for **49**, starting from 6-chloro-7-methoxy-3-methylsulfanyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide monohydrate (**48**) and cyclobutylamine (1 mL) in dioxane (5 mL) (62%). Mp >284 °C; IR (KBr); ^{1}H NMR (DMSO-d_6, 400 MHz). Anal. ($C_{12}H_{14}ClN_3O_3S$) C, H, N, S.

Biological Assays. Measurement of Insulin Release from Incubated Rat Pancreatic Islets. Pancreatic islets were isolated by the collagenase method from fed Wistar rats (180−220 g). Groups of 10 islets, each derived from the same batch of islets, were preincubated for 30 min at 37 °C in 1 mL of a physiological salt medium (in mM: NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24), supplemented with 2.8 mM glucose and 0.5% (w/v) dialyzed albumin (fracteom V, Sigma), and equilibrated against a mixture of O_2 (95%) and CO_2 (5%).

The islets were then incubated at 37 °C for 90 min in 1 mL of the same medium containing 16.7 mM glucose and the reference compound or the benzothiadiazine derivative. The release of insulin was measured radioimmunologically using rat insulin as a standard.[41]

Residual insulin release was expressed as a percentage of the value recorded in control experiments (100%), i.e., in the absence of drug and presence of 16.7 mM glucose.

Measurement of the Contractile Activity in Rat Aorta. All experiments were performed with aortae removed from fed Wistar rats (180−220 g). A section of the aorta was cleared of adhering fat and connective tissue and was cut into transverse rings (3−4 mm long). The endothelium was removed by rubbing the intimal surface with forceps. The segments were suspended under 1.5 g of tension by means of steel hooks in an organ bath containing 20 mL of a Krebs bicarbonate buffered solution of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5. The physiological solutions were maintained at 37 °C and bubbled continuously with a mixture of O_2 (95%) and CO_2 (5%). The isometric contractions of the aortic rings were measured with a force-displacement transducer. After 60 min of equilibration, the rings were exposed to 30 mM KCl. When the tension had stabilized, the drugs were added to the bath at increasing concentrations until maximal relaxation (or until 0.3 mM). The relaxation response was expressed as the percentage of the contractile response to KCl. The ED_{50} values (drug concentration evoking 50% inhibition of the plateau phase induced by KCl) were assessed from dose−response curves using Datanalyst software (EMKA Technologies, France).[55]

Measurement of ^{86}Rb and ^{45}Ca Outflow and Insulin Release from Perifused Rat Pancreatic Islets. Experiments were performed with pancreatic islets isolated from fed Wistar rats (180−220 g). The media used for incubating, washing, and perifusing the islets consisted of a physiological salt medium (in mM: NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24) supplemented with 0.5% (w/v) dialyzed albumin (fraction V, Sigma) and gassed with O_2 (95%)/CO_2 (5%).

The methods used to measure ^{86}Rb (^{42}K substitute) outflow, ^{45}Ca outflow, and insulin release from perifused pancreatic islets have been described previously.[22,51] Briefly, groups of 100 islets were incubated for 60 min in the physiological medium containing 16.7 mM glucose and either ^{86} Rb (0.15−0.25 mM, 50 μCi/mL) or ^{45}Ca (0.02−0.04 mM, 100 μCi/mL). After incubation, the islets were washed four times with a nonradioactive medium and then placed in a perifusion chamber. The perifusate was delivered at a constant rate (1.0 mL/min). From 31 to 90 min, the effluent was continuously collected over successive periods of 1 min each. An aliquot of the effluent (0.5 mL) was used for scintillation counting, while the remainder was stored at −20 °C for insulin radioimmunoassay. At the end of the perifusion, the radioactive content of the islets was also determined. The outflow of ^{86}Rb or ^{45}Ca (cpm/min) was expressed as a fractional outflow rate (% of instantaneous

islet content/min, FOR). Some media contained no $CaCl_2$ and were enriched with 0.5 mM EGTA (Sigma).

When high concentrations (50 mM) of extracellular K^+ were used, the concentration of extracellular NaCl was lowered to keep the osmolarity constant.

BPDZ 259 was dissolved in dimethyl sulfoxide, which was added to both control and test media at final concentrations not exceeding 0.1% (v/v).

Results are expressed as the mean (±SEM) together with the number of individual experiments (n). The inhibitory effect of BPDZ 259 on ^{45}Ca outflow and insulin release from islets perifused in the presence of 16.7 mM glucose was taken as the difference between the mean value for ^{45}Ca outflow or insulin output recorded in each individual experiment between the 40−44 and 60−68 min of perifusion.

The magnitude of the increase in ^{45}Ca outflow was estimated in each individual experiment from the integrated outflow of ^{45}Ca observed during stimulation (45−68 min) after correction for the basal value (40−44 min).

Measurement of Cytosolic Ca^{2+} Concentration from Single Rat Pancreatic Islet Cells. Pancreatic islets were disrupted in a Ca^{2+}-deprived medium and then centrifuged through an albumin solution to remove debris and dead cells. Cells were seeded onto glass coverslips and maintained in tissue culture for 72 h before use. The cells were then incubated with fura-2 AM (2 μmol/L) (Molecular Probes) for 1 h. The medium used to perifuse the cells contained the following (in mM): NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24, glucose 2.8. The medium was gassed with O_2 (95%)/CO_2 (5%). Fura-2 fluorescence of single-loaded cells was measured by use of dual-excitation microfluorimetry with a Spex photometric system (Optilas, Alphen aan den Rijn, Holland). The excitation and emission wavelengths were set at 340/380 and 510 nm, respectively.

$[Ca^{2+}]_i$ was calculated as previously described.[55] The experiment was repeated with different cell populations.

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Supporting Information Available: Elemental analysis results and IR and ^1H NMR spectra of the newly synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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