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# Synthesis by microwave irradiation of a substituted benzoxazine parallel library with preferential relaxant activity for guinea pig trachealis

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## Abstract

An efficient, facile, and practical parallel combinatorial synthesis of substituted-benzoxazines under microwave irradiation was described. The procedure involved the use of a microwave oven especially designed for organic synthesis suitable for parallel synthesis of solution libraries. A demonstration 19-membered library of substituted *N,N*-dimethyl- and *N*-methyl-benzoxazine amide derivatives, structurally related to the potassium channel opener cromakalim, was generated by both conventional and microwave procedures, achieving a reduction from 7 h to 30–36 min in library generation time for the microwave approach. All the synthesized compounds were tested using the *in vitro* models of rat aorta and guinea pig trachea rings pre-contracted with phenylephrine and carbachol, respectively. All *N,N*-dimethyl amide derivatives showed a relaxant activity higher on guinea pig trachea rings than on rat aorta rings.

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**Keywords:** Benzoxazine; Parallel synthesis; ATP-sensitive potassium channels; Microwave; Relaxant activity

## 1. Introduction

As part of a search for new relaxant agents, and in order to develop an economical, rapid and safe method, in this paper we reported the first parallel synthesis in solution of benzoxazine library using microwave irradiation. Traditional methods of organic synthesis are orders of magnitude too slow to satisfy the demand for these compounds. The efficiency of microwave flash-heating chemistry in dramatically reducing reaction times (reduced from days and hours to minutes and seconds) has recently been proven in several different fields of organic chemistry. We believe that the time saved by using microwaves is potentially important in traditional organic synthesis but could be of even greater importance in high-speed combinatorial and medicinal chemistry [1,2]. Clearly, the ability of microwave technology to rapidly synthesize

organic compounds would be of significant benefit for library generation and its potential as a feature tool for drug-discovery programs has recently been recognized [1,2].

Potassium channels represent a very diversified group of ionic channels [3–5]. Many hypotensive or myorelaxant agents such as aprikalim [6], cromakalim (CRK) [7], pinacidil [8] or diazoxide, have the properties to open the subtype of potassium channels called ATP-sensitive potassium channels ( $K_{ATP}$ ) [9]. Potassium channels have also been identified in airways, particularly it has been shown that small  $Ca^{2+}$  activated, delayed-rectifier and ATP-sensitive potassium channels play distinct roles in airway electrophysiology and pharmacology. Therefore, airway potassium channels represent a suitable pharmacological target for the development of new effective therapeutic options in the treatment of asthma and chronic obstructive pulmonary disease [10–12]. Using the bioisosterism concept, in previous works [13,14] we have synthesized some benzoxazine derivatives (A and B) variously substituted, related to CRK (Fig. 1).

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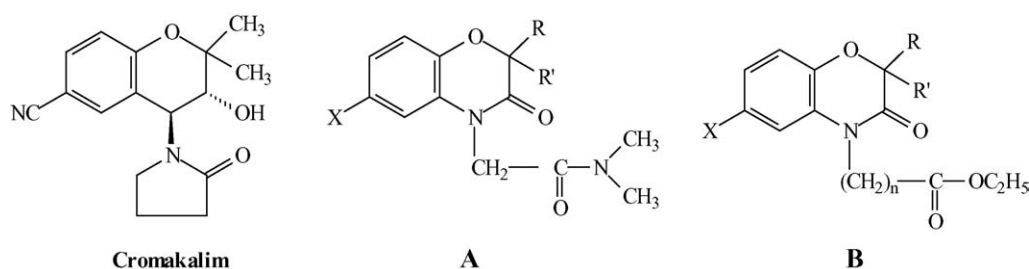


Fig. 1. The structure of cromakalim and 1,4-benzoxazine amide (A) and ester (B) derivatives.

We have previously shown that vasorelaxant activities of the synthesized compounds, evaluated on phenylephrine pre-contracted rat aorta ring deprived of endothelium, demonstrated that several ester derivatives **B** possess interesting biological properties producing vasorelaxation greater than 50% at concentration of  $10^{-4}$  M and some compounds were expected to be  $K_{ATP}$  channel openers, since their activity was blocked by glibenclamide. In contrast the amide derivatives **A** were found poorly active since their vasorelaxant activities fell below 50% at concentration of  $10^{-4}$  M [13].

Comparison of the crystal structures of compound 2-[3,4-dihydro-3-oxo-6-( $\Delta^2$ -thiazolin-2-yl)-2H-1,4-benzoxazin-4-yl]-*N,N*-dimethylacetamide with ethyl 2-[3,4-dihydro-3-oxo-6-2H-1,4-benzoxazin-4-yl]-acetate (series **A** and **B**, respectively) and **CRK** suggested that the relatively low activity of the amide derivatives, compared with the corresponding esters, depends on lack of adequate shape complementarity with the considered receptors rather than on unfavorable conformational characteristics [13].

On the basis of biological data reported in literature [15] on some *N,N*-disubstituted **CRK** analogs, in the present paper we have evaluated if the synthesized amide derivatives, devoid of the vasorelaxant activity on aorta, as previously shown by us [13], retain, on the contrary, the activity for the airway smooth muscle. Along these lines, some molecular modifications were conducted: different alkyl chains between benzoxazine nucleus and *N,N*-dimethyl amide group was introduced. We synthesized also a second series of compounds containing only one methyl group on the amide moiety to evaluate if the presence of dialkyl groups were fundamental for the selectivity of action on airway smooth muscle.

The substituents inserted at the 6-position of the 1,4-benzoxazine ring are only those previously reported displaying a higher vasorelaxant activity. Concerning the 2-position of the 1,4-benzoxazine ring, the introduction of two methyl groups was considered. These modifications provided the new 1,4-benzoxazine-*N,N*-dimethylamide (**5a–h**) and *N*-monomethylamide (**6a–l**) derivatives. Herein, we report on the preparation using microwave irradiation and the *in vitro* evaluation of novel benzoxazine derivatives.

## 2. Chemistry

The new *N,N*-dimethyl and *N*-methyl amide derivatives **5** and **6**, respectively, listed in Table 2 were prepared according

to the procedure showed in Scheme 1. The synthetic procedure was performed using both a conventional and microwave heating method. In the latter case a microwave oven was used (ETHOS 1600, Milestone) especially designed for organic synthesis, which can accommodate a rotor with 36 vessels (MultiPREP 36) suitable for parallel synthesis of solution libraries. All reactions were performed in closed vessels. The experimental conditions used in our work were similar to those used by conventional heating, with the same amount of starting reagent and volume of solvent. The temperature of the reaction mixtures was monitored directly by a microwave-transparent fluoroptic probe.

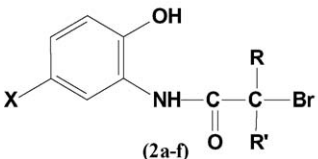
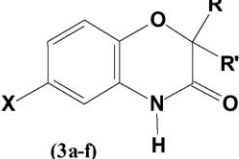
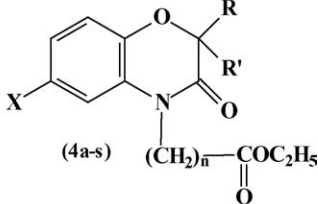




Reagents and solvent are loaded into the individual reaction chamber with the product outlet closed. Prior to library generation we undertook a study to optimize the chemical events associated with microwave library generation. In fact we always optimize a number of single reactions to determine a suitable irradiation power and time and the cooling times required between irradiations. We found the optimal microwave conditions to be a cycle of 2–4 min of irradiation at 200–400 W followed by 3 min off, repeated three times, giving a total reaction event time of 12–18 min, which had allowed all model reactions to reach completion. Upon completion of the reactions the products are dispensed out of the individual reaction vessels by opening the plug allowing the reaction products to be directly dispensed into a workup vessel.

Amides **2a–f** were obtained by acylation of 2-amino-4-*X*-phenol (**1**) with bromoacetyl bromide or 2-bromoisobutyryl bromide in  $\text{CHCl}_3$  in the presence of sodium hydrogencarbonate. Cyclization in the presence of potassium carbonate in DMF afforded benzoxazine derivatives **3a–f**. These were converted into the ester intermediates **4a–o** by condensation with ethyl 2-bromoacetate, ethyl 3-bromopropionate or ethyl 4-bromobutyrate in sodium hydride/DMF. The 6-( $\Delta^2$ -thiazolin-2-yl) derivatives **4p–s** were obtained by reaction of the corresponding 6-cyano derivatives with stoichiometric amounts of 2-aminoethanethiol hydrochloride in absolute ethanol and triethylamine solution. The ethyl ester derivatives **4a–s** were converted by microwave irradiation in the corresponding amide derivatives **5a–h**, **6a–l** by reaction in dimethylformamide solution with the appropriate amines. Conventional heating experimental conditions of compounds **2a–f**, **3a–f** and **4a–s**, was previously reported by us [13–14].

The final compounds **5a–h**, **6a–l** reported in Table 2, were purified by chromatography on a silica gel column and fur-

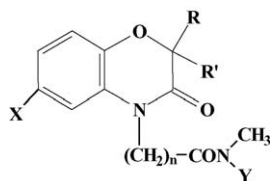
Table 1

Conventional heating versus microwave irradiation for intermediate compounds (2a–f, 3a–f and 4a–s)<sup>a</sup>

<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">  <p>(2a-f)</p> </div> <div style="text-align: center;">  <p>(3a-f)</p> </div> <div style="text-align: center;">  <p>(4a-s)</p> </div> </div>										
Compounds	X	R = R'	n	Conventional heating <sup>b</sup>			Microwave irradiation			
				Yield <sup>c</sup> (%)	Time (min)	Temperature (°C)	Yield <sup>c</sup> (%)	Time (min)	Power <sup>d</sup> (W)	Temperature (°C)
2a	Cl	H	–	–	180	40	–	12	200	40
2b	NO <sub>2</sub>	H	–	–	180	40	–	12	200	40
2c	CN	H	–	–	180	40	–	12	200	40
2d	Cl	CH <sub>3</sub>	–	–	180	40	–	12	200	40
2e	NO <sub>2</sub>	CH <sub>3</sub>	–	–	180	40	–	12	200	40
2f	CN	CH <sub>3</sub>	–	–	180	40	–	12	200	40
3a	Cl	H	–	62	180	80	92	15	300	80
3b	NO <sub>2</sub>	H	–	70	180	80	96	15	300	80
3c	CN	H	–	80	180	80	95	15	300	80
3d	Cl	CH <sub>3</sub>	–	65	180	80	90	15	300	80
3e	NO <sub>2</sub>	CH <sub>3</sub>	–	68	180	80	90	15	300	80
3f	CN	CH <sub>3</sub>	–	70	180	80	94	15	300	80
4a	Cl	H	1	40	180	40	87	18	400	40
4b	NO <sub>2</sub>	H	1	66	180	40	91	18	400	40
4c	CN	H	1	88	180	40	96	18	400	40
4d	Cl	CH <sub>3</sub>	1	62	180	40	90	18	400	40
4e	NO <sub>2</sub>	CH <sub>3</sub>	1	55	180	40	92	18	400	40
4f	CN	CH <sub>3</sub>	1	53	180	40	90	18	400	40
4g	Cl	H	2	50	180	40	90	18	400	40
4h	NO <sub>2</sub>	H	2	65	180	40	94	18	400	40
4i	CN	H	2	38	180	40	88	18	400	40
4k	CN	CH <sub>3</sub>	2	41	180	40	86	18	400	40
4l	Cl	H	3	55	180	40	85	18	400	40
4m	NO <sub>2</sub>	H	3	59	180	40	90	18	400	40
4n	CN	H	3	68	180	40	90	18	400	40
4o	CN	CH <sub>3</sub>	3	80	180	40	92	18	400	40
4p		H	1	67	180	70	94	18	400	70
4q		CH <sub>3</sub>	1	70	180	70	96	18	400	70
4r		H	3	60	180	70	92	18	400	70
4s		CH <sub>3</sub>	3	58	180	70	90	18	400	70

<sup>a</sup> The experimental conditions used on microwave irradiation were similar to those used by conventional heating, with the same amount of starting reagent and volume of solvent.<sup>b</sup> Oil bath.<sup>c</sup> Isolated purified yield.<sup>d</sup> The delivered microwave power was continuously and dynamically adjusted to precisely the defined temperature (±1 °C).

Table 2

Conventional heating versus microwave irradiation for final compounds (**5a–h** and **6a–l**)<sup>a</sup>

Compounds	X	Y	R = R'	n	Conventional heating <sup>b</sup>			Microwave irradiation			
					Yield <sup>c</sup> (%)	Time (min)	Temperature (°C)	Yield <sup>c</sup> (%)	Time (min)	Power <sup>d</sup> (W)	Temperature (°C)
<b>5a</b>	Cl	CH <sub>3</sub>	H	2	60	240	70	90	18	300	70
<b>5b</b>	NO <sub>2</sub>	CH <sub>3</sub>	H	2	58	240	70	86	18	300	70
<b>5c</b>	CN	CH <sub>3</sub>	H	2	50	240	70	84	18	300	70
<b>5d</b>	CN	CH <sub>3</sub>	CH <sub>3</sub>	2	52	240	70	80	18	300	70
<b>5e</b>	Cl	CH <sub>3</sub>	H	3	56	240	70	85	18	300	70
<b>5f</b>	NO <sub>2</sub>	CH <sub>3</sub>	H	3	61	240	70	92	18	300	70
<b>5g</b>		CH <sub>3</sub>	H	3	60	240	70	94	18	300	70
<b>5h</b>		CH <sub>3</sub>	CH <sub>3</sub>	3	60	240	70	90	18	300	70
<b>6a</b>	Cl	H	H	1	63	240	70	95	18	300	70
<b>6b</b>	NO <sub>2</sub>	H	H	1	58	240	70	90	18	300	70
<b>6c</b>	CN	H	H	1	59	240	70	88	18	300	70
<b>6d</b>	Cl	H	CH <sub>3</sub>	1	65	240	70	96	18	300	70
<b>6e</b>	NO <sub>2</sub>	H	CH <sub>3</sub>	1	62	240	70	94	18	300	70
<b>6f</b>	CN	H	CH <sub>3</sub>	1	58	240	70	88	18	300	70
<b>6g</b>	Cl	H	H	2	54	240	70	85	18	300	70
<b>6h</b>	NO <sub>2</sub>	H	H	2	52	240	70	85	18	300	70
<b>6i</b>	Cl	H	H	3	50	240	70	82	18	300	70
<b>6k</b>		H	H	1	68	240	70	93	18	300	70
<b>6l</b>		H	CH <sub>3</sub>	1	72	240	70	96	18	300	70

<sup>a</sup> The experimental conditions used on microwave irradiation were similar to those used by conventional heating, with the same amount of starting reagent and volume of solvent.

<sup>b</sup> Oil bath.

<sup>c</sup> Isolated purified yield.

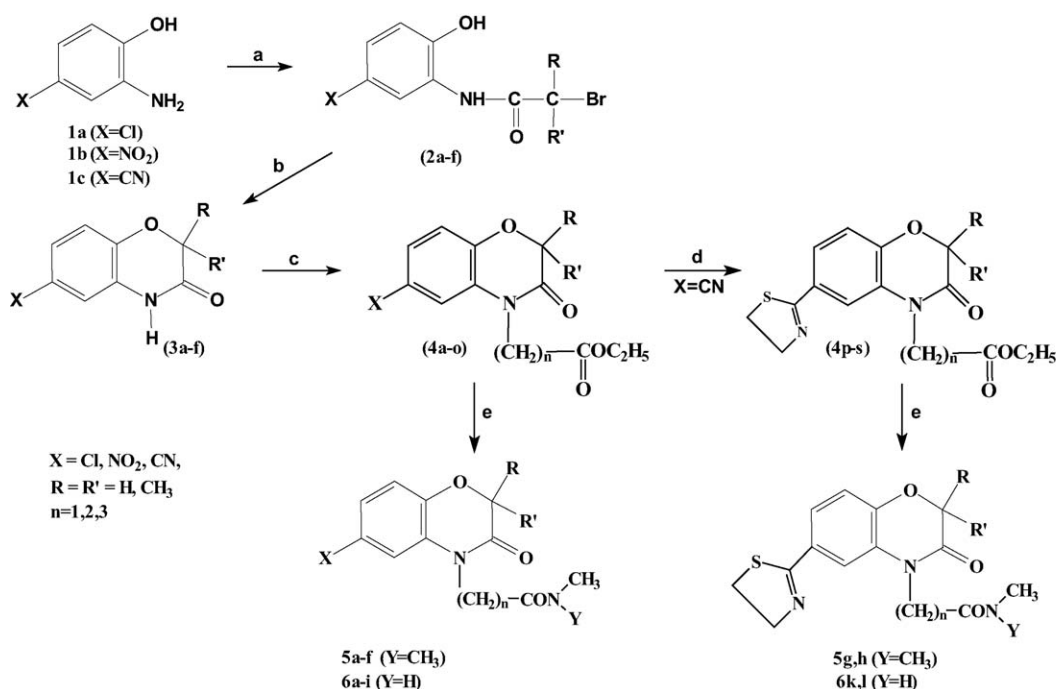
<sup>d</sup> The delivered microwave power was continuously and dynamically adjusted to precisely the defined temperature ( $\pm 1$  °C).

ther crystallized from an appropriate solvent (yields ranging between 80% and 96%).

Comparison of the microwave library with the conventional-generated library is favorable; in fact microwave approach gave the desired compounds in better yields than those obtained by conventional heating and the overall times for the synthesis were considerably reduced from 3–4 h to 12–18 min (Tables 1 and 2). All final compounds were characterized by <sup>1</sup>H NMR and MS and the data were consistent with the considered structures.

### 3. Biological assays

The pharmacological study was performed to investigate, firstly if new compounds showed a vasorelaxant and/or an airway smooth muscle relaxation-activity. This study was performed by using rat isolated aorta rings pre-contracted with phenylephrine (PE) and guinea pig trachea rings pre-contracted with carbachol. 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 ( $K_v$ ), to



Scheme 1. Reagents and conditions: (a) bromoacetyl bromide or 2-bromoisobutyl bromide,  $\text{NaHCO}_3$ ,  $\text{CHCl}_3$ ,  $\mu\text{v}$ , 40 °C; (b) anhydrous  $\text{K}_2\text{CO}_3$ , DMF,  $\mu\text{v}$ , 80 °C; (c) ethyl 2-bromoacetate or ethyl 3-bromopropionate or ethyl 4-bromobutyrate,  $\text{NaH}$ , DMF,  $\mu\text{v}$ , 40 °C; (d) 2-aminoethanethiol hydrochloride, triethylamine/absolute EtOH,  $\mu\text{v}$ , 70 °C; (e) methylamine or di methylamine, DMF,  $\mu\text{v}$ , 70 °C.

elucidate a putative mechanism of action of our compounds we performed *in vitro* assays using selective potassium channel blockers such as glibenclamide (GLY) for the  $\text{K}_{\text{ATP}}$ , and 4-aminopyridine (4-AMP) as unselective potassium channel blocker. Compounds that showed a vasorelaxation higher than 50% but with a different behavior to **CRK** were tested to investigate on probable calcium channels blocking activity.

#### 4. Results and discussion

The smooth muscle relaxant activity of new compounds, in the concentration range of 1–100  $\mu\text{M}$ , was determined by the effects on phenylephrine pre-contracted rat isolated aorta rings and on carbachol pre-contracted guinea pig isolated trachea rings, respectively. In the present paper we also reported the activity, on guinea pig isolated trachea rings, of the preceding synthesized compounds (**Aa–h**) which were devoid of appreciable vasorelaxant activity on aorta rings [13].

The relaxant activity of all tested compounds is summarized in Table 3, where the relaxation effect at 100  $\mu\text{M}$  was reported. For comparison the percentage of relaxation values of compounds (**Aa–h**) described in preceding work [13] are included in Table 3. Additionally, the percentage of relaxation values of the reference compound **CRK** is given.

The activity of compounds that showed a relaxation greater than 50% on rat aorta rings (**5g**, **5h**, **6e**, **6f**) or on guinea pig trachea rings (**Aa**, **Ab**, **5a**, **5e**, **5g**, **5h**, **6f**) was also expressed as  $\text{EC}_{50}$  ( $\mu\text{M}$ ) (Table 4).

Results confirmed data reported in literature, about the selectivity of action for the *N,N*-dimethyl amide derivatives

**Aa–h**. In fact, our results showed that these compounds were more active on trachea than on aorta rings and some of those have shown a selective activity to relax guinea pig trachea rings. The most favorable selectivity for trachea smooth muscle was showed by nitro derivatives (**Ab** and **Ae**).

Analog results were obtained for new synthesized compounds (**5a–h**) which generally exhibited more activity to relax trachea than aorta rings. Only compound **5d** showed a similar low vasorelaxant activity on both tissues.

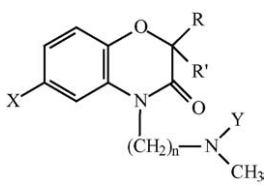
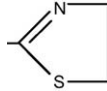
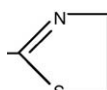
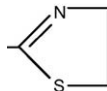
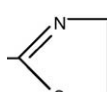
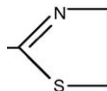
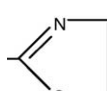
Analysis of the structures of *N,N*-dimethylamide derivatives endowed with highest vasorelaxant activity suggest that a chloro substituent or a thiazolidine ring in 6-position is favorable for the relaxant activity on trachea smooth muscle (i.e. compounds **Aa**, **5a**, **5e**, **5g** and **5h** with percent of relaxation values of 82.0, 64.0, 52.1, 95.4 and 76.5, respectively). Moreover, the two methyl groups, in several *N,N*-dimethyl derivatives, at the 2-position of the 1,4-benzoxazine ring seem to influence negatively the relaxant activity observed on trachea rings. Indeed the 2,2-dimethyl derivatives (**Ae**, **Af**, and **5d**) revealed to be less active than their corresponding dimethyl derivatives (**Ab–c** and **5c**) except for **Ah** that showed a similar low vasorelaxant activity than corresponding dimethyl derivative **Ag**. The results show that the length of the alkyl chain did not play an important role in the relaxant activity.

In conclusion the *N,N*-dimethyl compounds which were generally devoid of the vasorelaxant activity for the aorta, retain however, the activity for the trachea.

Conversion of the *N,N*-dimethyl to *N*-monomethyl derivatives (**6a–l**) gave compounds that exhibited major vasorelax-



Table 3  
Relaxant activity on rat aorta and guinea pig trachea rings

						
Compounds	X	Y	R = R'	n	% of relaxation (aorta) 10 <sup>-4</sup> M	% of relaxation (trachea) 10 <sup>-4</sup> M
<b>Aa</b>	Cl	CH <sub>3</sub>	H	1	45.8 ± 3.4 (n = 3)	82.0 ± 18.0 (n = 3) **
<b>Ab</b>	NO <sub>2</sub>	CH <sub>3</sub>	H	1	33.0 ± 6.2 (n = 3)	61.8 ± 9.0 (n = 6) *
<b>Ac</b>	CN	CH <sub>3</sub>	H	1	0 (n = 3)	31.6 ± 6 (n = 5) *
<b>Ad</b>	Cl	CH <sub>3</sub>	CH <sub>3</sub>	1	27.7 ± 4.6 (n = 3)	32.1 ± 10.4 (n = 4)
<b>Ae</b>	NO <sub>2</sub>	CH <sub>3</sub>	CH <sub>3</sub>	1	0 (n = 2)	41.1 ± 7.8 (n = 4) **
<b>Af</b>	CN	CH <sub>3</sub>	CH <sub>3</sub>	1	4.3 ± 2.0 (n = 3)	20.1 ± 5.3 (n = 4) *
<b>Ag</b>		CH <sub>3</sub>	H	1	8.9 ± 2.5 (n = 4)	35.1 ± 2.6 (n = 3) ***
<b>Ah</b>		CH <sub>3</sub>	CH <sub>3</sub>	1	16.8 ± 1.4 (n = 4)	41.4 ± 8.0 (n = 4) *
<b>5a</b>	Cl	CH <sub>3</sub>	H	2	19.3 ± 11.6 (n = 4)	64.0 ± 9.0 (n = 6) **
<b>5b</b>	NO <sub>2</sub>	CH <sub>3</sub>	H	2	11.7 ± 2.1 (n = 3)	41.0 ± 11.6 (n = 4)
<b>5c</b>	CN	CH <sub>3</sub>	H	2	13.5 ± 6.0 (n = 4)	30.2 ± 4.3 (n = 4) *
<b>5d</b>	CN	CH <sub>3</sub>	CH <sub>3</sub>	2	23.1 ± 4.1 (n = 3)	19.9 ± 5.8 (n = 3)
<b>5e</b>	Cl	CH <sub>3</sub>	H	3	4.0 ± 1.5 (n = 3)	52.1 ± 12 (n = 6) **
<b>5f</b>	NO <sub>2</sub>	CH <sub>3</sub>	H	3	13.6 ± 5 (n = 3)	39.8 ± 10.1 (n = 4) *
<b>5g</b>		CH <sub>3</sub>	H	3	68.6 ± 7.6 (n = 6)	95.4 ± 3.0 (n = 6) **
<b>5h</b>		CH <sub>3</sub>	CH <sub>3</sub>	3	97.6 ± 0.8 (n = 8)	76.5 ± 8.4 (n = 10) *
<b>6a</b>	Cl	H	H	1	29.6 ± 7.6 (n = 6)	24.3 ± 7.6 (n = 4)
<b>6b</b>	NO <sub>2</sub>	H	H	1	37.5 ± 14.4 (n = 3)	40.4 ± 8.0 (n = 4)
<b>6c</b>	CN	H	H	1	28.7 ± 12.1 (n = 5)	17.4 ± 5.4 (n = 3)
<b>6d</b>	Cl	H	CH <sub>3</sub>	1	43.7 ± 11.6 (n = 5)	27.9 ± 9.1 (n = 3)
<b>6e</b>	NO <sub>2</sub>	H	CH <sub>3</sub>	1	60.8 ± 10.0 (n = 4)	26.7 ± 2.8 (n = 3) *
<b>6f</b>	CN	H	CH <sub>3</sub>	1	58.4 ± 18.1 (n = 3)	67.2 ± 8.2 (n = 6)
<b>6g</b>	Cl	H	H	2	2.7 ± 1.8 (n = 3)	19.3 ± 3.7 (n = 3)
<b>6h</b>	NO <sub>2</sub>	H	H	2	5.1 ± 2 (n = 3) *	34.4 ± 6.6 (n = 6) **
<b>6i</b>	Cl	H	H	3	19.9 ± 4.9 (n = 3)	37.8 ± 10.8 (n = 7)
<b>6k</b>		H	H	1	29.1 ± 9 (n = 5)	13.6 ± 3.8 (n = 3)
<b>6l</b>		H	CH <sub>3</sub>	1	39.1 ± 11.1 (n = 5)	15.1 ± 6 (n = 3)
<b>CRK</b>					83.1 ± 2 (n = 35)	49.5 ± 7.2 (n = 4)

Results are expressed as mean ± S.E.M. (number of separate experiments) of percent relaxation. Significance between percent relaxation observed on aorta and trachea rings was calculated by GraphPad Calculation Program using a Student's *t*-test at two way, significance are expressed as follow.

\* *P* < 0.05.; \*\* *P* < 0.01; \*\*\* *P* < 0.001.

Table 4

Vasorelaxant activity expressed as EC<sub>50</sub> (μM) of active compounds and cromakalim (**CRK**) on phenylephrine-induced contraction (**a**) in rat thoracic aorta rings and (**b**) on carbachol-induced contraction in guinea pig trachea rings

Compounds ( <b>a</b> )	EC <sub>50</sub>	Compounds ( <b>b</b> )	EC <sub>50</sub>
<b>5g</b>	41 (29–70)	<b>Aa</b>	51 (35–101)
<b>5h</b>	47 (33–65)	<b>Ab</b>	100 (53–200)
<b>6e</b>	69 (30–119)	<b>5a</b>	74 (51–103)
<b>6f</b>	90 (45–180)	<b>5e</b>	200 (174–240)
		<b>5g</b>	63 (35–100)
		<b>5h</b>	60 (28–100)
		<b>6f</b>	76 (41–103)
<b>CRK</b>	0.7 (0.4–1)		

Results are expressed as mean (95% confidential limits) of several experiments (4–8).

ant activity on aorta rings in comparison to preceding *N,N*-dimethyl derivatives. In fact compounds **Ae** and **Ae** were unable to relax vascular smooth muscles, while the respective *N*-monomethyl derivatives **6c** and **6e** showed a significant relaxant activity (28.7% and 60.8%, respectively). All these compounds showed no selectivity for the smooth muscle trachea; in fact they generally showed similar activity for both tissues (**6a**, **6b**, **6c**, **6f**, **6k**). Only compounds **6e** and **6h** revealed an activity significantly different on aorta and trachea.

In conclusion these results show that introduction of the second methyl group on the amide function was detrimental to the vasorelaxant activity on the aorta ring while it is mandatory for the activity–selectivity for the trachea.

As an extension to these investigations subsequent mechanistic study of the more interesting compounds that showed a relaxation higher than 60% on guinea pig trachea (**Aa**, **Ab**, **5a**, **5g**, **5h**, **6f**), revealed that the relaxant activity in guinea pig trachea was not inhibited by either glibenclamide (GLY; 0.1 mM), a potent and selective blocker of ATP-sensitive K<sup>+</sup> channels, or 4-aminopiridine (4-AMP; 10 mM); only the activity of compound **5a** was significantly inhibited by both inhibitors tested; in fact the relaxation of compound **5a** (100 μM) in presence of GLY or 4-AMP was 30.3 ± 10% and 45.7 ± 13.9%, respectively. On the other hand **CRK** relaxation of trachea was significantly inhibited by either GLY or 4-AMP. These data suggest that the compounds are not K<sub>ATP</sub> channel openers. For these compounds (**Aa**, **Ab**, **5g**, **5h**, **6f**) to investigate on a possibility to block calcium voltage-dependent channels, we performed some experiments to evaluate the capability to inhibit the KCl (60 mM) induced contraction by the selected compounds. Only compounds **5g** and **5h**, characterized by a thiazoline ring at the 6-position and a length of the alkyl chain *n* = 3, at concentration of 30 μM significantly reduced 60 mM KCl induced contraction of rat aorta rings; on the other hand vehicle (DMSO 1:100) did not modify KCl induced contraction (Fig. 2).

The obtained biological data let us to hypothesize that the structural modifications performed in this new series, were able to influence their action; in fact, probably, some compounds act by inhibition of calcium channels (**5g**, **5h**) instead of opening potassium channels.

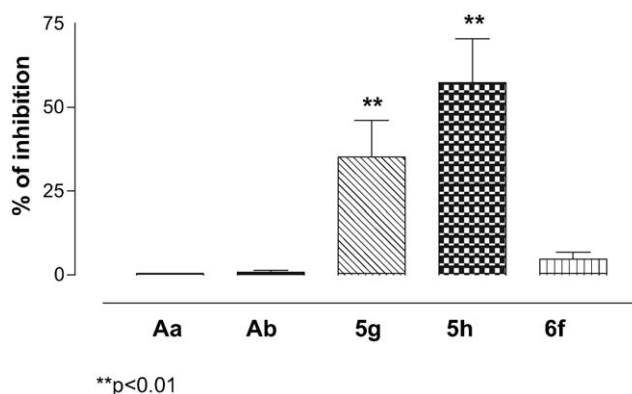


Fig. 2. Inhibition effect of compounds **Aa**, **Ab**, **5g**, **5h** and **6f** (30 μM) of KCl (60 mM) induced contraction. Data are expressed as mean ± S.E.M. inhibition (%) of four to six single rat aorta rings.

In conclusion, our data indicates that several of the new *N,N* dimethylamide derivatives can represent potential candidates as lead structures in designing new compounds with preferential activity on guinea pig trachealis, some of them may be acting on calcium channels. It is known that asthma is a very common pathology. The possibility to have a preferential action on airway and not on vascular smooth muscle could be useful when it is necessary to have a relaxation of the airway smooth muscle without effect on vessel and then on blood pressure.

Instead, regarding the microwave heating, we conclude that for this library the principal advantage gained by the microwave procedure is a significant reduction in library generation time. We are currently using our microwave methodology to develop larger libraries for biological screening.

## 5. Experimental protocols

### 5.1. Chemistry

Synthesis was performed using a microwave oven especially designed for organic synthesis. The experiments were carried out in standard Pyrex glassware chamber. The temperature of the reaction mixture was monitored directly by a microwave-transparent fluoroptic probe. All melting points (m.p.) were determined on a capillary m.p. apparatus and are uncorrected. <sup>1</sup>H NMR spectra were recorded on a Bruker WM 500 spectrometer. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane. The following abbreviations are used: bs = broad single, d = doublet, t = triplet, q = quartet, m = multiplet, s = singlet. Mass spectrometric detection was performed using a MicroMass (Manchester, UK) QuattroII LC triple-quadrupole mass spectrometer, equipped with a heated nebulizer as the APCI source. Silica gel F<sub>254</sub> (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, N, S,) and the results were within ±0.4% of



the theoretical values. Anhydrous  $\text{Na}_2\text{SO}_4$  was used as drying agent for organic extraction. All solvent evaporation was performed under reduced pressure. 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.

*5.1.1. General procedure for the synthesis of 3,4-dihydro-3-oxo-2H-1,4-benzoxazine derivatives (3a–f)*

The selected alkyl bromide (15 mmol) was slowly added to an ice-bath cooled solution of 2-amino-4X-phenol (**1**) (10 mmol) in saturated solution of sodium hydrogencarbonate (15 ml) and  $\text{CHCl}_3$  (20 ml). The reaction mixture for every compound was introduced into the single reaction vessel and simultaneously the desired parameters (microwave power, temperature and time) were set as reported in Table 1. The reaction mixture was monitored by TLC (diethyl ether/*n*-hexane 1:1 v/v, as eluent). After 12 min the layers were separated and the organic phase was (washed with  $\text{H}_2\text{O}$ ) dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated in vacuum to provide crude products **2a–f** as brown oils, which were used without further purification. The solution of crude products and anhydrous  $\text{K}_2\text{CO}_3$  (10 mmol) in 25 ml of DMF was introduced into the single reaction vessel and simultaneously the desired parameters (microwave power, temperature and time) were set as reported in Table 1. The reaction mixture was monitored by TLC (diethyl ether/*n*-hexane 1:1 v/v, as eluent). After 15 min the reaction mixture was poured into  $\text{H}_2\text{O}$  (100 ml) and extracted several times with  $\text{CHCl}_3$ . The combined organic extracts were dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated under reduced pressure. The resulting solid was crystallized from appropriate solvents.

*5.1.1.1. 3a.* Compound **3a** was synthesized starting from **2a**, yield 92% m.p. 90–92° C (diethyl ether).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  8.45 (bs, 1H, NH), 6.96 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.94 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.82 (s, 1H<sub>5</sub>, Ar–H) and 4.63 ppm (s, 2H, O–CH<sub>2</sub>C=O).

*5.1.1.2. 3b.* Compound **3b** was synthesized starting from **2b**, yield 96%, m.p. 96–98° C (diethyl ether).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  10.9 (bs, 1H, NH), 7.92 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.83 (s, 1H<sub>5</sub>, Ar–H), 7.22 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), and 4.85 ppm (s, 2H, O–CH<sub>2</sub>C=O).

*5.1.1.3. 3c.* Compound **3c** was synthesized starting from **2c**, yield 95%, m.p. 106–108° C (diethyl ether, *n*-hexane).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  8.82 (bs, 1H, NH), 7.35 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.30 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.98 (s, 1H<sub>5</sub>, Ar–H) and 4.82 ppm (s, 2H, O–CH<sub>2</sub>C=O).

*5.1.1.4. 3d.* Compound **3d** was synthesized starting from **2d**, yield 90%, m.p. 76–78° C (diethyl ether).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  9.7 (bs, 1H, NH), 6.95 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.90 (s,

1H<sub>5</sub>, Ar–H), 6.82 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), and 1.53 ppm (s, 6H, CH<sub>3</sub>).

*5.1.1.5. 3e.* Compound **3e** was synthesized starting from **2e**, yield 90%, m.p. 84–86° C (diethyl ether, *n*-hexane).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  9.4 (bs, 1H, NH), 7.94 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.80 (s, 1H<sub>5</sub>, Ar–H), 7.06 (d, 1H<sub>7</sub> Ar–H,  $J$  = 8.4 Hz), and 1.62 ppm (s, 6H, CH<sub>3</sub>).

*5.1.1.6. 3f.* Compound **3f** was synthesized starting from **2f**, yield 94%, m.p. 97–99° C (diethyl ether, *n*-hexane).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  9.90 (bs, 1H, NH), 7.02 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.95 (s, 1H<sub>5</sub>, Ar–H), 6.92 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), and 1.53 ppm (s, 6H, CH<sub>3</sub>).

*5.1.2. General procedure for the synthesis of benzoxazine ester derivatives (4a–o)*

The selected intermediate **3** (11 mmol) was dissolved in DMF (30 ml). This solution was cooled with an ice bath and NaH (60% in oil dispersion 0.16 mol) was slowly added. After 10 min the appropriate bromo ester (17 mmol) was added. The reaction mixture for every compound was introduced into the single reaction vessel and simultaneously the desired parameters (microwave power, temperature and time) were set as reported in Table 1. Then the reaction was poured into cold water (250 ml) and the mixture extracted with  $\text{CHCl}_3$ . The organic layer was washed several times with  $\text{H}_2\text{O}$ , dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated under reduced pressure. The resulting solid was crystallized from appropriate solvents.

*5.1.2.1. 4a.* Compound **4a** was synthesized starting from **3a** and ethyl 2-bromoacetate, yield 87%, m.p. 116–118° C (diethyl ether, *n*-hexane).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  6.91 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.89 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.67 (s, 1H<sub>5</sub>, Ar–H), 4.60 (s, 2H, CH<sub>2</sub>N), 4.55 (s, 2H, O–CH<sub>2</sub>C=O), 4.21 (q, 2H, OCH<sub>2</sub>) and 1.24 ppm (t, 3H, CH<sub>3</sub>,  $J$  = 7.3 Hz).

*5.1.2.2. 4b.* Compound **4b** was synthesized starting from **3b** and ethyl 2-bromoacetate, yield 91%, m.p. 117–119° C (diethyl ether).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.90 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.60 (s, 1H<sub>5</sub>, Ar–H), 7.11 (d, 1H<sub>7</sub> Ar–H,  $J$  = 8.4 Hz), 4.78 (s, 2H, CH<sub>2</sub>N), 4.70 (s, 2H, O–CH<sub>2</sub>C=O), 4.27 (q, 2H, OCH<sub>2</sub>) and 1.30 ppm (t, 3H, CH<sub>3</sub>,  $J$  = 7.3 Hz).

*5.1.2.3. 4c.* Compound **4c** was synthesized starting from **3c** and ethyl 2-bromoacetate, yield 96%, m.p. 144–146° C (ethyl alcohol).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.32 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.10 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 7.00 (s, 1H<sub>5</sub>, Ar–H), 4.72 (s, 2H, CH<sub>2</sub>N), 4.63 (s, 2H, O–CH<sub>2</sub>C=O), 4.24 (q, 2H, OCH<sub>2</sub>) and 1.29 ppm (t, 3H, CH<sub>3</sub>,  $J$  = 7.3 Hz).

*5.1.2.4. 4d.* Compound **4d** was synthesized starting from **3d** and ethyl 2-bromoacetate, yield 90%, m.p. 86–88° C (diethyl ether, *n*-hexane).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  6.98 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.80 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.66 (s, 1H<sub>5</sub>,

Ar–H), 4.57 (s, 2H, CH<sub>2</sub>N), 4.24 (q, 2H, OCH<sub>2</sub>), 1.51 (s, 6H, CH<sub>3</sub>) and 1.27 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.5. 4e.** Compound **4e** was synthesized starting from **3e** and ethyl 2-bromoacetate, yield 92%, m.p. 132–134 °C (isopropylether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.92 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.65 (s, 1H<sub>5</sub>, Ar–H), 7.20 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.74 (s, 2H, CH<sub>2</sub>N), 4.32 (q, 2H, OCH<sub>2</sub>), 1.50 (t, 6H, CH<sub>3</sub>) and 1.30 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.6. 4f.** Compound **4f** was synthesized starting from **3f** and ethyl 2-bromoacetate, yield 90%, m.p. 63–64 °C (diethyl ether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.34 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.06 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 6.94 (s, 1H<sub>5</sub>, Ar–H), 4.462 (s, 2H, CH<sub>2</sub>N), 4.27 (q, 2H, OCH<sub>2</sub>), 1.57 (s, 6H, CH<sub>3</sub>) and 1.30 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.7. 4g.** Compound **4g** was synthesized starting from **3a** and ethyl 3-bromopropionate, yield 90%, m.p. 87–88 °C (diethyl ether, dichloromethane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.01 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.93 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 6.85 (s, 1H<sub>5</sub>, Ar–H), 4.60 (s, 2H, O–CH<sub>2</sub>C=O), 4.25 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 4.15 (q, 2H, OCH<sub>2</sub>), 2.70 (t, 2H, CH<sub>2</sub>C=O, *J* = 7.3 Hz) and 1.25 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.8. 4h.** Compound **4h** was synthesized starting from **3b** and ethyl 3-bromopropionate, yield 94%, m.p. 103–104 °C (diethyl ether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.98 (s, 1H<sub>5</sub>, Ar–H), 7.90 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.00 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.70 (s, 2H, O–CH<sub>2</sub>C=O), 4.25 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 4.10 (q, 2H, OCH<sub>2</sub>), 2.70 (t, 2H, CH<sub>2</sub>C=O, *J* = 7.3 Hz) and 1.25 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.9. 4i.** Compound **4i** was synthesized starting from **3c** and ethyl 3-bromopropionate, yield 88%, m.p. 85–87 °C (*n*-hexane, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.15 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.00 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 6.94 (s, 1H<sub>5</sub>, Ar–H), 4.72 (s, 2H, O–CH<sub>2</sub>C=O), 4.33 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 4.24 (q, 2H, OCH<sub>2</sub>), 2.84 (t, 2H, CH<sub>2</sub>C=O, *J* = 7.3 Hz) and 1.25 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.10. 4k.** Compound **4k** was synthesized starting from **3f** and ethyl 3-bromopropionate, yield 86%, m.p. 63–64 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.35 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.25 (s, 1H<sub>5</sub>, Ar–H), 7.00 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.25 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 4.10 (q, 2H, OCH<sub>2</sub>), 2.60 (t, 2H, CH<sub>2</sub>C=O, *J* = 7.3 Hz) 1.51 (s, 6H, CH<sub>3</sub>) and 1.25 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.11. 4l.** Compound **4l** was synthesized starting from **3a** and ethyl 4-bromobutyrate, yield 85%, m.p. 66–67 °C, diethyl ether). <sup>1</sup>H NMR: δ 7.13 (s, 1H<sub>5</sub>, Ar–H), 6.96 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.91 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.58 (s, 2H, O–CH<sub>2</sub>C=O), 4.17 (q, 2H, OCH<sub>2</sub>), 3.95 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 2.42 (t, 2H, CH<sub>2</sub>C=O, *J* = 7.3 Hz), 1.98 (m, 2H, CH<sub>2</sub>) and 1.25 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.12. 4m.** Compound **4m** was synthesized starting from **3b** and ethyl 4-bromobutyrate, yield 90%, m.p. 94–96 °C (diethyl ether). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 8.10 (s, 1H<sub>5</sub>, Ar–H), 8.07 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.08 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.72 (s, 2H, O–CH<sub>2</sub>C=O), 4.19 (q, 2H, OCH<sub>2</sub>), 4.05 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 2.45 (t, 2H, CH<sub>2</sub>C=O, *J* = 7.3 Hz), 2.01 (m, 2H, CH<sub>2</sub>) and 1.26 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.13. 4n.** Compound **4n** was synthesized starting from **3c** and ethyl 4-bromobutyrate, yield 90% m.p. 92–94 °C (diethyl ether). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.48 (s, 1H<sub>5</sub>, Ar–H), 7.32 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.04 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.67 (s, 2H, O–CH<sub>2</sub>C=O), 4.17 (q, 2H, OCH<sub>2</sub>), 3.97 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 2.42 (t, 2H, CH<sub>2</sub>C=O, *J* = 7.3 Hz), 1.96 (m, 2H, CH<sub>2</sub>) and 1.26 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.2.14. 4o.** Compound **4o** was synthesized starting from **3f** and ethyl 4-bromobutyrate, yield 92%, m.p. 89–90 °C (diethyl ether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.34 (s, 1H<sub>5</sub>, Ar–H), 7.25 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.98 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.12 (q, 2H, OCH<sub>2</sub>), 3.93 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 2.39 (t, 2H, CH<sub>2</sub>C=O, *J* = 7.3 Hz), 1.93 (m, 2H, CH<sub>2</sub>), 1.49 (s, 6H, CH<sub>3</sub>) and 1.26 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

### 5.1.3. General procedure for the preparation of benzoxazine derivatives (**4p–s**)

A mixture of the appropriate ester (10 mmol) and 2-aminoethanethiol hydrochloride (10 mmol) in absolute ethanol and triethylamine (10 mmol) solution was introduced into the single reaction vessel and simultaneously the desired parameters (microwave power, temperature and time) were set as reported in Table 3. After 18 min the reaction was cooled and the ethanol removed under reduced pressure. 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 (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under reduced pressure. The resulting solid was crystallized from appropriate solvents.

**5.1.3.1. 4p.** Compound **4p** was synthesized starting from **4c**, yield 94%, m.p. 107–109 °C (diethyl ether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 6.88 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.75 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 6.50 (s, 1H<sub>5</sub>, Ar–H), 4.65 (s, 2H, CH<sub>2</sub>N), 4.61 (s, 2H, O–CH<sub>2</sub>C=O), 4.45 (t, 2H, CH<sub>2</sub>N=, *J* = 8.2 Hz), 4.23 (q, 2H, OCH<sub>2</sub>, *J* = 7.3 Hz), 3.43 (t, 2H, CH<sub>2</sub>S, *J* = 8.2 Hz) and 1.21 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.3.2. 4q.** Compound **4q** was synthesized starting from **4f**, yield 96%, m.p. 63–64 °C (diethyl ether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 6.78 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.68 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 6.48 (s, 1H<sub>5</sub>, Ar–H), 4.62 (s, 2H, CH<sub>2</sub>N), 4.41 (t, 2H, CH<sub>2</sub>N=, *J* = 8.2 Hz), 4.24 (q, 2H, OCH<sub>2</sub>, *J* = 7.3 Hz), 3.23 (t, 2H, CH<sub>2</sub>S, *J* = 8.2 Hz), 1.51 (s, 6H, CH<sub>3</sub>) and 1.21 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.3.3. 4r.** Compound **4r** was synthesized starting from **4n**, yield 92%, m.p. 108–109 °C (diethyl ether). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.70 (s, 1H<sub>5</sub>, Ar–H), 7.55 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.10 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.81 (s, 2H, O–CH<sub>2</sub>C=O), 4.60 (s, 2H, CH<sub>2</sub>N), 4.35 (t, 2H, CH<sub>2</sub>N=, *J* = 8.2 Hz), 4.21 (m, 2H, CH<sub>2</sub>), 4.15 (q, 2H, OCH<sub>2</sub>, *J* = 7.3 Hz), 3.52 (t, 2H, CH<sub>2</sub>S, *J* = 8.2 Hz), 2.44 (t, CH<sub>2</sub>C=O, *J* = 7.3 Hz) and 1.26 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.3.4. 4s.** Compound **4s** was synthesized starting from **4o**, yield 90%, m.p. 60–61 °C (diethyl ether). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.68 (s, 1H<sub>5</sub>, Ar–H), 7.49 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.00 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.57 (s, 2H, CH<sub>2</sub>N), 4.32 (t, 2H, CH<sub>2</sub>N=, *J* = 8.2 Hz), 4.15 (m, 2H, CH<sub>2</sub>), 4.02 (q, 2H, OCH<sub>2</sub>, *J* = 7.3 Hz), 3.48 (t, 2H, CH<sub>2</sub>S, *J* = 8.2 Hz), 2.42 (t, CH<sub>2</sub>C=O, *J* = 7.3 Hz), 1.54 (s, 6H, CH<sub>3</sub>) and 1.26 ppm (t, 3H, CH<sub>3</sub>, *J* = 7.3 Hz).

**5.1.4. General procedure for the synthesis of N,N'-dimethylamide (5a–h) and N-methylamide (6a–l) benzoxazine derivatives**

In a solution of the appropriate ester (**4a–s**) (10 mmol) in DMF (14 ml) were added the selected amine (2.0 M solution in tetrahydrofuran, 10 mmol). The mixture was introduced into the single reaction vessel and simultaneously the desired parameters (microwave power, temperature and time) were set as reported in Table 3. After 18 min the reaction was cooled and the DMF 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 (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under reduced pressure. The resulting solid was crystallized from appropriate solvents.

**5.1.4.1. 5a.** Compound **5a** was synthesized starting from **4g** and dimethylamine, yield 90%, m.p. 104–105 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.10 (s, 1H<sub>5</sub>, Ar–H), 6.98 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.90 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.60 (s, 2H, O–CH<sub>2</sub>C=O), 4.22 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 3.00 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.96 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>) and 2.68 ppm (t, 2H, CH<sub>2</sub>CON, *J* = 8.1 Hz). ESI-MS: *m/z* = 283.1 [M + H]<sup>+</sup>.

**5.1.4.2. 5b.** Compound **5b** was synthesized starting from **4h** and dimethylamine, yield 86%, m.p. 160–161 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 8.01 (s, 1H<sub>5</sub>, Ar–H), 7.91 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.06 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.70 (s, 2H, O–CH<sub>2</sub>C=O), 4.28 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 2.98 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.94 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>) and 2.72 ppm (t, 2H, CH<sub>2</sub>CON, *J* = 8.1 Hz). ESI-MS: *m/z* = 294.2 [M + H]<sup>+</sup>.

**5.1.4.3. 5c.** Compound **5c** was synthesized starting from **4i** and dimethylamine, yield 84%, m.p. 120–121 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.40 (s, 1H<sub>5</sub>, Ar–H), 7.31 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.05 (d, 1H<sub>7</sub>, Ar–H,

*J* = 8.4 Hz), 4.70 (s, 2H, O–CH<sub>2</sub>C=O), 4.25 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 2.98 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.90 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>) and 2.71 ppm (t, 2H, CH<sub>2</sub>CON, *J* = 8.1 Hz). ESI-MS: *m/z* = 274.2 [M + H]<sup>+</sup>.

**5.1.4.4. 5d.** Compound **5d** was synthesized starting from **4k** and dimethylamine, yield 80%, m.p. 185–186 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.74 (s, 1H<sub>5</sub>, Ar–H), 7.57 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.00 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.29 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 2.99 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.91 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.68 (t, 2H, CH<sub>2</sub>CON, *J* = 8.1 Hz) and 1.48 ppm (s, 6H, CH<sub>3</sub>). ESI-MS: *m/z* = 302.3 [M + H]<sup>+</sup>.

**5.1.4.5. 5e.** Compound **5e** was synthesized starting from **4l** and dimethylamine, yield 85%, m.p. 89–90 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.34 (s, 1H<sub>5</sub>, Ar–H), 6.97 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.88 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.60 (s, 2H, O–CH<sub>2</sub>C=O), 3.95 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 3.01 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.98 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.40 (t, 2H, CH<sub>2</sub>CON, *J* = 8.1 Hz) and 2.00 ppm (m, 2H, CH<sub>2</sub>). ESI-MS: *m/z* = 297.8 [M + H]<sup>+</sup>.

**5.1.4.6. 5f.** Compound **5f** was synthesized starting from **4m** and dimethylamine, yield 92%, m.p. 120–121 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 8.29 (s, 1H<sub>5</sub>, Ar–H), 7.91 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 7.04 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.69 (s, 2H, O–CH<sub>2</sub>C=O), 4.04 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 2.97 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.96 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.42 (t, 2H, CH<sub>2</sub>CON, *J* = 8.1 Hz) and 2.03 ppm (m, 2H, CH<sub>2</sub>). ESI-MS: *m/z* = 308.2 [M + H]<sup>+</sup>.

**5.1.4.7. 5g.** Compound **5g** was synthesized starting from **4r** and dimethylamine, yield 94%, m.p. 142–144 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.72 (s, 1H<sub>5</sub>, Ar–H), 7.44 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.98 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.62 (s, 2H, O–CH<sub>2</sub>C=O), 4.43 (t, 2H, CH<sub>2</sub>N=, *J* = 8.2 Hz), 4.05 (t, 2H, CH<sub>2</sub>N, *J* = 8.2 Hz), 3.41 (t, 2H, CH<sub>2</sub>S, *J* = 8.2 Hz), 2.95 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.94 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.39 (t, 2H, CH<sub>2</sub>CO, *J* = 8.1 Hz) and 2.03 ppm (m, 2H, CH<sub>2</sub>). ESI-MS: *m/z* = 348.2 [M + H]<sup>+</sup>.

**5.1.4.8. 5h.** Compound **5h** was synthesized starting from **4s** and dimethylamine, yield 90%, m.p. 105–106 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.65 (s, 1H<sub>5</sub>, Ar–H), 7.42 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.92 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 4.05 (t, 2H, CH<sub>2</sub>N=, *J* = 8.2 Hz), 3.42 (t, 2H, CH<sub>2</sub>S, *J* = 8.2 Hz), 2.94 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.93 (s, 3H, N(CH<sub>3</sub>)<sub>2</sub>), 2.38 (t, 2H, CH<sub>2</sub>CO, *J* = 8.1 Hz), 2.10 (m, 2H, CH<sub>2</sub>) and 1.51 ppm (m, 6H, CH<sub>3</sub>). ESI-MS: *m/z* = 376.5 [M + H]<sup>+</sup>.

**5.1.4.9. 6a.** Compound **6a** was synthesized starting from **4a** and methylamine, yield 95%, m.p. 202–203 °C (diethyl ether). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.26 (s, 1H<sub>5</sub>, Ar–H), 7.12 (d, 1H<sub>8</sub>, Ar–H, *J* = 8.2 Hz), 6.99 (d, 1H<sub>7</sub>, Ar–H, *J* = 8.4 Hz), 6.19



(bs, 1H, NH), 4.66 (s, 2H, CH<sub>2</sub>N), 4.47 (s, 2H, O–CH<sub>2</sub>C=O) and 2.82 ppm (m, 3H, NCH<sub>3</sub>). ESI-MS:  $m/z$  = 255.7 [M + H]<sup>+</sup>.

**5.1.4.10. 6b.** Compound **6b** was synthesized starting from **4b** and methylamine, yield 90%, m.p. 105–106 °C (diethyl ether). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.97 (s, 1H<sub>5</sub>, Ar–H), 7.94 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.07 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.14 (bs, 1H, NH), 4.78 (s, 2H, CH<sub>2</sub>N), 4.56 (s, 2H, O–CH<sub>2</sub>C=O) and 2.84 ppm (m 3H, NCH<sub>3</sub>). ESI-MS:  $m/z$  = 266.2 [M + H]<sup>+</sup>.

**5.1.4.11. 6c.** Compound **6c** was synthesized starting from **4c** and methylamine, yield 88%, m.p. 194–195 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.45 (s, 1H<sub>5</sub>, Ar–H), 7.34 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.08 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.10 (bs, 1H, NH), 4.78 (s, 2H, CH<sub>2</sub>N), 4.50 (s, 2H, O–CH<sub>2</sub>C=O) and 2.91 ppm (m, 3H, NCH<sub>3</sub>). ESI-MS:  $m/z$  = 246.2 [M + H]<sup>+</sup>.

**5.1.4.12. 6d.** Compound **6d** was synthesized starting from **4d** and methylamine, yield 96%, m.p. 201–203 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 6.99 (s, 1H<sub>5</sub>, Ar–H), 6.91 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.83 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 5.95 (bs, 1H, NH), 4.38 (s, 2H, CH<sub>2</sub>N), 2.75 (m, 3H, NCH<sub>3</sub>) and 1.45 ppm (s, 6H, CH<sub>3</sub>). ESI-MS:  $m/z$  = 283.6 [M + H]<sup>+</sup>.

**5.1.4.13. 6e.** [16]Compound **6e** was synthesized starting from **4e** and methylamine, yield 94%, m.p. 210–212 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.88 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.86 (s, 1H<sub>5</sub>, Ar–H), 6.99 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 5.95 (bs, 1H, NH), 4.62 (s, 2H, CH<sub>2</sub>N), 2.79 (m, 3H, NCH<sub>3</sub>) and 1.50 ppm (s, 6H, CH<sub>3</sub>). ESI-MS:  $m/z$  = 294.2 [M + H]<sup>+</sup>.

**5.1.4.14. 6f.** Compound **6f** was synthesized starting from **4f** and methylamine, yield 88%, m.p. 244–245 °C (diethyl ether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.01 (s, 1H<sub>5</sub>, Ar–H), 6.98 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.92 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.01 (bs, 1H, NH), 4.42 (s, 2H, CH<sub>2</sub>N), 2.78 (m, 3H, NCH<sub>3</sub>) and 1.48 ppm (s, 6H, CH<sub>3</sub>). ESI-MS:  $m/z$  = 274.3 [M + H]<sup>+</sup>.

**5.1.4.15. 6g.** Compound **6g** was synthesized starting from **4g** and methylamine, yield 85%, m.p. 143–144 °C (diethyl ether). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.15 (s, 1H<sub>5</sub>, Ar–H), 6.95 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.86 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.10 (bs, 1H, NH), 4.56 (s, 2H, O–CH<sub>2</sub>C=O), 4.20 (t, 2H, CH<sub>2</sub>N,  $J$  = 8.2 Hz), 2.81 (m, 3H, NCH<sub>3</sub>) and 2.55 ppm (m, 2H, CH<sub>2</sub>CO). ESI-MS:  $m/z$  = 269.1 [M + H]<sup>+</sup>.

**5.1.4.16. 6h.** Compound **6h** was synthesized starting from **4h** and methylamine, yield 85%, m.p. 195–196 °C (diethyl ether). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 8.09 (s, 1H<sub>5</sub>, Ar–H), 7.91 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.08 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 5.80 (bs, 1H, NH), 4.69 (s, 2H, O–CH<sub>2</sub>C=O), 4.32 (t, 2H, CH<sub>2</sub>N,

$J$  = 8.2 Hz), 2.80 (m, 3H, NCH<sub>3</sub>) and 2.58 ppm (m, 2H, CH<sub>2</sub>CO). ESI-MS:  $m/z$  = 280.3 [M + H]<sup>+</sup>.

**5.1.4.17. 6i.** Compound **6i** was synthesized starting from **4i** and methylamine, yield 82%, m.p. 127–128 °C (diethyl ether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.15 (s, 1H<sub>5</sub>, Ar–H), 6.95 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.89 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 5.93 (bs, 1H, NH), 4.58 (s, 2H, O–CH<sub>2</sub>C=O), 3.95 (t, 2H, CH<sub>2</sub>N,  $J$  = 8.2 Hz), 2.83 (m, 3H, NCH<sub>3</sub>), 2.28 (m, 2H, CH<sub>2</sub>CO) and 2.00 (m, 2H, CH<sub>2</sub>). ESI-MS:  $m/z$  = 283.1 [M + H]<sup>+</sup>.

**5.1.4.18. 6k.** Compound **6k** was synthesized starting from **4p** and methylamine, yield 93%, m.p. 227–228 °C (diethyl ether, ethyl alcohol). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.67 (s, 1H<sub>5</sub>, Ar–H), 7.45 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 7.00 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.37 (bs, 1H, NH), 4.74 (s, 2H, O–CH<sub>2</sub>C=O), 4.56 (s, 2H, CH<sub>2</sub>N), 4.41 (t, 2H, CH<sub>2</sub>N=,  $J$  = 8.2 Hz), 3.41 (t, 2H, CH<sub>2</sub>S,  $J$  = 8.2 Hz) and 2.81 ppm (m, 3H, NCH<sub>3</sub>). ESI-MS:  $m/z$  = 306.4 [M + H]<sup>+</sup>.

**5.1.4.19. 6l.** Compound **6l** was synthesized starting from **4q** and methylamine, yield 96%, m.p. 158–159 °C (diethyl ether, *n*-hexane). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.52 (s, 1H<sub>5</sub>, Ar–H), 7.37 (d, 1H<sub>8</sub>, Ar–H,  $J$  = 8.2 Hz), 6.90 (d, 1H<sub>7</sub>, Ar–H,  $J$  = 8.4 Hz), 6.24 (bs, 1H, NH), 4.46 (s, 2H, CH<sub>2</sub>N), 4.33 (t, 2H, CH<sub>2</sub>N=,  $J$  = 8.2 Hz), 3.32 (t, 2H, CH<sub>2</sub>S,  $J$  = 8.2 Hz) 2.79 (m, 3H, NCH<sub>3</sub>) and 1.46 ppm (s, 6H, CH<sub>3</sub>). ESI-MS:  $m/z$  = 334.4 [M + H]<sup>+</sup>.

## 5.2. Pharmacology

### 5.2.1. Vasorelaxant activity on rat aorta ring (in vitro assay)

Vasorelaxant activity was performed using a previously described method [17] with minor modifications. Male Wistar rats (200–250 g; Nossan, Italy) were housed in an environment with controlled temperature (21–24 °C) and lighting (12:12 h light–darkness cycle). Standard chow and drinking water were provided ad libitum. A period of 7 days was allowed for acclimatization of rats before any experimental manipulation was undertaken. All the experiments were conducted following the principles of laboratory animal care (law N.86/609/CEE), as well as specific national law (N.116/1992). Animals were anesthetized by inhalation of isoflurane and after exsanguinations 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 intimate 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<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 1.3; KH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25.0; and glucose 11.7. The solution was maintained at 37 °C and bubbled with 95% O<sub>2</sub>–5% CO<sub>2</sub> (pH 7.4). Developed tension was measured using an isometric force transducer

(7003 transducer, Ugo Basile, Comerio, Italy) connected to a recorder (Graphtec Linearecorder, 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 vasorelaxants on phenylephrine (PE; 1  $\mu$ M) induced contraction and data were expressed as mean  $\pm$  S.E.M. of PE induced relaxation percentage. To assess the probable activity of compounds to interact with calcium channels activity, we performed another set of experiments where rat aorta rings, after standardization, were contracted twice with KCl 60 mM. The first contraction was induced in absence of drugs while the second contraction was performed in presence of vehicle (2.5  $\mu$ l DMSO) or tested compounds (30  $\mu$ M). Data were calculated in percent of inhibition; the first KCl contraction represented the 100% value.

#### 5.2.2. Airway smooth muscle relaxant activity on guinea pig trachea rings (in vitro assay)

Trachea smooth muscle relaxation was performed using a previously described method [18] with minor modifications. Briefly guinea pigs (250–350 g; Charles River, Italy) were anesthetized by inhalation of isoflurane and after exsanguinations the trachea removed, cleaned of adherent connective tissue, and cut into rings 3 mm in length. The epithelium was removed by gently rubbing with moistened filter paper. Epithelium-denuded rings were mounted under 0.5 g of tension on 2.5 ml organ baths containing Krebs salt solution. The solution was maintained at 37 °C and bubbled with 95% O<sub>2</sub>–5% CO<sub>2</sub> (pH 7.4). Developed tension was measured using an isometric force transducer (7003 transducer, Ugo Basile) connected to a recorder (Graphtec Linearecorder, 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 carbachol-induced contraction (1  $\mu$ M).

Either for aorta then for trachea rings drugs were dissolved in DMSO and added to the organ bath in cumulative manner in the range 2.5–15  $\mu$ l (maximum final concentration of DMSO 0.6%). Drugs that showed a relaxation in concentration-dependent manner with a relaxation over than 50% at the highest used concentration (100  $\mu$ M), were considered for the experiments performed 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 (10  $\mu$ M), or 4-AMP (10 mM).

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