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Synthesis, biological activity and conformational study of 1,4-benzoxazine derivatives as potassium channel modulators

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Abstract – With the aim of discovering new molecules with K^+-channel activating properties, we have synthesized derivatives of cromakalim (CRK), an important molecule which shows specific affinity towards K^+ channels, by replacing the benzopyrane ring of this reference compound with a 1,4-benzoxazine moiety. A different number of substituents showing a good discrimination between hydrophobic and electronic properties have been inserted at the 6-position of the 1,4-benzoxazine ring. We describe here the synthesis and discuss the solid state conformation of these new molecules. When tested on rat aorta ring precontracted with phenylephrine, two compounds (**2c** and **2d**) showed a concentration-dependent relaxation similar to that measured for cromakalim but less potent than this reference drug. © Elsevier, Paris

potassium channel modulators / crystal structures / conformational study / 1,4-benzoxazine derivatives / SAR / cromakalim

1. Introduction

Potassium channels are exceptionally diverse both in variety and function. They play an important and complex role in the basic electrical and mechanical function of a wide variety of tissues, including smooth muscle, cardiac muscle and glands [1]. A wide variety of K^+-channel subtypes have been identified and characterized (e.g. voltage-gated channels (K_v), Ca^{+2}-actived-channels (K_{Ca}) [2]. An ATP-dependent K^+ channel (K_{ATP}) has been found [3] in vascular tissues and cromakalim is provided with specific affinity towards the target channel [4, 5]. The major clinical utility of the ATP-sensitive potassium channel opener was originally thought to be for the treatment of hypertension, primarily due to their potent peripheral vasodilating properties [6]. Although experimental studies, show that the first agents (cromakalim, pinacidil) may be effective for the treatment of a variety of disease as (asthma, urinary incontinence, etc.) besides hypertension [7].

Studies on the mechanism of action of the antihypertensive drug cromakalim suggested that its relaxant activity is associated with cell membrane hyperpolarization due to an increase in outward K^+ conductance. In this work we show that modification of cromakalim molecule can lead to new compounds with K^+ channel opener activity.

Taking into account previously structure–activity investigations [8, 9], we have synthesized structural analogues of cromakalim by replacing the benzopyrane ring system of cromakalim with the 1,4-benzoxazine nucleus and the 4-pyrrolidinone moiety with acyclic groups represented by N,N-dimethylacetamide or ethylacetate *(figure 1)*.

A limited number of substituents spanning a reasonable range of electronic and hydrophobic properties have been inserted at the 6-position of the 1,4-benzoxazine ring. These modifications provided the 1,4-benzoxazine de-

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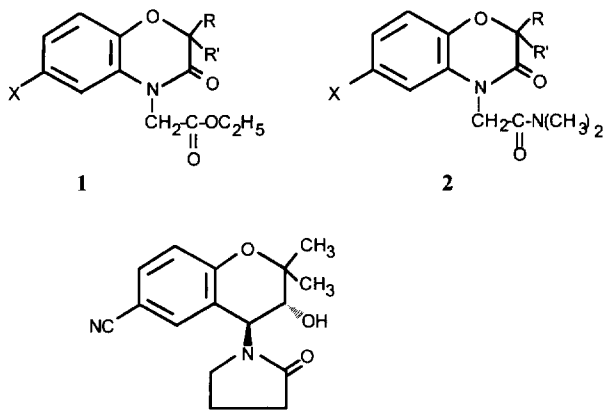


Figure 1. The structure of cromakalim and 1,4-benzoxazine ester and amide derivatives **1** and **2**, respectively.

rivatives **1a–n** and **2a–n** *(table I)*. Koga et al. [10] have recently developed a pharmacophore model for potassium channel openers based on the structures of chemically diverse drugs such as cromakalim, nicorandil, pinacidil, diazoxide and minoxidil. Preliminary molecular modeling studies performed by us on a few structures representative of the amides **1a–n** and esters **2a–n**, using the crystal structure of cromakalim [11] as template, suggested that the designed compounds fulfilled the pharmacophore requirements proposed by Koga et al. [10]. Our modeling study was carried out following a computational protocol identical to that described by Koga et al. [10]. A similar procedure was subsequently applied using the crystal structures of cromakalim as a template and those of compounds **1n** and **2e** (see below). Concerning the 6-substituent on the 1,4-benzoxazine ring, it is worth mentioning that extensive investigations in the class of benzopyrane analogues have revealed that this substituent influences activity through electronic and lipophilic effetcs [12]. To highlight the effects of the 6-substituent in our series of 1,4-benzoxazine we have therefore decided to vary its electronic and lipophilic character. The 6-cyano group has been selected also because it has proved to be the most favourable one in the class of cromakalim analogues. A simple conversion of the 6-cyano into a 6-(Δ^2-thiazolin-2-yl) group *(figure 2)* was realized in order to explore the effect of a high lipophilic moiety at this position.

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. Since several outward K^+ channels have been identified, we tested the active analogues in presence of selective channel blockers. For this purpose we used several blockers such as glibenclamide for the K_{ATP} channel, tetraethylammonium for the K_{Ca} channel, barium chloride for the K_v or 4-aminopyridine for the unselective potassium channel blockers.

2. Chemistry

The 2-(3,4-dihydro-3-oxo-2H-1,4-benzoxazin-4-yl)-N,N-dimethylacetamide **1a–n** and 2-(3,4-dihydro-3-oxo-2H-1,4-benzoxazin-4-yl)-ethylacetate **2a–n** derivatives have been listed in *table I* and were synthesized following the steps outlined in *figure 2*. The starting compounds 2-amino-4-substituted-phenols for the synthesis of general compounds **3** were commercially available, only the 2-amino-4-cyanophenol was prepared by selective reduction of 4-hydroxy-3-nitrobenzonitrile with tin (II) chloride in 37% hydrogen chloride. Acylation of 2-aminophenol **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 **2a–e**, **2g–m** by condensation with 2-chloro-N,N-dimethylacetamide or ethyl bromoacetate, respectively, in sodium hydride/DMF at room temperature. Compounds **2l** which was described independently by Matsumoto et al. [13], was synthesized according to the same general procedure for the preparation of compounds **2**.

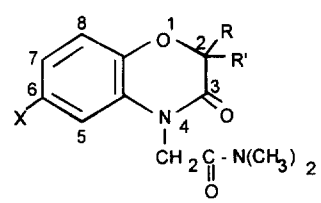
The 6-(Δ^2-thiazolin-2-yl) derivatives **1f,n** and **2f,n** were obtained by reaction of the corresponding 6-cyano derivatives **1e,m** and **2e,m**, respectively, with stechiometric amounts of 2-aminoethanethiol hydrochloride in absolute ethanol and triethylamine solution. All products were isolated by chromatography, further purified by crystallization from appropriate solvents (yields ranging between 30–85%) and characterized by ^{1}H-NMR spectroscopy and GLC-MS.

Structures, physicochemical data and % of relaxation activity of all compounds synthesized are summarized in *table I*.

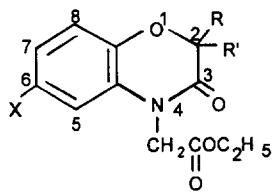
3. Results and discussion

All the compounds were tested in vitro for their vasorelaxant activity using rat thoracic aorta rings pre-

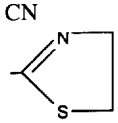
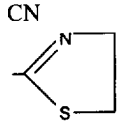
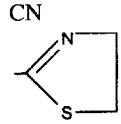
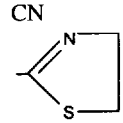
Table I. Physicochemical properties and vasorelaxant activity of 1,4-benzoxazine derivatives (**1a–n**) and (**2a–n**).



1a–n



2a–n

Compound	X	R = R'	Formula [a]	M.W.	Yield (%)	M.p. (°C)	Recryst. solvent [b]	MS (*m/z*)	% of relaxation (10^{-4} M)
1a	H	H	$C_{12}H_{14}N_2O_3$	234.25	76	172–173	a	234; 189; 134; 77	15.4
1b	CH_3	H	$C_{13}H_{16}N_2O_3$	248.28	60	161–163	d	248; 176; 148; 72	16.3
1c	Cl	H	$C_{12}H_{13}ClN_2O_3$	268.70	70	185–187	e	268; 168; 141; 72	45.8
1d	NO_2	H	$C_{12}H_{13}N_3O_5$	279.25	70	166–167	f	299; 236; 179; 133; 72	33
1e	CN	H	$C_{13}H_{13}N_3O_3$	259.26	58	206–207	c	259; 159; 102; 72	5.0
1f		H	$C_{15}H_{17}N_3O_3S$	319.38	67	108–109	d	319; 248; 219; 150; 72	8.5
1g	H	CH_3	$C_{14}H_{18}N_2O_3$	262.31	65	160–161	d	262; 162; 134; 172	2.5
1h	CH_3	CH_3	$C_{15}H_{20}N_2O_3$	276.33	34	118–120	e + b	276; 176; 148; 72	14.5
1i	Cl	CH_3	$C_{14}H_{17}ClN_2O_3$	296.75	81	147–149	f + b	296; 196; 154; 221; 72	27.7
1l	NO_2	CH_3	$C_{14}H_{17}N_3O_5$	307.29	69	168–170	d	307; 207; 191; 121; 72	0
1m	CN	CH_3	$C_{15}H_{17}N_3O_3$	287.32	60	166–167	g	287; 288; 187; 145; 72	4.3
1n		CH_3	$C_{17}H_{21}N_3O_3S$	347.43	85	195–197	a + b	347; 247; 210; 187; 72	8.7
2a	H	H	$C_{12}H_{13}NO_4$	235.24	30	80–82	a + b	235;162; 107; 77	15.8
2b	CH_3	H	$C_{13}H_{15}NO_4$	249.27	61	102–103	a + b	250; 219; 204; 176	36
2c	Cl	H	$C_{12}H_{12}ClNO_4$	269.68	40	116–118	a + b	269; 271; 196; 141; 29	70.3
2d	NO_2	H	$C_{12}H_{12}N_2O_6$	280.24	66	117–119	a	280; 207; 133; 29	71.7
2e	CN	H	$C_{13}H_{12}N_2O_4$	260.25	88	144–146	d	260; 187; 132; 29	85.1
2f		H	$C_{15}H_{16}N_2O_4S$	320.37	67	107–109	a + b	321; 219; 185	89.4
2g	H	CH_3	$C_{14}H_{17}NO_4$	263.29	53	68–70	a + b	263; 220: 192; 162; 120; 77	20
2h	CH_3	CH_3	$C_{15}H_{19}NO_4$	277.32	48	74–76	e	277; 176; 134; 149	20.4
2i	Cl	CH_3	$C_{14}H_{16}ClNO_4$	297.74	62	86–88	a + b	297; 226; 196; 154; 41	68.5
2l	NO_2	CH_3	$C_{14}H_{16}N_2O_6$	308.29	55	83–85	a + b	308; 207; 179; 121; 41	70.0
2m	CN	CH_3	$C_{15}H_{16}N_2O_4$	288.30	53	132–134	g + b	288; 245; 187; 145; 41	48.6
2n		CH_3	$C_{17}H_{20}N_2O_4S$	348.42	70	63–64	a + b	348; 247; 187; 145; 41	91.8

[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) methyl alcohol; (d) ethyl alcohol; (e) ethyl acetate; (f) acetone; (g) isopropylether.

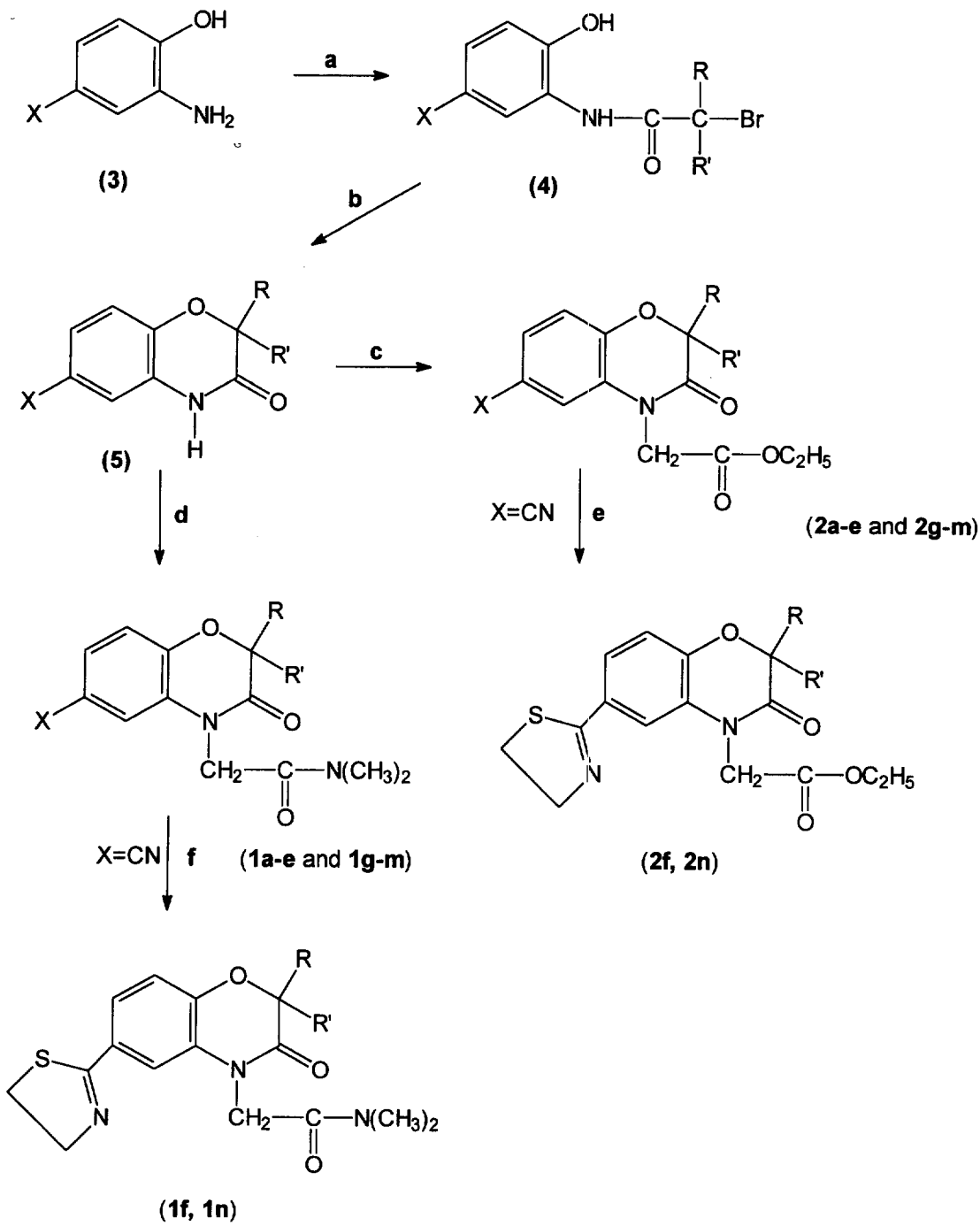


Figure 2. (a) Bromoacethyl bromide or 2-bromoisobutyryl bromide, $NaHCO_3$, $CHCl_3$, room temperature; (b) anhydrous K_2CO_3, DMF, 80 °C; (c) ethylbromoacetate, NaH, DMF, room temperature; (d) 2-chloro-N,N-dimethylacetamide, NaH, DMF, room temperature; (e)–(f) 2-aminoethanethiol hydrochloride, triethylamine/absolute EtOH, reflux.

contracted with phenylephrine. The synthesized compounds were always compared for their activity with cromakalim, taken as a reference drug.

The more active compounds and the reference drug were tested in presence of glibenclamide (0.1 mM), tetraethylammonium (1 mM) and $BaCl_2$ (5 mM) which block K_{ATP}, K_{Ca} and K_v channels respectively. Additionally, 4-aminopyridine (10 mM) was used as unspecific blocker of all mentioned K^+ channels [14].

The vasorelaxant activities of compounds **1a–n** and **2a–n** were measured on phenylephrine precontracted rat aorta rings deprived of endothelium in a concentration–range 0.1–100 μM. Effects obtained with concentration 100 μM of compounds, expressed as percentage of relaxation, are summarised in *table I*. Cromakalim, employed as reference drug, produced a relaxation of 86.5 ± 3.3% (n = 18) at a concentration of 100 μM.

All the amide derivatives **1a–n** (100 μM) were found poorly active since their vasorelaxant activities felt below 50% *(table I)*; thus, they were excluded from further experimentation. In contrast, several ester derivatives (**2c–f, 2i,l,n**) produced vasorelaxation greater than 50% and were submitted to additional pharmacological assays.

It is interesting to note that the other esters derivatives bearing an hydrogen or a methyl group at the 6-position of the 1,4-benzoxazine ring (**2a,b,g,h**) are devoid of appreciable activity. Such a finding seems consistent with structure–activity relationships (SARs) relative to cromakalim analogues indicating the need of electron-withdrawing substituents at the 6-position of the benzopyrane nucleus [8, 9]. Moreover the two methyl groups at the 2-position of the 1,4-benzoxazine ring are devoid of influence on the vasorelaxant activity. Only compound **2m** showed vasorelaxation lower than corresponding demethyl derivative **2e**.

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

Compound X	EC_{50}
2c	60.2 (43.8–76.5)
2d	41.2 (21.4–61.0)
2e	21.8 (3.4–40.3)
2f	13.6 (4.3–23.0)
2i	70.9 (37.0–104.8)
2l	51.1 (17.1–85.0)
2n	15.9 (9.9–21.8)
CRK [a]	0.7 (0.4–1.1)

Results are expressed as mean (95% confidence limits) of several experiments (10–19).
[a] Cromakalim.

Figure 3 shows the concentration–response curves of rat aorta rings precontracted by phenylephrine of compounds **2c–f, 2i,l,n**, and cromakalim. Relaxations induced by the active compounds are concentration-dependent.

The concentration–response curve of analysed compounds are not superimposable to those obtained with cromakalim. Indeed, all the active compounds show a 50% effective concentration (EC_{50}) higher than cromakalim *(table II)*; hence, they are less active than cromakalim.

On *table III* we reported the percent of relaxation induced by active compounds in presence of the above mentioned K^+ channel blockers. In this table we compared their effects at a concentration of 100 μM. At this concentration cromakalim, the reference drug, produced the maximum relaxation of phenylephrine(1 μM) precontracted aorta.

In our experimental model cromakalim induced relaxation of aorta rings ($P < 0.001$) inhibited by glibencla-

Table III. Relaxation of phenylephrine precontracted rat aorta rings induced by active compounds (100 μM) in the presence or absence of K^+ channel blockers.

Compound	Control (vehicle)	Glibenclamide (0.1 mM)	Tetraethylammonium (1 mM)	Barium chloride (5 mM)	4-Aminopyridine (10 mM)
2c	70.3 ± 2.5 (25)	32.8 ± 9.4 (4) [e]	67.8 ± 2.5 (6)	100 ± 1.0 (6) [e]	24.6 ± 3.2 (3) [e]
2d	71.7 ± 3.4 (18)	26.1 ± 2.1 (6) [e]	73 ± 6.9 (3)	97.8 ± 1.0 (6) [e]	11.7 ± 1.6 (3) [e]
2e	85.1 ± 5.0 (15)	90.0 ± 1.7 (3)	69 ± 8.2 (3)	31.5 ± 6.1 (4) [e]	7.3 ± 2.4 (3) [e]
2f	89.4 ± 3.3 (17)	100 ± 1.0 (3) [d]	90.8 ± 7.3 (3)	68.6 ± 6.1 (8) [d]	49.7 ± 6.6 (3) [e]
2i	68.5 ± 4.5 (20)	50.9 ± 6.2 (6) [b]	64.4 ± 9.7 (3)	27.8 ± 11.7 (3) [d]	56.1 ± 3.2 (3) [b]
2l	70.0 ± 5.6 (16)	52.5 ± 4.9 (9) [d]	70.1 ± 2.5 (3)	82 ± 18.0 (5)	54.1 ± 0.9 (3) [d]
2n	91.8 ± 2.2 (16)	96.7 ± 3.3 (3)	90.1 ± 5.7 (6)	100 ± 1.3 (6) [d]	55.4 ± 2.7 (3) [e]
CRK [a]	86.5 ± 3.37 (18)	46.7 ± 5.1 (5) [e]	90.7 ± 5.8 (3)	100 ± 1.2 (3) [e]	43.5 ± 2.0 (3) [e]

[a] Cromakalim.
Results are expressed as mean ±S.E.M. and (n). Statistical analysis was performed between the effect of each compound in the presence of K^+ channel blockers versus own control and they are expressed as follows: [b]: $P < 0.05$; [c]: $P < 0.01$; [d]: $P < 0.005$; [e]: $P < 0.001$.

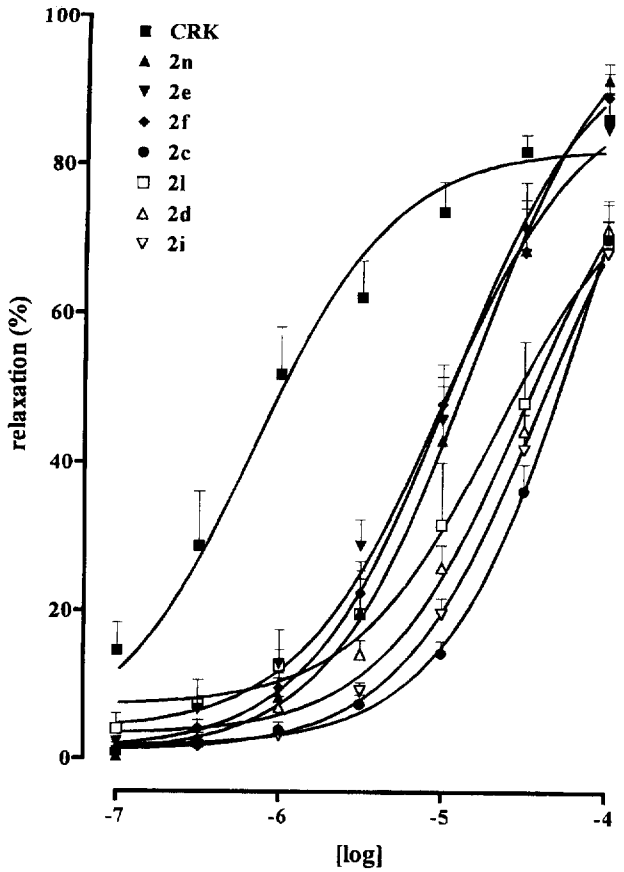


Figure 3. Concentration–response curve of rat aorta rings precontracted by phenylephrine for **2c–f,i,l,n** and cromakalim.

mide and 4-aminopyridine but not by tetraethylammonium and barium chloride at concentration we used *(table III)*.

Compounds **2c** and **2d** (EC_{50} = 60.2 and 41.2 μM), although less active than cromakalim (EC_{50} = 0.7 μM), display an overall pharmacological behaviour similar to that of the reference drug. Since their vasorelaxant activity is inhibited by glibenclamide and potentiated by $BaCl_2$ treatment. It may be hypothesised that both compounds interact mainly with the K_{ATP} channels.

Compounds **2e** and **2f** (EC_{50} = 21.8 and 13.6 μM respectively) are strongly inhibited by $BaCl_2$ and blocked by the action of 4-aminopyridine *(table II)*. We can hypothesise that these compounds act mainly on K_v channels.

Compounds **2i** and **2l** (EC_{50} = 70.9 and 51.1 μM respectively) revealed to be less active than their corresponding 2,2-dimethyl analogues **2c** and **2d**. This fact appears to contradict the SARs developed for cromakalim-like structures [8, 9] whose activity is favoured by geminal methyl groups at the 2-position of the benzopyrane ring. However, since the vasorelaxant activity of **2i** and **2l** is statistically modified by the presence of glibenclamide, this could suggest an action on K_{ATP} channels. This hypothesis can be ascribe only to the compound **2l** since nor tetraethylammonium or $BaCl_2$ can inhibit its relaxation. In contrast compound **2i** is also heavily inhibited by $BaCl_2$, suggesting that this compound is mainly a K_v opener.

The relaxation induced by **2n** (EC_{50} = 15.9 μM) was inhibited only by the unspecific potassium channel blocker 4-aminopyridine *(table III)* thus making problematic the identification of the principal target of this latter compound.

To sum up, the pharmacological assays conducted on the synthesized compounds show that the ester derivatives **2c–f, 2i,l,n** are provided with significant vasorelaxant activity profiles whereas the whole set of amides **1a–n** are overall poorly active. Within the ester series, subtle structural modifications at the 2- and 6-positions of the 1,4-benzoxazine nucleus lead to relevant changes in the mechanism of action. While compounds **2c** and **2d** appear to behave much like cromakalim, the main targets of the other ester derivatives differ one from another and deserve further investigations to be elucidated. 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.

4. X-Ray and modeling

The crystal structures of the amide derivative **1n** and of the ester derivative **2e** were determined by X-ray diffraction analysis in order to investigate possible geometric/conformational effects as basis of the observed differences in activity between the amide and ester series. The unit cell of **1n** contains two independent molecules with very similar geometries, labeled A and B, for which the fitting of the non-hydrogen atoms yields a root mean square (RMS) distance of 0.078 Å.

Observed bond distances and bond angles are in agreement with literature data for similar compounds [15] and with the geometry observed for the cromakalim [12] molecule.

Compound **1n** can be described as formed by two ortho condensated rings linked to a third thiazolinic ring in ortho position with a quasi-planar disposition; this overall shape is very similar to that observed for compound **2e**, where the thiazolinic ring is substituted by a cyano group. The 1,4-benzoxazine moiety and the ortho condensated

phenyl ring are in fact almost co-planar, while the third ring, formed by the S1, C17, N5, C18 ad C19 atoms, is, in both molecules present in the asymmetric unit, only slightly out from the plane formed by the others. The torsion angle C7–C6–C17–N5, describing the disposition of the thiazolinic ring with respect to the other cyclic moiety, is *trans*, with an identical value of 172° for both the A and B molecules. The molecule presents a lateral chain substituent on the N4 atom of the benzoxazinic ring. The C10–N4–C11–C12 torsion angle, describing the disposition of this lateral chain with respect to the cyclic moiety, is –87° and –88° for the A and B molecules, respectively, indicating a quasi *g*-disposition of the chain. The torsion angles describing the conformation of the lateral chain are all nearly *trans*. The only difference between the two molecules in the asymmetric unit of **1n** concerns with the disposition of the two methyl groups C15 and C16 insisting on the 1,4-benzoxazine ring. The N-substituted amido moiety is nearly perpendicular to the plane of the carbonyl C3=O2 of the bicyclic moiety. Compound **2e** presents a similar disposition of the bicyclic ring system. In this compound the C10–N4–C11–C12 torsion angle, describing the disposition of the lateral chain insisting on the N4 nitrogen atom, is –79.4°, indicating again a *g*-disposition of the chain. The torsion angles of the lateral chain are all *trans* and, similarly to what found for compound **1n**, the ester terminal moiety on the nitrogen atom N4 is in near orthogonal disposition with respect to the plane of the carbonyl group C3=O2 of the bicyclic moiety.

The conformations of **1n** and **2e** are reminiscent of the crystal structure of cromakalim [11] whose pyrrolidinone carbonyl group is similarly orthogonal to the main plane passing through the benzopyrane ring. In both compounds the carbonyl group, a key chemical function for K_{ATP} opening activity [8, 9], has an arrangement orthogonal with respect to the 1,4-benzoxazine nucleus and points outward the ring system.

Figure 4 illustrates the X-ray structures of **1n** and **2e** (as capped-stick models) overlayed on the molecular model of 3*S*,4*R*-cromakalim built from available crystallographic data [12] (see the section on molecular modeling). The three molecular models were superimposed about the following atoms: 2-, 3-, 6- and 7-positions of the bicyclic frames, C and O of the carbonyl group. It can be noticed that the carbonyl oxygen, the ring systems and the 6-substituents are fairly close in space.

We assume that the conformational and geometric properties of **1n** and **2e** are sufficiently representative of the amide and ester series, respectively, as far as the conformational and geometric properties of the 4-substituents are concerned. On the basis on this assumption, the inactivity of the amide derivatives as K_{ATP} openers does not depend on unfavourable conformational properties since in the solid phase the 4-substituent of **1n** is characterized by a three-dimensional arrangement similar to that of the 4-substituents of **2e** and cromakalim. A plausible explanation for the relatively low activities of the amides is that one of the N-methyl groups of the side chain (indicated by an arrow in *figure 4*) interferes sterically with ligand–receptor binding. This hypothesis is consistent with the fact that neither **2e** neither cromakalim occupy the 'forbidden' region occupied by the

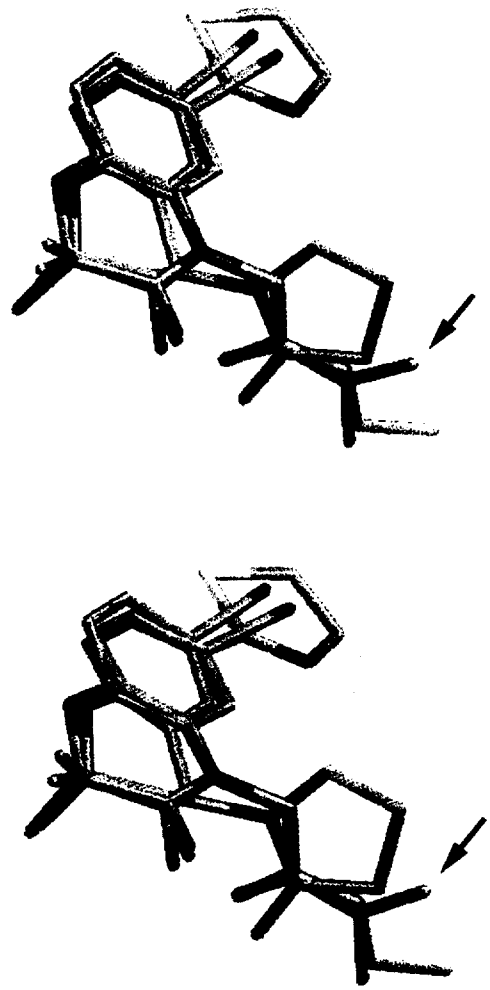


Figure 4. Stereopair picture of the crystal structures of **1n** and **2e** overlayed on the crystal structure of cromakalim. An arrow indicates the N-methyl group hypothesized to be responsible of the relatively low vasorelaxant activity of **1n** as well as of its amide congeners.

above mentioned N-methyl group. On the basis of these results we have recently undertaken the synthesis and biological evaluation of N-monomethylamide analogues which will be the subject of a forthcoming paper.

In conclusion, several 1,4-benzoxazine derivatives of type **1a–n** and **2a–n**, designed as analogues of cromakalim, have been synthesized and tested in functional pharmacological assays. While **1a–n** were found provided by poor activity, compounds **2a–n** revealed interesting vasorelaxant properties. In particular, **2c** and **2d** exhibit an overall pharmacological profile similar of that shown by cromakalim. Comparisons of the crystal structures of **1n**, **2e** and cromakalim suggested that the relatively low activity of the amide derivatives **1a–n**, compared with the corresponding esters **2a–n**, depends on lack of adequate shape complementarity with the considered receptors rather than on unfavourable conformational characteristics.

5. Experimental protocols

5.1. Chemistry

Melting points were determined using a Kofler melting points apparatus and are uncorrected. Proton NMR spectra were taken in $CDCl_3$ and recorded at 500 MHz on a Bruker WM 500 instrument. Chemical shifts are given in ppm relative to $(Me)_4Si$ as the internal standard (abbreviations: brs, broad signal; d, doublet; t, triplet; m, multiplet; s, singlet; chemical shifts in ppm). 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. Crystal data were collected on a diffractometer at the 'Centro di Biocristallografia del C.N.R.' of the Department of Chemistry of the University 'Federico II' in Naples equipped with a Micro Vax 3100 Digital Computer (running under VMS) where all calculations were performed.

Chromatography was performed using Carlo Erba silica-gel (0.05–0.20 mm) for column chromatography and Merck Kieselgel 60 (F_{254}, 0.25 mm) plates for TLC. Evaporation was carried out in vacuo on a rotary evaporation. The following experimental methods represent general procedures for the synthesis of each of the compounds presented in the text. 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. Reagent grade materials were purchased from Aldrich Chemical Co. and were used without further purification.

5.1.1. 2-Amino-4-cyanophenol 3

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%), m.p. 158–159 °C. IR (KBr): 1295, 1517, 2224 cm^{-1}. ^{1}H-NMR (*d*-DMSO): δ = 6.87 (d, 1H, Ar-H, *J* = 8.1 Hz), 6.95 (d, 1H, Ar-H, *J* = 8.8 Hz), 7.01 (s, 1H, Ar-H) and 10.35 ppm (brs, 2H, NH_2).

5.1.2. 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-amino-phenol **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%).

5.1.3. General procedure for the preparation of 2-[3,4-dihydro-3-oxo-2H-1,4-benzoxazin-4-yl]-N,N-dimethylacetamide 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 2-Chloro-N,N-dimethylacetamide (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**) as colourless solid (yields 61–81%).

Spectral data of title compound **1b**: ^{1}H-NMR ($CDCl_3$) δ = 6.88 (d, $1H_8$, Ar-H, J = 8.2 Hz), 6.78 (d, $1H_7$, Ar-H, J = 8.4 Hz), 6.54 (s, $1H_5$, Ar-H), 4.75 (s, 2H, CH_2N), 4.65 (s, 2H, O–CH_2C=O), 3.15 (s, 3H, $N(CH_3)_2$), 2.96 (s, 3H, $N(CH_3)_2$) and 2.28 ppm (s, 3H, CH_3), Similar ^{1}H-NMR data occur in all derivatives of general formula **1**.

*5.1.4. General procedure for the preparation of 2-[3,4-dihydro-3-oxo-2H-1,4-benzoxazin-4-yl]-ethylacetate **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 ethylbromoacetate (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**: ^{1}H-NMR ($CDCl_3$) δ 6.90 (d, $1H_8$, Ar-H, J = 8.2 Hz), 6.78 (d, $1H_7$, Ar-H, J = 8.4 Hz), 6.52 (s, $1H_5$, Ar-H), 4.65 (s, 2H, CH_2N), 4.61 (s, 2H, O–CH_2C=O), 4.25 (m, 2H, OCH_2, J = 7.3 Hz), 2.29 (s, 3H, CH_3) and 1.22 ppm (t, 3H, CH_3, J = 7.3 Hz). Similar ^{1}H-NMR data occur in all derivatives of general formula **2**.

*5.1.5. General procedure for the preparation of 2-[3,4-dihydro-3-oxo-6-(Δ^2-thiazolin-2-yl)-2H-1,4-benzoxazin-4-yl]-N, N-dimethylacetamide **1f, n***

A mixture of 2-(3,4-dihydro-3-oxo-6-cyano-2H-1,4-benzoxazin-4-yl)-N,N-dimethyl-acetamide (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 ethanol to give analytically pure products **1f** and **1n** as white crystals in 67 and 85% yields, respectively.

Spectral data of title compound **1f**: ^{1}H-NMR ($CDCl_3$) δ = 6.87 (d, $1H_8$, Ar-H, J = 8.2 Hz), 6.75 (d, $1H_7$, Ar-H, J = 8.4 Hz), 6.52 (s, $1H_5$, Ar-H), 4.73 (s, 2H, CH_2N), 4.65 (s, 2H, O–CH_2C=O), 4.42 (t, 2H, CH_2N=, J = 8.2 Hz), 3.40 (t, 2H, CH_2S, J = 8.2 Hz), 3.14 (s, 3H, $N(CH_3)_2$), and 2.95 ppm (s, 3H, $N(CH_3)_2$). Similar ^{1}H-NMR data occur in compound **1n**.

*5.1.6. General procedure for preparation of 2-[3,4-dihydro-3-oxo-6-(Δ^2-thiazolin-2-yl)-2H-1,4-benzoxazin-4-yl]-ethylacetate **2f, n***

Compounds **2f** and **2n** were prepared from 2-(3,4-dihydro-3-oxo-6-ciano-2H-1,4-benzoxazin-4-yl)-ethyl-acetate (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 I.*

Spectral data of title compound **2f**: ^{1}H-NMR ($CDCl_3$) δ 6.88 (d, $1H_8$, Ar-H, J = 8.2 Hz), 6.75 (d, $1H_7$, Ar-H, J = 8.4 Hz), 6.50 (s, $1H_5$, Ar-H), 4.65 (s, 2H, CH_2N), 4.61 (s, 2H, O–CH_2C=O), 4.45 (t, 2H, CH_2N=, J = 8.2 Hz), 4.23 (m, 2H, OCH_2, J = 7.3 Hz), 3.43 (t, 2H, CH_2S, J = 8.2 Hz) and 1.21 ppm (t, 3H, CH_3, J = 7.3 Hz). Similar ^{1}H-NMR data occur in compound **2n**.

5.2. Structural studies

5.2.1. X-Ray diffraction analysis

Single crystals 2-[3,4-dihydro-3-oxo-2,2-dimethyl-6-(Δ^2-thiazolin-2-yl)-2H-1,4-benzoxazin-4-yl]-N, N-dimethylacetamide **1n** and 2-(3,4-dihydro-3-oxo-6-cyano-2H-1,4-benzoxazin-4-yl)-ethylacetate **2e** were obtained by slow evaporation at room temperature from ethanol/ethyl ether (1:1 v/v) solutions. Compound **1n,** molecular formula $C_{17}H_{21}N_3O_3S$, M_w = 347.4 D, crystallizes in the monoclinic system, $P2_1/c$ space group, with Z = 8, a = 12.67(3), b = 16.19(4), c = 18.04(2), β = 106.70(5), V = 3543.2 Å^3, d_{exp} = 1.30 g/cm^3 (d_{calc} = 1.303 g/cm^3). Compound **2e**, molecular formula $C_{13}H_{12}N_2O_4$, M_w = 260.2 D, crystallizes in the triclinic system, P-1 space group, with Z = 2, a = 7.992(3), b = 8.650(4), c = 9.958(5), α = 89.28(4), β = 101.00(3), γ = 110.80(5), V = 630.4 Å^3, d_{exp} = 1.37 g/cm^3 (d_{calc} = 1.371 g/cm^3).

X-ray intensity data collection were performed on an automated four-circle CAD4 Enraf-Nonius single crystal X-ray diffractometer at T = 295 K. Measured reflections were 6734 and 2390 for **1n** and **2e**, respectively, using graphite monochromated, Nickel filtered, CuK_α radiation. All reflections were corrected for Lorentz and polarization effects [16]. The structures were solved by direct methods as programmed in SIR92 [17], and completed by analyzing subsequent difference Fourier maps. For the anisotropic final refinement a full-matrix least-squares procedure [17] was used, minimizing the quantity $\Sigma w(F_o - F_c)^2$, with a weight w equal to $1/\sigma(F_o^2)$; the reflections considered 'observed' and used in the calculations, with $F_o > 3.0\sigma(F_o)$, are 5251 and 1653 for **1n** and **2e**, respectively. Hydrogen atoms were located by difference Fourier techniques and included in the structure

factor calculation with a B_{eq} thermal parameter equal to that of the corresponding carrier atom, but non refined. The atomic scattering factors, with the real and imaginary dispersion corrections for all atomic species, were calculated according to Cromer and Waber [16]. The final R indexes were 0.075 (R_w 0.070) and 0.059 (R_w 0.055) for **1n** and **2e**, respectively. Final positional parameters, equivalent thermal factor for non-hydrogen atoms, bond distances, bond angles and torsion angles have been deposited with the Cambridge Crystallographic Data Centre as Supplementary Material. Copies of the data can be obtained by application to the Director, CCDC, 12 Union Road, Cambridge obtained, free of charge, on CB2 1EZ, UK (Fax: + 44-1223-336-033 or E-mail: teched@chemcrys.cam.ac.uk).

5.2.2. Molecular modeling

Molecular models of **1n** and **2e** were constructed using the CRYSIN command of the software package SYBYL [18] starting from the crystallographic fractional coordinates of the two molecules. The crystal structure of 4*S*,3*R*-cromakalim [12] was retrieved from the October 1996 version 5.12 release of the Cambridge Crystallographic Database (CSD) [19] using the CSD/QUEST routine interfaced with SYBYL (refcode of 4*S*,3*R*-cromakalim: JAHMEK). For molecular modeling purposes, the retrieved structure was converted into its degenerate 4*R*,3*S*-geometry through the SYBYL/INVERT option (this command inverts the sign of all the torsional angles leading to a geometry having unmodified interatomic distances and internal energy). The invertion of chirality was performed because it has been reported that the 4*R*,3*S* is 100–200 times more active than the 4*S*,3*R*-enantiomer of cromakalim [20]. The molecular models of **1n** and **2e** were superimposed on that of cromakalim by minimizing the root mean square (RMS) distance relative to the following six atoms: C2, C3, C6, C7 of the bicyclic frames, C and O of the carbonyl group. The fitting procedure was accomplished through the SYBYL/FIT command. SYBYL and CSD/QUEST were run on a Silicon Graphics Indigo XS24 workstation.

5.3. 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 ≈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 on 2.5 mL organ baths containing Krebs salt solution of 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 or barium chloride-induced contraction (1 μM and 5 mM respectively). Drugs were dissolved in DMSO and added to the organ bath in cumulative manner in the range 1–25 nL (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 antagonists. Each tissue was used only for one concentration–response curve of tested compound in presence or absence of inhibitors such as glibenclamide (0.1 mM), or tetraethylammonio (1 mM) or 4-aminopyridine (10 mM), or barium chloride (5 mM). When the activity of these compounds was observed in the presence of barium chloride we did not use the phenylephrine to induce a contraction because the barium concentration used contracted by itself the aorta rings.

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