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Synthesis and Vasorelaxant Activity of New 1,4-benzoxazine Derivatives Potassium Channel Openers

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Abstract—As part of a search for new potassium channel openers, the synthesis and vasorelaxant activity of new 1,4-benzoxazine derivatives derived from transformation of the benzopyran skeleton of cromakalim were described. Several new 1,4-benzoxazine derivatives were provided with significant vasorelaxant activity with an overall pharmacological behavior similar to CRK (**1f**, **1i**, **2d**, **2e**, **2f** and **2i**).

Introduction

Potassium channel openers are recently discovered as a novel class of compounds.[1,2] Their pharmacological action involves the relaxation of smooth muscle by the opening of potassium channels, suggesting a number of therapeutic targets for this class of compounds such as hypertension, angina pectoris, coronary heart disease, asthma, urinary incontinence and alopecia.[3–6] Additionally, these agents may afford cells a measure of protection against ischemia, independent of their vasodilating actions[7] and have antilipidemic effects, lowering low-density lipoprotein (LDL) cholesterol and triglycerides while increasing high density lipoprotein (HDL) cholesterol.[8]

There are several prototypes of this class of compounds with a diverse range of chemical structure such as cromakalim,[9] pinacidil,[10] nicorandil,[11] and aprikalim[12] that act as openers of ATP-dependent potassium channel (K_{ATP}). Among these compounds, many structural modification studies have focused on cromakalim, a benzopyran derivative, because it possesses the most potent activity. As part of a program to develop new compounds by modifying cromakalim, the synthesis and the biological activity of a new series of 2-[3,4-dihydro-3-oxo-2H-1,4-benzoxazin-4-yl]-ethylacetate derivatives, which exhibited a good channel opening activity, was previously reported.[13]

On the basis of these results we decide to evaluate the effect on their activity by introducing different alkyl chain between benzoxazine nucleus and the ester group. These modifications provided the new 1,4-benzoxazine-ethyl propionate (**1**) and ethylbutyrate derivatives (**2**) (Table 1). The substituents inserted at the 6-position of the 1,4-benzoxazine ring are those previously reported displayng a good discrimination between electronic and hydrophobic properties. In this way we could more easily compare the effects of the different alkyl chain on biological activity.

The pharmacological study was performed to investigate firstly a vasorelaxant activity of new compounds and then to elucidate a putative mechanism of action using pharmacological tools. For this purpose, we performed in vitro assays using rat thoracic aorta rings precontracted with phenylephrine (PE). Since several outward potassium channels have been identified such as ATP-dependent potassium channels (K_{ATP}), calcium-dependent potassium channel (K_{Ca}), voltage-dependent potassium channels (K_V), we tested the active analogues

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in presence of selective channel blockers. We used Glibenclamide (GLY) for the K_{ATP}, tetraethylammonium (TEA) for the K_{Ca}, and 4-aminopyridine (4-AMP) as unselective potassium channel blockers. Compounds that showed a vasorelaxation higher than 50% but with a different behavior to CRK were tested to investigated on probable calcium blocking activity.

Chemistry

The synthesis strategy for the preparation of the new 1,4-benzoxazine derivatives **1** and **2** was outlined in Scheme 1. The starting compounds 2-amino-4-substituted-phenols (**3**) for the synthesis of general compounds (**5**) were commercially available, only the 2-amino-4-cyanophenol was obtained by a procedure previously reported.[13] Acylation of 2-amino-4-X-phenol (**3**) with bromoacetyl bromide or 2-bromoisobutyryl bromide in $CHCl_3$ in the presence of sodium bicarbonate gave the corresponding amides **4**. Cyclization in the presence of potassium carbonate in DMF afforded benzoxazine derivatives **5**.

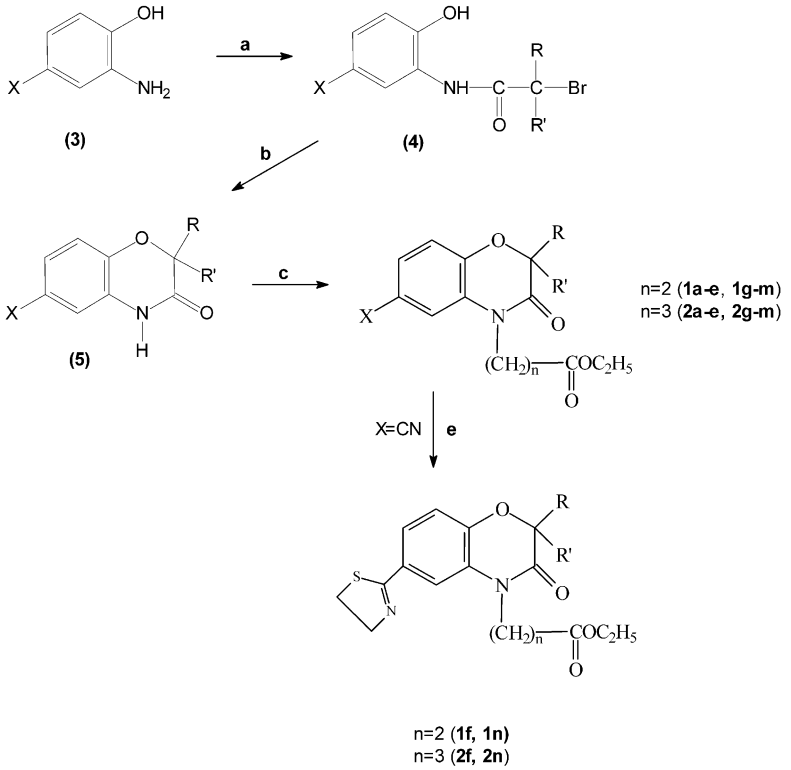
These latters were converted into the desired compounds **1a–e**, **1g–m** and 2**a–e, 2g–m** by condensation with ethyl-3-bromopropionate (series **1**) or ethyl-4-bromobutyrate (series **2**) in sodium hydride/DMF at room temperature.

The 6-(Δ^2-thiazolin-2-yl) derivatives **1f**, **1n, 2f** and **2n** were obtained by reaction of the corresponding 6-cyano derivatives **1e**, **1m, 2e** and **2m** respectively, with stoichiometric amounts of 2-aminoethanethiol hydrochloride in absolute ethanol and triethylamine solution. All products were isolated by column chromatography, further purified by crystallization from appropriate solvents and characterized by ^{1}H NMR spectroscopy and GLC-MS. Structures, physicochemical data and % of relaxation activity of all synthesized compounds was summarized in Table 1.

Results and Discussion

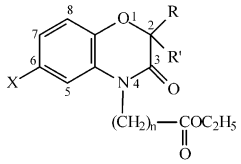
The vasorelaxant activities of compounds (series **1** and **2**) were evaluated in vitro using rat aorta rings pre-contracted with PE (1 μM) in a concentration range 1–100 μM. Results obtained with compounds at concentration of 100 μM, expressed as percentage of relaxation, are summarized in Table 1. Data indicate that several of the new ester derivatives are provided with significant vasorelaxant activity profile.

Figure 1 shows the concentration–response curves of compounds, which exhibited a vasorelaxation activity higher than 50%. Vasorelaxations induced by the active compounds are concentration-dependent. The concentration–response curve of analyzed compounds was not superimposable to those obtained with cromakalim. Indeed, all the active compounds show an EC_{50} (μM) higher than cromakalim (Table 2). Cromakalim (0.1–100 μM), employed as reference drug, produced a relaxation of 83.1 ± 2% ($n = 20$) when concentration of



Scheme 1. Reagents: (a) bromoacethyl bromide or 2-bromoisobutyryl bromide, $NaHCO_3$, $CHCl_3$, room temperature; (b) anhydrous K_2CO_3, DMF, 80 °C; (c) ethyl-3-bromopropionate or ethyl 4-bromobutyrate, NaH, DMF, room temperature; (d) 2-aminoethanethiol hydrochloride, triethylamine/absolute EtOH, reflux.

Table 1. Physicochemical properties and vasorelaxant activity of 1,4-benzoxazine derivatives (**1a**–**n**) and (**2a**–**n**).



Compd	X	*n*	R = R′	Formula[a]	M_r	Yield (%)	Mp (°C)	Recrystallization solvents[b]	MS (*m*/*z*)	% of relaxation (10^{-4} M)
1a	H	2	H	$C_{13}H_{15}NO_4$	249.26	45	Oil	—	249; 220; 205; 177	12.3
1b	CH_3	2	H	$C_{14}H_{17}NO_4$	263.29	47	42–44	a + b	263; 218; 188; 91	14.3
1c	Cl	2	H	$C_{13}H_{14}ClNO_4$	283.71	50	87–88	a + d	283; 183; 174	42.7
1d	NO_2	2	H	$C_{13}H_{14}N_2O_6$	294.26	65	103–104	a + b	294; 193; 165; 107	43.4
1e	CN	2	H	$C_{14}H_{14}N_2O_4$	274.26	38	85–87	b + c	274; 159; 145	25.9
1f		2	H	$C_{16}H_{18}N_2O_4S$	334.37	63	96–97	a + b	334; 289; 261; 174	87.1
1g	H	2	CH3	$C_{15}H_{19}NO_4$	277.27	73	Oil	—	277; 234; 162; 134	29.7
1h	CH_3	2	CH3	$C_{16}H_{21}NO_4$	291.34	45	Oil	—	291; 248; 230; 176	28.3
1i	Cl	2	CH3	$C_{15}H_{18}ClNO_4$	311.76	71	Oil	—	311; 211; 196; 101	63.2
1l	NO_2	2	CH3	$C_{15}H_{18}N_2O_6$	322.31	40	66–67	a + b	322; 220; 187	20.5
1m	CN	2	CH3	$C_{16}H_{18}N_2O_4$	302.35	41	63–64	a + c	302; 187; 173	43.0
1n		2	CH3	$C_{18}H_{22}N_2O_4S$	362.44	35	92–93	a	362; 307; 279; 192	69.0
2a	H	3	H	$C_{14}H_{17}NO_4$	263.29	43	41–43	b	263; 218; 190; 134	48.0
2b	CH_3	3	H	$C_{15}H_{19}NO_4$	277.30	54	39–40	b	277; 204; 148; 29	42.0
2c	Cl	3	H	$C_{14}H_{16}ClNO_4$	297.72	55	66–67	a	297; 197; 177; 88	69.5
2d	NO_2	3	H	$C_{14}H_{16}N_2O_6$	308.27	59	94–96	a	308; 207; 179; 121	71.1
2e	CN	3	H	$C_{15}H_{16}N_2O_4$	288.30	68	92–94	a	288; 201; 187; 145	62.8
2f		3	H	$C_{17}H_{20}N_2O_4S$	348.24	60	108–109	a	348; 261; 174; 115	73.7
2g	H	3	CH3	$C_{16}H_{21}NO_4$	291.33	53	69–71	a + b	291; 230; 177; 162	77.3
2h	CH_3	3	CH3	$C_{17}H_{23}NO_4$	305.37	30	Oil	—	305; 232; 176; 87	81.8
2i	Cl	3	CH3	$C_{16}H_{20}ClNO_4$	325.77	55	79–81	e	325; 225; 205; 110	70.7
2l	NO_2	3	CH3	$C_{16}H_{20}N_2O_6$	336.32	48	88–89	a + b	336; 236; 208; 140	56.9
2m	CN	3	CH3	$C_{17}H_{20}N_2O_4$	316.35	80	89–90	a + b	316; 229; 180; 124	80.0
2n		3	CH3	$C_{19}H_{24}N_2O_4S$	376.48	58	60–61	a	376; 289; 247; 202	83.9

[a]All compounds were analyzed for C, H, Cl, N and S and the analytical results were within ±0.4% of the calculated values for the formulae shown.
[b]Crystallization solvents: (a) diethyl ether; (b) *n*-hexane; (c) ethyl alcohol; (d) dichloromethane; (e) petroleum ether.

100 μM was reached, while the EC_{50} calculated was 0.4 μM and 0.25–0.65 as 95% confidence limits, hence the considered compounds are less active than cromakalim.

The 1,4-benzoxazine ethylbutyrate derivatives (series **2**) resulted generally more actives than corresponding ethylpropionate derivatives (series **1**). In fact only two compounds of series **2** produced vasorelaxation less than 50% while nine compounds of series **1** showed a relaxation less than 50%. These results shows that the length of the alkylchain plays an important role in the vasorelaxant activity and allowed us to conclude that the propylene chain was the better to furnish analogues with a good activity.

Subsequently, we attempted the modification of the nature of the substituents at the 6-position on the 1,4-benzoxazine ring. Analysis of the structures endowed with highest vasorelaxant activity suggest that a thiazoline ring is favorable for the activity (**1f**, **1n**, **2f**, **2n**). This finding is consistent with data relative to other analogue derivatives.[13] Note compounds **2g** and **2h** with a methyl or no substituent also exhibited high value of vasorelaxant activity (81.8 and 77.3%, respectively). Moreover in the serie **1** the two methyl groups at the 2-position of the 1,4-benzoxazine ring did not seem greatly influenced on the vasorelaxant activity. However, in the series **2** the 2,2-dimethyl derivatives (**2g**–**2n**) revealed to be more active than their corresponding demethyl derivatives (**2a**–**2f**) except for **2l** that showed a low vasorelaxant activity than corresponding demethyl derivative **2d**.

To evaluate the activity of these compounds as putative openers of potassium channels were performed experiments using Glibenclamide (GLY; 10 μM) and tethraethylammonium (TEA, 1 mM) as inhibitors of ATP- and calcium- potassium channels respectively and 4-aminopyridine (4-AMP, 10 mM) as unselective inhibitor. Experiments with the inhibitors above stated, were performed only for compounds that produced vasorelaxation greater than 50%. In Table 3 are summarized all the results obtained for series **1** and **2**.

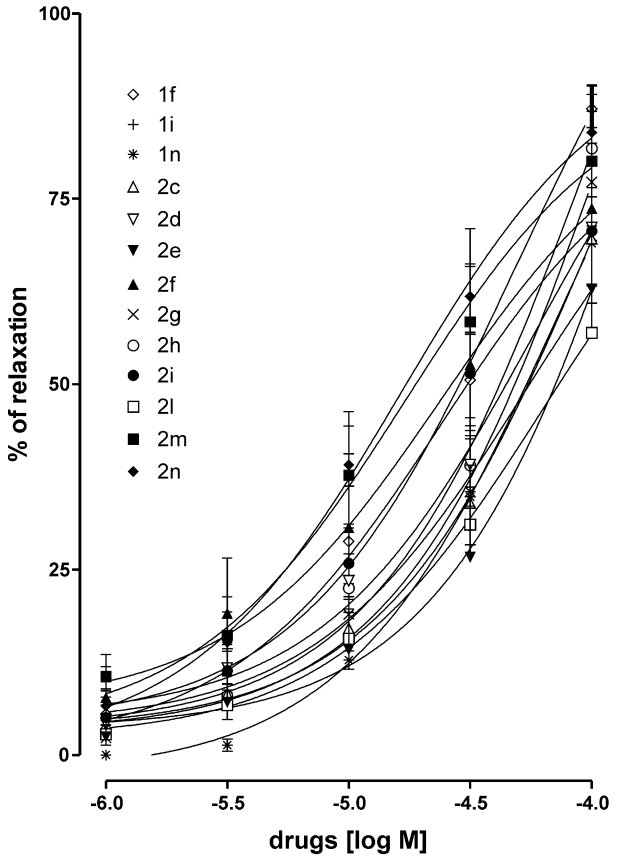


Figure 1. Concentration–response curve of compounds **1f**, **1i**, **1n**, **2c**–**2n** (1–100 mM) on rat aorta rings pre-contracted with phelylephrine. Data are expressed as mean ± SEM of 6–12 at aorta rings.

Table 2. Calculated EC_{50} (μM) of active compounds and cromakalim on phenylephrine-induced contraction in rat aorta rings

Compound	EC_{50} (95% confidence limits)
1f	35 (21–59)
1i	82 (41–162)
1n	77 (69–86)
2c	71 (45–113)
2d	48 (31–73)
2e	63 (23–140)
2f	32 (10–93)
2g	59 (31–110)
2h	53 (30–97)
2i	27 (14–51)
2l	72 (41–126)
2m	20 (11–37)
2n	19 (11–33)
CRK[a]	0.4 (0.25–0.65)

Results are expressed as mean (95% confidence limits) of several experiments (3–16).
[a]Cromakalim.

In our experimental model cromakalim, induced relaxation of aorta rings was significantly inhibited by GLY and 4-AMP ($p < 0.001$) but not by TEA at concentration used (Table 3).

The results obtained testing the series 1 showed that only the compounds **1f** and **1i** had displayed a behaviour similar to CRK. All the other compounds did not shown an activation of calcium-dependent potassium channels because TEA did not block significantly the relaxation observed. We can exclude an activity on voltage potassium channels since 4-AMP did not inhibit the relaxation induced by these compounds. Conversely, 4-AMP and TEA enhanced significantly the relaxation of compound **1n**.

As stated above, series **2** compounds were all more active if compared to series **1** except for the compound **2f**, which exhibited a low vasorelaxant activity respect to its analogue **1f**. GLY and 4-AMP significantly inhibited the relaxation induced by compounds **2d**, **2e**, **2f** and **2i**. TEA at a concentration of 1 mM, that as been shown to block only calcium activated potassium channel, inhibited significantly the relaxation mediated by compound **2h**. In contrary TEA enhanced significantly the relaxation induced by compounds **2c**, **2d** and **2l**.

These results suggest that structural modification at 2- and 6-positions of the 1,4-benzoxazine nucleus lead to relevant changes in the mechanism of action. In fact, the presence of some electrons with-drawing substituents such as nitro and cyano groups or thyazoline ring associated to the absence of the geminal methyl groups at the 2-position lead to compounds (**1f**, **2f**, **2d** and **2e**) which, although less active than CRK ($EC_{50} = 0.4\mu M$), displayed overall pharmacological behavior similar to that of the reference drug, since their vasorelaxant activity was inhibited by GLY and 4-AMP but not by TEA. It may be hypothesized that these compounds interact mainly with the K_{ATP} channels.

Results of the corresponding 2,2-dimethyl analogues (**1n**, **2n**, **2l**, and **2m**) suggest that these compounds may act by the involvement of a different mechanism of action.

The influence of the geminal methyl groups was inverse with compounds **1i** and **2i** characterized by a presence of a chlorine atom at the 6-position which displayed a behavior similar to CRK. Results of the corresponding demethyl derivative **2c** showed that the blockers of potassium channels GLY and 4-AMP did not inhibit the vasorelaxation whereas the effect observed in presence of TEA was increased, indicating a possible involvement of another mechanism of action. Also the unsubstituted or methyl substituted at the 6-position led to compounds **2g** and **2h** with a different behavior to CRK; compound **2h** seems to act inhibiting the calcium-dependent potassium channels.

These results suggest that some structural modification operated have caused a shift in the biological activity. Indeed, blockers of potassium channels did not inhibit the vasorelaxation and in some cases increased the vasorelaxation activity, indicating a possible involvement of another mechanism of action. In this regard we have investigated on a possible involvement of calcium activated channels. We choose some compounds that showed a vasorelaxant activity higher than 50% (**1n**, **2c**, **2g**, **2h**, **2l**, **2m** and **2n**), that were not inhibited by potassium channels blockers, to investigate on a probable ability to block calcium voltage-dependent channels. The calcium blocking activity was investigated as

Table 3. Relaxation % of compounds (100 mM), with or without potassium channels inhibitors on rat aorta rings pre-contracted by PE 1 μM

Compd	Control (vehicle)	Glibenclamide (10 mM)	Tetraethylammonium (1 mM)	4-Aminopyridine (10 mM)
1f	87.1±3.2 (6)	59.7±10.5* (5)	61.9±14.8 (3)	34.7±6.8‡ (5)
1i	63.2±6.1 (4)	48.7±1.5* (4)	55.9±17 (3)	35.6±8.3* (3)
1n	69±1.8 (4)	84.8±9.3 (3)	90.1±5.7**(3)	95.7±2.3‡ (3)
2c	69.5±5.6 (13)	59.9±4.6 (3)	100±0† (3)	58.4±5.2 (3)
2d	71.1±4 (11)	52.5±6.6**(4)	87.9±6.6* (4)	39.2±7† (5)
2e	62.8±8 (12)	25±18.7* (3)	88.7±6.6 (3)	18.1±5.2**(3)
2f	73.7±8.8 (6)	52.7±3.4* (4)	66.2±19.4 (4)	31.7±15* (4)
2g	77.3±9.5 (5)	62.4±8.7 (8)	76.5±9.0 (3)	73.9±4.1 (3)
2h	81.8±7.2 (5)	79.7±3.8 (5)	58.2±2.1* (3)	60.5±8.7* (4)
2i	70.7±5.8 (13)	54.6±4* (4)	84.6±5.2 (4)	50.2±3.9* (4)
2l	56.9±4 (8)	67.7±1.0 (3)	97.4±2.6‡ (3)	49.4±4.5 (4)
2m	80.0±4.6 (13)	65.9±8.3 (5)	89.2±7.3 (3)	67.2±4.5 (7)
2n	83.9±6.2 (13)	66.6±5.9 (5)	92.5±6.8 (4)	70.3±7.9 (4)
CRK[a]	83.1±2 (20)	38.5±7.9D (14)	73.0±10.2 (3)	43.5±1.9D (3)

Results are expressed as mean±SEM and (n). Statistical analysis was performed comparing the % of relaxation (100 mM) in presence of inhibitor versus control value.
$p<0.05$ was considered significant. * = $p<0.05$; ** $p<0.01$; † = $p<0.005$; ‡ = $p<0.0001$.

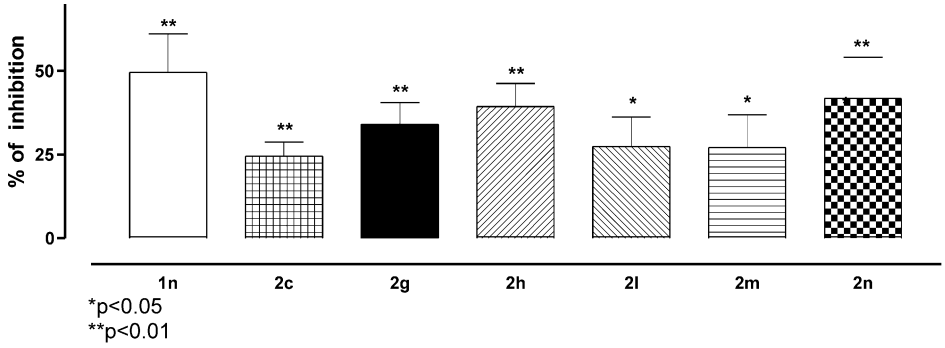


Figure 2. Inhibition effect of compounds **1n**, **2c**, **2g**, **2h**, **2l**, **2m**, **2n** (30 mM) of KCl (60 mM) induced contraction. Data are expressed as mean±SEM of 3–4 rat aorta rings.

ability to inhibit the KCl (60 mM) induced contraction. Compounds **1n**, **2c**, **2g**, **2h**, **2l**, **2m** and **2n**, at concentration of 30 μM significantly reduced KCl induced contraction of rat aorta rings (Fig. 2). Vehicle (DMSO 1:1000) did not modify KCl induced contraction; the % of inhibition value observed was 0.6±0.4 ($n=7$).

In conclusion, our data indicates that the new ester derivatives are provided with significant vasorelaxant activity profile. Compounds **1f**, **1i**, **2d**, **2e**, **2f** and **2i** have a similar activity to that observed for cromakalim. Subtile structural modifications at the 2- and 6-positions of the 1,4-benzoxazine nucleus lead to relevant changes in the mechanism of action. In fact, pharmacological data led us to hypothesize that structural changes of some compounds have brought the synthesis of drugs that can act as calcium voltage potassium channels without exclude the possibility to act on other calcium channels. Moreover the increase in vasorelaxation observed for some compounds in presence of potassium channels inhibitors, such as TEA or 4-AMP, could be justify by the fact that probably these compounds have still preserved some ability to interact with potassium channels without explicate the pharmacological activity. Hence, in presence of potassium channels inhibitors the concentration reached is higher to act as calcium channels blockers than in absence of these inhibitors. Our results implying that these structural modification may act as template for deriving new compounds with a different profile.

The complexity of the pharmacological data, mostly resulting from the involvement of different receptors each to different extent, impedes the development of reliable SARs for the present series of compounds.

Experimental

Chemistry

General. All melting points were determined on a capillary melting points apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM 500 spectrometer using tetramethylsilane as an internal standard. The following abbreviations are used: brs = broad single, d = doublet, t = triplet, m = multiplet, s = singlet. Combined GLC–MS analyses were performed on Hewlett-Packard and 5890 Gas Chromatograph with a mass selective detector MDS HP 5970. A column 25 m×0.20 mm HP-5 (cross-linked PhMe silicone 5%) with a 0.33 mm film tickness was employed. Silica gel F_{254} (Merck) plates were used for thin-layer chromatography (TLC). Column chromatography was performed using Carlo Erba silica-gel (0.05–0.20 mm). Elemental analyses were carried out on

a Carlo Erba Model 1106. Elemental analyses (C, H, Cl, N, S) and the results were within ± 0.4% of the theoretical values. Anhydrous Na_2SO_4 was used as drying agent for organic extraction. All solvent evaporation was performed under vacuum. Reagent grade materials were purchased from Aldrich Chemical Co. and were used without further purification. The following experimental methods represent general procedures for the synthesis of each of the compounds presented in the text.

2-Amino-4-cyanophenol. To a stirred solution of tin (II) chloride (0.433 mol) in 37% hydrogen chloride (60 mL) was added 4-hydroxy-3-nitrobenzonitrile. After the addition, the resulting mixture was allowed to stir at room temperature and carefully monitored by TLC. After 3 h, the reaction mixture was cooled to 5 °C, alkalinized to pH = 8 with 30% NaOH and than with $NaHCO_3$ solution until to white precipitate was formed and finally extracted with ethyl acetate. The organic layer was separated and dried over anhydrous Na_2SO_4. The solvents were evaporated and the crude residue was purified using a silica gel column chromatography and acetone–NH_4OH (9.5:0.5 v/v) as eluent. Recrystallization from ethanol give the required compound as brown pale solid (yield 84%), mp 158–159 °C. IR (KBr): 1295, 1517, 2224 cm^{-1}. ^1H NMR (DMSO-*d*): δ 10.35 (brs, 2H, NH2), 7.01 (s, 1H, Ar–H), 6.95 (d, 1H, Ar–H, $J = 8.8$ Hz) and 6.87 ppm (d, 1H, Ar–H, $J = 8.1$ Hz).

General procedure for the preparation of 3,4-dihydro-3-oxo-2H-1,4-benzoxazines (5). Bromoacetyl bromide or 2-bromoisobutyryl bromide (0.15 mol) was added dropwise to an ice-bath cooled solution of 2-aminophenol **3** (0.1 mol) and 350 mL of saturated solution of sodium carbonate in 600 mL of $CHCl_3$. The reaction mixture was stirred at room temperature for 3 h and monitored by TLC (diethylether/*n*-hexane 1:1 v/v, as eluent). The layers were separated and the organic phase was (washed with H_2O) dried over anhydrous Na_2SO_4 and concentrated in vacuo to provide crude product **4** as brown oil, which was used without further purification. The solution of crude product **4** and anhydrous K_2CO_3 (0.1 mol) in 250 mL of DMF was heated at 80 °C and stirred for 3 h (TLC diethylether/*n*-hexane 1:1 v/v, as eluent). After cooling the reaction mixture was poured into H_2O (250 mL) and extracted several times with $CHCl_3$. The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated in vacuo. Recrystallization from appropriate solvents gave compound 5 as white solid (yields ranging from 62 to 80%). ^1H NMR data are in according with the proposed structures.

General procedure for the preparation of 3-[3,4-dihydro-3-oxo-2H-1,4-benzoxazin-4-yl]-ethyl propionate (1a–e and 1g–m). To a solution of compound **5** (0.11 mol) in 70 mL of DMF cooled with an ice bath was added NaH (60% in oil dispersion 0.16 mol) in portions and after 10 min ethyl-3-bromopropionate (0.17 mol) was added. The reaction was stirred at room temperature for 3 h and then poured into cold water (250 mL) and extracted with $CHCl_3$. The organic phase was washed several times with H_2O, dried and evaporated. Recrystallization from appropriate solvents gave final products **1a–e** and **1g–m** (yields ranging 38–73%). Spectral data of title compound **1b**: ^1H NMR ($CDCl_3$) δ 6.95 (d, $1H_8$, Ar–H, $J = 8.2$ Hz), 6.80 (d, $1H_7$, Ar–H, $J = 8.4$ Hz), 6.52 (s, $1H_5$, Ar–H), 4.61 (s, 2H, O-CH_2C=O), 4.28 (t, 2H, CH_2N $J = 7.3$ Hz), 4.15 (m, 2H, OCH_2), 2.70 (t, 2H, CH_2C=O) 2.30 (s, 3H, CH_3) and 1.25 ppm (t, 3H, CH_3, $J = 7.3$ Hz). Similar ^1H NMR data occur in all derivatives of general formula **1**.

General procedure for the preparation of 4-[3,4-dihydro-3-oxo-2H-1,4-benzoxazin-4-yl]-ethyl butyrate (2a–e and 2g–m). Compounds **2a–e** and **2g–m** were prepared from 5 (0.11 mol) with NaH (60% in oil dispersion, 0.16 mol) and ethyl-4-bromobutyrate (0.17 mol) in DMF (70 mL) by the same procedure used for the preparation of **1a–e** and **1g–m** from **5**. The products were crystallized from appropriate solvents. Spectral data of title compound **2b**: ^1H NMR ($CDCl_3$) δ 7.10 (d, $1H_8$, Ar–H, $J = 8.2$ Hz), 6.90 (d, $1H_7$, Ar–H, $J = 8.4$ Hz), 6.86 (s, $1H_5$, Ar–H), 4.91 (s, 2H, O–CH_2C=O), 4.55 (t, 2H, CH_2N $J = 7.3$ Hz), 4.15 (m, 2H, OCH_2), 4.01(m, 2H, CH_2), 2.44 (t, 2H, CH_2C=O), 2.36 (s, 3H, CH_3) and 1.26 ppm (t, 3H, CH_3, $J = 7.3$ Hz). Similar ^1H NMR data occur in all derivatives of general formula **2**.

General procedure for the preparation of 3-[3,4-dihydro-3-oxo-6-(Δ^2-thiazolin-2-yl)-2H-1,4-benzoxazin-4-yl]ethyl propionate (1f, 1n). A mixture of appropriate 2-(3,4-dihydro-3-oxo-6-ciano-1,4-benzoxazin-4-yl)-ethyl propionate **1e** or **1m** (0.1 mol) and 2-aminoethanethiol hydrochloride (0.1 mol) in absolute ethanol and triethylamine (0.1 mol) solution was heated to reflux for 3 h and monitored by TLC. After cooling the ethanol was removed under reduced pressure and the residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane 6:4 v/v, as eluent). Fractions containing the product were combined, dried in vacuo, and recrystallized from appropriate solvents to give, respectively, analytically pure products **1f** (yield 63%) and **1n** (yield 35%) as white crystals.

Spectral data of title compound **1f**: ^1H NMR ($CDCl_3$) δ 7.60 (s, $1H_5$, Ar–H), 7.50 (d, $1H_8$, Ar–H, $J = 8.2$ Hz), 7.00 (d, $1H_7$, Ar–H, $J = 8.4$ Hz), 4.70 (s, 2H, O–CH_2C=O), 4.55 (s, 2H, CH_2N), 4.30 (t, 2H, CH_2N=, $J = 8.2$ Hz), 4.10 (m, 2H, OCH_2, $J = 7.3$ Hz), 3.43 (t, 2H, CH_2S, $J = 8.2$ Hz), 2.70 (t, CH_2C=O) and 1.25 ppm (t, 3H, CH_3, $J = 7.3$ Hz). Similar ^1H NMR data occur in compound **1n**.

General procedure for the preparation of 4-[3,4-dihydro-3-oxo-6-(Δ^2-thiazolin-2-yl)-2H-1,4-benzoxazin-4-yl]ethyl butyrate (2f and 2n). Compounds **2f** and **2n** were prepared from appropriate 2-(3,4-dihydro-3-oxo-6-ciano-1,4-benzoxazin-4-yl)ethyl butyrate **2e** or **2m** (0.1 mol) with 2-aminoethanethiol hydrochloride (0.1 mol) in absolute ethanol and triethylamine (0.1 mol) according to the procedure used for the preparation of **1f** and **1n**. The products were crystallized from appropriate solvents as reported in Table 1. **2f**: ^1H NMR ($CDCl_3$) δ 7.70 (s, $1H_5$, Ar–H), 7.55 (d, $1H_8$, Ar–H, $J = 8.2$ Hz), 7.10 (d, $1H_7$, Ar–H, $J = 8.4$ Hz), 4.81 (s, 2H, O–CH_2C=O), 4.60 (s,

2H, CH_2N), 4.35 (t, 2H, CH_2N, $J=8.2$ Hz), 4.21 (m, 2H, CH_2), 4.15 (m, 2H, OCH_2, $J=7.3$ Hz), 3.52 (t, 2H, CH_2S, $J=8.2$ Hz), 2.44 (t, $CH_2C{=}O$) and 1.26 ppm (t, 3H, CH_3, $J=7.3$ Hz). Similar ^1H NMR data occur in compound **2n**.

Pharmacology

Vasorelaxant activity in vitro assay. Male Wistar rats (200–250 g; Nossan, Italy) were killed by exsanguination after exposition to CO_2 and the thoracic aorta was removed, cleaned of adherent connective tissue, and cut into rings $\sim$3 mm in length. The endothelium was removed by gently rubbing the intimal surface with moistened filter paper. Endothelium-denuded rings were mounted under 0.5 g of tension in 2.5 mL organ baths containing Krebs salt solution at the following composition (in mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 1.3; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; and glucose 11.7. The solution was maintained at 37 °C and bubbled with 95% O_2–5% CO_2 (pH 7.4). Developed tension was measured using an isometric force transducer (7003 transducer, Ugo Basile, Comerio, Italy) connected to a recorder (Graphtec Linearcorder, WR 3310). Rings were allowed to equilibrate for 60 min and the Krebs solution was replaced each 15 min. After equilibration drugs were tested as vasorelaxant on phenylephrine-induced contraction (1 μM). Drugs were dissolved in DMSO and added to the organ bath in cumulative manner in the range 1–25 μL (maximum final concentration of DMSO 1%). Drugs that showed a vasorelaxation in concentration-dependent manner with a relaxation over than 50% of contraction, at the highest used concentration (100 μM), were considered for the test in presence of inhibitors. Each tissue was used only for one concentration–response curve of tested compound in presence or absence of inhibitors such as GLY (0.1 mM), or TEA (1 mM) or 4-AMP (10 mM).

To investigate on the ability of some compounds to interfere with calcium channels, we performed experiments using KCl (60 mM)[14] as contracting agent. After tissue stabilization, KCl was added to the organ bath (100% contraction) and than washed-out. Compounds, at concentration of 30 μM, were added to the organ bath; 15 min after, KCl 60 mM was added again. Analogous experiment was performed in presence of vehicle (DMSO 0.1%) as control. Inhibition of calcium channels activity was expressed as% of inhibition of first KCl induced contraction.

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