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Il Farmaco 56 (2001) 827–834

IL FARMACO

New 5-substituted-1-(2-hydroxybenzoyl)-benzotriazoles, potassium channel activators. IV

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Received 20 January 2001; accepted 12 June 2001

Abstract

This paper reports the synthesis of a series of new 5-substituted-1-(2-hydroxybenzoyl)-benzotriazoles, which have been tested for their activity as possible activators of potassium channels. In rat aortic rings, the 'opened' derivatives **1a**–**f**, intermediates of synthesis, showed vasorelaxing properties, with appreciable values of potency. However, the most remarkable effects were recorded for the 2-hydroxybenzoylbenzotriazoles **3a**–**f**, which showed full vasorelaxing efficacy and high potency values. The introduction of a 2-hydroxybenzyl substituent in the 1 position of the benzotriazole ring (compound **7**) strongly decreased the activity, showing the importance of the electron-acceptor carbonyl function. The best compound, **3b**, was further investigated, in order to evaluate the possible mechanism of action involved in the vasodilator activity. In the vascular model, different potassium channel blockers inhibited the effects of the compound, and an increase of the levels of membrane depolarisation induced a significant reduction of the recorded responses. Compound **3b** was also tested in a model of isolated rat heart, retroperfused through the aorta and submitted to a global ischemia/reperfusion cycle. In such an experimental condition, **3b** showed an interesting cardioprotective activity. All the above observations are in agreement with the hypothesis of a mechanism linked to the activation of potassium channels.

Keywords: Salicylanilides; Benzotriazoles; Vasodilator activity; Cardioprotection; Potassium channels

1. Introduction

In a previous paper [1], we had reported the synthesis and pharmacological evaluation, as potential potassium channel activators, of a series of 1,2,3-triazolyl-benzimidazolone (**A**) and 1,2,3-triazolyl-benzotriazole (**B**) derivatives (Fig. 1). These compounds had been studied for their structural relationships with the benzimidazolone derivatives NS 004 and NS 1619 (Fig. 1), effective activators of the high conductance calcium activated potassium channels (BK_{Ca}) [2]. None of the triazolyl-benzimidazolones and triazolyl-benzotriazoles showed any improvement, when compared with the reference compound NS 1619. However, the triazolyl-benzotriazole derivatives showed higher vasodilator activity and potency than those of the corresponding benzimidazolone derivatives, indicating the replacement of the benzimidazolone heterocycle with the benzotriazole nucleus as an encouraging choice, for the development of new potential potassium channel openers. A further paper [3] concerning some modifications of the structure and/or substituents of the above mentioned benzimidazolone and benzotriazole derivatives showed that the removal of the benzotriazole or benzimidazolone heterocycles and their replacement by different moieties, such as the benzotriazinone ring, decreased the activity of the compounds. In this work, the investigation of new potential potassium channel activators focused on the preparation of a series of 5-substituted-1-(2-hydroxybenzoyl)-benzotriazoles with structural

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0014-827X/01/$ - see front matter
PII: S0014-827X(01)01146-6

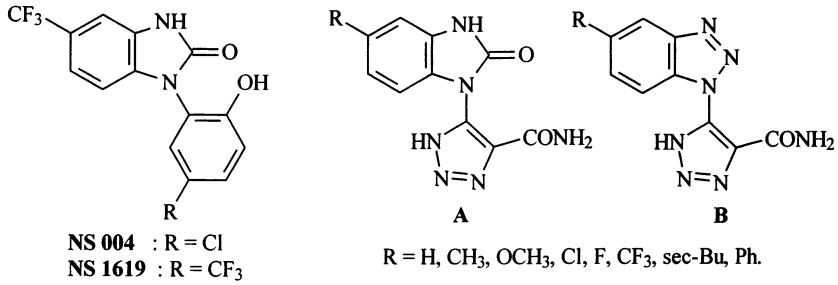


Fig. 1. Comparison between the reference benzimidazolones NS 004 and NS 1619, and the previously synthesised triazolylbenzimidazolones (**A**) and triazolylbenzotriazoles (**B**).

characteristics indicated by the previous experimental evidence as important requirements for such a pharmacological activity.

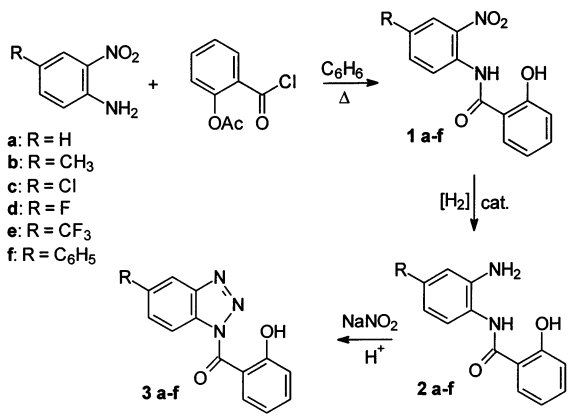
2. Chemistry

The pharmacological evaluation indicated that the activity of the benzotriazole ring was higher than that of the benzimidazolone one [1,3]; therefore, a new structural change involved the triazolyl-benzotriazole derivatives and consisted of the replacement of the 1,2,3-triazole ring with a 2-hydroxybenzoyl substituent, as reported in Scheme 1. This change combined the adoption of a 2-hydroxyphenyl substituent, which is present in the reference compounds NS 004 and NS 1619, with the estrangement of the same substituent from the bicyclic ring and with the shift of the carbonyl function out of the benzimidazolone ring. Thus, the whole structure acquired greater flexibility because the two molecular parts were connected by a bridge consisting of the carbonyl function. In addition, the carbonyl placed in this position could behave as a full hydrogen bond acceptor, whilst the behaviour was different in the benzimidazolone ring because a tautomeric equilibrium might be present.

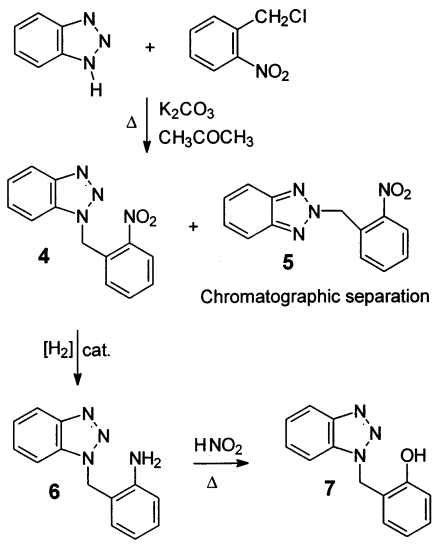
The salicylic acid chloride [4], obtained from the acetylsalicylic acid sodium salt and thionyl chloride, was reacted with the appropriate *ortho*-nitroaniline in benzene solution to give the corresponding *N*-(4-substituted-2-nitrophenyl)-salicylamides **1a**–**f** in good yields. Reduction of the nitro group by catalytic hydrogenation at room temperature and pressure provided the salicyl-(2-amino-anilides) **2a**–**f**, which were cyclised to the expected 5-substituted-1-(2-hydroxybenzoyl)-benzotriazoles **3a**–**f**, by treatment with nitrous acid in 50% acetic acid solution (Scheme 1).

A structural change, useful to evaluate the importance of the carbonyl function, was achieved by the replacement of the 2-hydroxybenzoyl substituent with a 2-hydroxybenzyl group, as reported in Scheme 2. In fact, the introduction of a methylenic group to form a bridge between the two parts of the molecule preserved the structural flexibility, but removed a hydrogen bond acceptor site.

Thus, the benzotriazole was reacted with 2-nitrobenzyl chloride in acetone solution in the presence of anhydrous potassium carbonate to give in 82% yield the expected mixture of the two isomeric benzotriazoles **4** and **5**, substituted in the 1 and 2 positions, respectively.



Scheme 1.



Scheme 2.

Table 1
Physicochemical properties of compounds **1**–**3**

Comp.	Yield (%)	Crystallisation solvent	M.p. (°C)	Mass		Elemental analysis
				M^+	Base	
1a	84	MeOH	154–157	258	121	$C_{13}H_{10}N_2O_4$
1b	79	MeOH	184–186	272	121	$C_{14}H_{12}N_2O_4$
1c	88	AcOEt	189–193	292	121	$C_{13}H_9N_2O_4Cl$
1d	95	MeOH	166–167	276	121	$C_{13}H_9N_2O_4F$
1e	70	MeOH	190–192	326	121	$C_{14}H_9N_2O_4F_3$
1f	85	MeOH/H_2O	178–180	334	214	$C_{19}H_{14}N_2O_4$
2a	89	MeOH	138–140	228	121	$C_{13}H_{12}N_2O_2$
2b	96	EtOH/H_2O	156–162	242	121	$C_{14}H_{14}N_2O_2$
2c	77	MeOH/H_2O	160–163	262	142	$C_{13}H_{11}N_2O_2Cl$
2d	67	EtOH/H_2O	153–156	246	126	$C_{13}H_{11}N_2O_2F$
2e	72	MeOH	195–197	296	121	$C_{14}H_{11}N_2O_2F_3$
2f	93	MeOH/H_2O	172–174	304	65	$C_{19}H_{16}N_2O_2$
3a	52	MeOH	63–66	239	121	$C_{13}H_9N_3O_2$
3b	96	MeOH	88–90	253	121	$C_{14}H_{11}N_3O_2$
3c	92	MeOH	133–135	273	121	$C_{13}H_8N_3O_2Cl$
3d	87	MeOH	93–95	257	121	$C_{13}H_8N_3O_2F$
3e	81	MeOH	110–112	307	121	$C_{14}H_8N_3O_2F_3$
3f	83	MeOH/H_2O	105–107	315	121	$C_{19}H_{13}N_3O_2$

Table 2
^{1}H NMR data (δ, ppm, J, Hz) and ^{13}C NMR data (δ, ppm) for compounds **1e**, **2c**, and **3f** in DMSO-d_6

Comp.	H-3	H-5	H-6	$J_{3,5}$	$J_{5,6}$	H-3′	H-4′	H-5′	H-6′				
^{1}H NMR													
1e	8.41	8.11	8.90	2.5	8.8	7.03	7.46	6.97	7.98				
2c	6.82	6.60	7.25	2.4	8.4	6.95	7.43	6.93	8.00				
3f	8.54	8.14	8.30	2.2	8.4	7.03	[a]	6.97	[a]				
^{13}C NMR													
Comp.	C-1	C-2	C-3	C-4	C-5	C-6	C=O	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
1e [b]	137.51	136.55	122.58	122.49	130.70	123.86	164.14	117.46	156.37	116.54	133.98	119.18	130.70
2c	130.23	144.34	114.28	120.80	115.06	127.61	166.92	116.12	158.74	116.73	133.25	118.34	128.60
3f [c]	129.53	145.93	113.66	120.22	116.78	127.51	158.08	116.94	155.63	115.99	133.06	118.39	129.76

[a] Signal submerged by phenyl resonances (from 7.86 to 7.44 δ).
[b] Other signals: 125.50 (CF_3).
[c] Other signals: 138.49, 128.64, 128.53, and 126.82 (phenyl).

The flash-chromatography of the mixture on silica gel provided 13% of the isomer 2-(2-nitrobenzyl)-benzotriazole (**5**) followed by the 1-(2-nitrobenzyl)substituted isomer **4** in 48% yield.

The nitrobenzyl derivative **4** was reduced by catalytic hydrogenation to the corresponding amino derivative **6**, which was converted to the hydroxy derivative **7** by diazotisation in 10% sulphuric acid and thermal decomposition of the diazonium salt.

The structures of all the new compounds were assigned on the basis of the well-known reaction mechanisms [5] and our previous evidence [1,3,6] and were confirmed by analytical and spectroscopic methods (Table 1). The ^{1}H and ^{13}C NMR spectra of some significant compounds are reported in Table 2. In particular, the structures of compounds **4** and **5** were assigned on the basis of the UV and ^{13}C NMR data. The UV spectra showed the bathochromic shift of the benzenoid band (^1L_b in the Platt notation [7]) described for the 2-substituted-benzotriazoles with respect to the 1-substituted ones [8]: the experimental values are 253 nm for **4** and 273 nm for **5**. The ^{13}C NMR data led to the same conclusions; in fact, the equivalence of the three couples of carbons, 3a:7a, 4:7 and 5:6 in the spectrum of **5** decreases the total number of the signals to 10, whilst in the spectrum of **4** the signals are 13. In addition, downfield shifts of $\cong$11 ppm for C-7a, of $\cong$8 ppm for CH_2 and C-7 and of $\cong$2.5 ppm for C-5 in the spectrum of the 1-substituted compound **4**, due to the aminic nature of the 1-nitrogen, with respect to the iminic nature of the same nitrogen in the 2-substituted compound **5**, are observed. This difference can establish

a good method for the structural attribution to the 1 and 2 substituted benzotriazole isomers, also in the presence of a second substituent on the benzotriazole phenyl ring. In this case, the second substituent causes loss of symmetry to the 2-substituted benzotriazole isomer, invalidating the above-mentioned assignment criterion.

3. Pharmacology

Large conductance calcium-activated potassium channels (BK_{Ca}) are involved in the modulation of vascular smooth muscle tone in different circulatory districts, such as the rabbit pulmonary artery [9], the rat portal vein [10] and the rat aorta [11]. Indeed, the membrane hyperpolarisation induced by an opening of outward potassium-channels causes the inactivation of voltage-operated calcium channels, the lowering of the concentration of free intracellular calcium and consequently a vasorelaxing effect. Thus, the functional evaluation of a vasorelaxing activity of the compounds tested on isolated rat aortae can be considered a reliable preliminary screening method, to detect a possible potassium channel opening effect.

Table 3
Vasorelaxing potency and efficacy exhibited by the tested compounds in aortic preparations pre-contracted by KCl 20 mM

Comp.	pIC_{50}	Efficacy (%)
1a	5.51 ± 0.034	100
1b	5.60 ± 0.015	100
1c	5.16 ± 0.029	100
1d	4.96 ± 0.053	100
1e	5.46 ± 0.052	100
1f	5.23 ± 0.092	100
3a	5.81 ± 0.047	100
3b	6.84 ± 0.098	100
3c	5.61 ± 0.056	100
3d	5.52 ± 0.030	100
3e	5.67 ± 0.089	100
3f	5.48 ± 0.036	100
7	4.71 ± 0.015	100
NS 1619	5.30 ± 0.068	100

Table 4
Potency and efficacy parameters recorded for the selected compound **3b** in control condition, in the presence of increased membrane depolarisation (60 mM KCl) or after the administration of several potassium channel blockers

Treatment	pIC_{50}	Efficacy (%)
Control	6.84 ± 0.098	100
60 mM KCl	5.63 ± 0.016	65 ± 6
+1 mM TEA	6.45 ± 0.014	100
+200 μM Quinine	5.60 ± 0.017	95 ± 3
+3 mM 4-AP	5.38 ± 0.019	97 ± 2
+1 μM Glibenclamide	6.78 ± 0.082	100

Besides, the role of the heart potassium channel in the protection of the myocardium against the ischemia-induced injury has been well assessed [12]. Therefore, also the model of rat isolated and perfused heart, submitted to appropriate ischemia/reperfusion cycles, has been chosen as a pharmacological test.

4. Results and discussion

The pharmacological study was aimed at investigating the potential vasorelaxing properties of the benzotriazole derivatives (**3a**–**f** and **7**), representing the target compounds of the whole synthetic work; however, it seemed interesting to screen also the 2-nitro-salicylanilides (**1a**–**f**), because of their close similarity with the 5-(2′-nitroanilino)-1,2,3-triazoles, 'opened' structure molecules which showed profiles of possible potassium channel activators [13].

As indicated in Table 3, all the tested compounds possessed full vasorelaxing efficacy. The order of magnitude of the potency parameters for the 'opened' compounds **1a**–**f** was interesting and almost comparable with that exhibited by the reference compound. The potency values observed for the salicylbenzotriazoles **3a**–**f** was very satisfactory; indeed, they were all higher than that of NS 1619. The replacement of the carbonyl group with a methylene bridge led to a significant decrease of potency, as revealed by the comparison between compound **7** (possessing the methylene bridge) and its analogue **3a** (possessing the carbonyl bridge).

The best compound, **3b**, underwent further experimental protocols to understand both its possible mechanism of action and to evaluate its possible protective effects in the ischemic myocardium.

In the vascular model, the responses induced by **3b** on aortic smooth muscle were reduced by increased levels of depolarisation. The consequent hypothesis of a potassium channel opening mechanism was further strengthened by the antagonism exerted by TEA, quinine and 4-aminopyridine (4-AP), compounds able to induce a significant rightward shift of the concentration–vasorelaxing response curves. The substantial ineffectiveness of glibenclamide to antagonise the effects of **3b** seemed to exclude the involvement of the ATP-sensitive potassium channel type (these results are summarised in Table 4).

In control hearts, the residual inotropic tension developed after an ischemia/reperfusion cycle dramatically decreased ($14 \pm 8\%$ of the pre-ischemic inotropic tension). Compound **3b** exhibited cardio-protective properties against the ischemic injury: the hearts reached levels of post-ischemic inotropic tension of 45 ± 10 and $62 \pm 11\%$ (of the pre-ischemic inotropic tension), in the organs treated with 0.1 and 1 μM of **3b**, respectively. These values were almost equivalent to

47 ± 12 and 56 ± 13%, recorded in the hearts treated with 0.1 and 1 μM, respectively, of the reference cardioprotective drug aprikalim.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Kofler hot-stage and are uncorrected. IR spectra in Nujol mulls were recorded in a Mattson Genesis series FTIR spectrometer. UV spectra were obtained in a Varian Cary 1 UV–Vis spectrophotometer. ^1H and ^{13}C NMR spectra were recorded with a Bruker AC 200 spectrometer (compounds **1e**, **2c**, **3f**, **4** and **5**) and with a Varian Gemini 2000 spectrometer (other compounds) in DMSO-d_6 in δ units, using TMS as an internal standard. Mass spectra were performed with a Hewlett Packard MS/System 5988. Elemental analyses (C, H, N) were within ± 0.4% of the theoretical values and were performed in a Carlo Erba Elemental Analyser Mod. 1106 apparatus. TLC data were obtained with a Riedel de Haen, 37360 DC-Karten F_{254}, 0.2 mm, eluting with a 1:3 AcOEt–petroleum ether mixture. Petroleum ether corresponds to the fraction boiling at 40–60 °C.

5.1.1. N-(4-Substituted-2-nitrophenyl)-salicylamides *1a–f*

To 6 ml of $SOCl_2$, ice-cooled and stirred, 1.6 g (7.92 mmol) of sodium acetylsalicylate was added. After 20 min the ice-bath was removed and stirring was continued at room temperature for 2 h. The solvent was evaporated in vacuo and the gelatinous residue, consisting of the acid chloride, was dissolved in 40 ml of anhydrous benzene. The appropriate 2-nitroaniline (7.92 mmol) (2-nitroaniline, 4-methyl-2-nitroaniline, 4-chloro-2-nitroaniline, 4-fluoro-2-nitroaniline, 4-trifluoromethyl-2-nitroaniline or 2-nitrobiphenylamine [14]) was added to the solution and the mixture was heated under reflux for 16 h. The solvent was evaporated and the residue was dissolved again in $CHCl_3$. This solution was extracted with 4% $NaHCO_3$, 5% HCl and 10% NaOH. By acidification of the aqueous alkaline extract the title compounds were precipitated, which were collected by filtration and washed with H_2O (Table 1). During the extraction with 10% NaOH a variable amount of the title compounds could precipitate as a sodium salt; in this case the precipitate was collected by filtration, suspended or dissolved in H_2O and acidified to give the desired compound. The remaining product, as a salt soluble in NaOH, was isolated in the usual manner.

5.1.2. N-(2-Aminophenyl)-salicylamide (*2a*), N-(4-fluoro-2-aminophenyl)-salicylamide (*2d*), N-(4-trifluoromethyl-2-aminophenyl)-salicylamide (*2e*) and N-(2-aminobiphenyl)-salicylamide (*2f*)

Ten percent of Pd/C (30–35 mg) was added to a solution of 1.0 mmol of the suitable nitro derivative (**1a**, **1d**, **1e** or **1f**) in 60 ml of AcOH and the mixture was hydrogenated at room temperature and pressure. The catalyst was filtered off, washed with AcOH and the filtrate was evaporated in vacuo. The crude residue was treated with 5% NaOH, paper filtered and the filtrate acidified to give the title compounds (Table 1).

5.1.3. N-(4-Methyl-2-aminophenyl)-salicylamide (*2b*)

Ten percent of Pd/C (150 mg) was added to a solution of 0.850 g (3.12 mmol) of the nitro derivative **1b** in 500 ml of MeOH and the mixture was hydrogenated at room temperature and pressure. The catalyst was filtered off, washed with boiling MeOH and the filtrate was evaporated in vacuo to give the title compound (Table 1).

5.1.4. N-(4-Chloro-2-aminophenyl)-salicylamide (*2c*)

Wet Ni-Raney (100 mg) was added to a solution of 0.500 g (1.70 mmol) of the nitro derivative **1c** in 100 ml of MeOH and the mixture was hydrogenated and worked up as described for the preparation of **2a** (Table 1).

5.1.5. 5-Substituted-1-(2-hydroxybenzoyl)-benzotriazoles *3a–f*

To an ice-cooled (≅ 5 °C) and stirred solution of 2.0 mmol of the appropriate amino derivative (**2a**, **2b**, **2c**, **2d**, **2e** or **2f**) in 20–30 ml of 50% AcOH, 0.165 g (2.40 mmol) of $NaNO_2$ was added portionwise. The ice-bath was removed and stirring was continued for 2 h. The suspension obtained was further diluted with H_2O and the precipitate, consisting of the title compounds, was collected by filtration and washed with H_2O (Table 1).

5.1.6. 1-(2-Nitrobenzyl)-benzotriazole (*4*) and 2-(2-nitrobenzyl)-benzotriazole (*5*)

Anhydrous K_2CO_3 (≅ 2.0 g) was added to a solution of benzotriazole (1.00 g, 8.40 mmol) and 2-nitrobenzyl chloride (1.45 g, 8.45 mmol) in 40 ml of anhydrous acetone and the mixture was heated under reflux for 20 h. The solvent was evaporated in vacuo and the residue was treated with H_2O and extracted with $CHCl_3$. The organic layer was dried and evaporated to give a solid white residue (2.09 g, yield 82%), consisting of a mixture of the two isomers **4** and **5** (TLC analysis: R_f = 0.34 and 0.54, respectively). The mixture was separated by flash-chromatography through silica gel, eluting with a 2:5 mixture of AcOEt–petroleum ether (40–60 °C), at first the isomer **5** and then the isomer **4**.

4: 1.227 g, yield 48%, m.p. 114–116 °C from MeOH. *Anal.* $C_{13}H_{10}N_4O_2$ (C, H, N). MS; *m/z*: 254 [M^+], 106 [100]. UV (ethanol): λ_{max} 216 nm (log ε 3.73) ^1L_a band; λ_{max} 253 nm (log ε 3.70) ^1L_b band. ^{1}H NMR (DMSO, δ, ppm): 6.35 (2H, s, CH_2); 6.84 (1H, H-6′); 7.42 (1H, H-6); 7.55 (1H, H-5); 7.59 (1H, H-4′); 7.65 (1H, H-5′); 7.85 (1H, H-4); 8.09 (1H, H-7); 8.17 (1H, H-3′). ^{13}C NMR (DMSO, δ, ppm): 48.4 (CH_2); 110.7 (C-7); 119.3 (C-4); 124.2 (C-5); 125.2 (C-3′); 127.7 (C-6); 129.3 (C-4′); 129.5 (C-6′); 130.9 (C-1′); 133.2 (C-7a); 134.3 (C-5′); 145.2 (C-3a); 147.5 (C-2′).

5: 0.324 g, yield 13%, m.p. 86–88 °C from MeOH; *Anal.* $C_{13}H_{10}N_4O_2$ (C, H, N). UV (ethanol): λ_{max} 216 nm (log ε 3.78) ^1L_a band; λ_{max} 273 nm (log ε 3.80) ^1L_b band. ^{1}H NMR (DMSO, δ, ppm): 6.36 (2H, s, CH_2); 7.29 (1H, H-6′); 7.43 (2H, H-5 and H-6); 7.65 (1H, H-4′); 7.74 (1H, H-5′); 7.91 (2H, H-4 and H-7); 8.16 (1H, H-3′). ^{13}C NMR (DMSO, δ, ppm): 56.6 (CH_2); 117.9 (C-4 and C-7); 125.1 (C-3′); 126.7 (C-5 and C-6); 129.5 (C-1′); 130.4 (C-4′); 131.6 (C-6′); 134.3 (C-5′); 143.8 (C-3a and C-7a); 147.9 (C-2′).

*5.1.7. 1-(2-Aminobenzyl)-benzotriazole (**6**)*

Ten percent of Pd/C (0.100 g) was added to a solution of 1.20 g (4.72 mmol) of the nitro isomer **4** in 200 ml of MeOH and the mixture was hydrogenated at room temperature and pressure. The catalyst was filtered off, washed with MeOH and the combined filtrate was evaporated in vacuo to give the title compound: 0.986 g, yield 93%, m.p. 104–106 °C from H_2O. *Anal.* $C_{13}H_{12}N_4$ (C, H, N). IR (cm^{-1}): 3434 and 3351 (NH_2). MS; *m/z*: 224 [M^+], 106 [100].

*5.1.8. 1-(2-Hydroxybenzyl)-benzotriazole (**7**)*

To a stirred solution of 0.500 g (2.23 mmol) of the amino derivative **6** in 10 ml of 75% H_2SO_4, a solution of $NaNO_2$ (0.185 g, 2.68 mmol) in 5 ml of H_2O was added dropwise. After 30 min the solution was gradually heated up to 90–95 °C to complete the evolution of N_2 gas (begun at temperature >60 °C). The solution assumed a red colour and the brown oil that separated was extracted with $CHCl_3$. The chloroform layer was extracted with 10% NaOH and the alkaline solution was paper filtered and acidified (pH $\cong$ 3) to precipitate the title compound which was collected by filtration and washed with H_2O: 0.302 g, yield 60%, m.p. 165–167 °C from H_2O. *Anal.* $C_{13}H_{11}N_3O$ (C, H, N). IR (cm^{-1}): 3376 (OH). MS; *m/z*: 225 [M^+], 107 [100].

5.2. Pharmacology

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609.

The pharmacological study was performed with the following vascular procedure. Furthermore, a selected compound was also tested as cardioprotective agent in an ischemia/reperfusion protocol on isolated rat heart.

5.2.1. Vascular protocol

To determine a possible vasodilator mechanism of action, the compounds were tested on isolated thoracic aortae of male normotensive Wistar rats (250–350 g).

The rats were killed by cervical dislocation under light ether anaesthesia and bled. The aortae were immediately excised, freed of extraneous tissues and the endothelium was removed by gently rubbing the intimal surface of the vessels. Aortic rings were suspended, under a preload of 2 g, in 10 ml organ baths, containing Tyrode solution (composition of saline in mM: NaCl, 136.8; KCl, 2.95; $CaCl_2$, 1.80; $MgSO_4 \cdot 7H_2O$, 1.05; NaH_2PO_4, 0.41; $NaHCO_3$, 11.9; glucose, 5.5), thermostated at 37 °C and continuously bubbled with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Basile mod. 7005), connected with a unirecord microdynamometer (Basile mod. 7050).

After an equilibration period of 60 min, the endothelial integrity was confirmed by acetylcholine (ACh) (55 μM)-induced relaxation of norepinephrine (NE, 1 μM)-precontracted tissues. A relaxation $<20\%$ of the NE-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation $\geq 20\%$ (i.e. significant presence of the endothelium), were not used in the experimental procedures. Thirty to forty minutes after confirmation of the endothelium removal, the aortic preparations were contracted by treatment with a single concentration of KCl (20 mM) and when the contraction reached a stable *plateau*, threefold increasing concentrations of the compounds (10 nM–1 mM) were added cumulatively. In parallel sets of experiments, to investigate the influence of a higher level of depolarisation on the responses evoked by the compound tested, the aortic preparations were contracted by 60 mM KCl. Then, threefold increasing concentrations of the compounds (10 nM–1 mM) were added cumulatively.

Preliminary experiments showed that both the KCl (20 and 60 mM)-induced contractions remained constant in a stable tonic state for at least 40 min.

In other sets of experiments, the potassium channel blockers tetraethylammonium chloride (TEA, 1 mM), quinine hydrochloride (200 μM), 4-aminopyridine (4-AP, 3 mM) or glybenclamide (1 μM) were added, before the KCl (20 mM)-induced contraction, followed by the administration of selected compounds.

Norepinephrine hydrochloride, acetylcholine chloride, TEA, quinine hydrochloride, 4-AP and KCl were dissolved in bi-distilled water. Glibenclamide was dissolved by sonication in aqueous NaOH (0.1 N). All the

other synthesised derivatives and the reference compound NS 1619 (RBI) were dissolved (10 mM) in aqueous NaOH (0.1 N). All compounds were dissolved at the concentration of 1 mM and further dilutions were performed in bi-distilled water. All solutions were prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicle.

5.2.2. Cardiac protocol

Normotensive male Wistar rats (250–350 g) were killed by cervical dislocation, under light ether anaesthesia. Immediately, the heart (connected with a portion of thoracic aorta) was excised from the mediastinum and rapidly perfused through a retrograde cannulation of the aorta. The pressure of the perfusion flow was constantly kept at 90 mmHg, to ensure the opening of the aortic valve. The perfusion solution was Tyrode buffer (composition in mM: NaCl, 136.8; KCl, 2.95; $CaCl_2$, 1.80; $MgSO_4 \cdot 7H_2O$, 1.05; NaH_2PO_4, 0.41; $NaHCO_3$, 11.9; glucose, 5.5), thermostated at 37 °C and continuously and richly bubbled with O_2.

The inotropic tension developed by the spontaneously beating hearts was recorded by means of an isometric transducer (Basile mod. 7005), connected with a unirecord microdynamometer (Basile mod. 7050).

After 1 h of equilibration time (pre-ischemic period), the perfusion was suspended and the hearts underwent a 30 min period of global ischemia. Then, the organs were re-perfused for 30 min. The hearts, showing significant arrhythmic episodes during the equilibration time, were discarded.

Together with the control group of heart (receiving the standard Tyrode solution), a second group received the selected synthesised compound (1 or 0.1 μM, dissolved in the perfusion Tyrode solution). A third group of hearts underwent the administration of the reference drug aprikalim (1 or 0.1 μM, dissolved in the perfusion Tyrode solution), a K_{ATP} potassium channel opener, possessing well-established cardioprotective properties [15].

5.2.3. Data analysis

5.2.3.1. Vascular protocol. The efficacy of the vasorelaxing responses was expressed as a maximal relaxant effect (E_{max}), calculated as a percentage of the contractile tone developed by the smooth muscle preparation, after the depolarising stimulus induced by KCl 20 mM. The above parameters were calculated by means of non-linear regression analysis of the sigmoidal concentration–response curves (computer program: GRAPHPAD PRISM), and were expressed as the mean of six experiments. Previous experiments demonstrated an almost complete quantitative equivalence between the contractile responses evoked by the two different concentrations of KCl, since the concentration 20 mM could substantially induce a maximal effect, in endothelium denuded aortic rings.

The parameter of potency of the vasorelaxing effects was expressed as pIC_{50}, representing the negative logarithm of the vasodilator molar concentration determining a half reduction of the contractile tone induced by the contractile agent. The above parameters were calculated by means of non-linear regression analysis of the sigmoidal concentration–response curves (computer program: GRAPHPAD PRISM), and were expressed as the mean ± SEM of six experiments.

The statistical comparison of experimental data was performed by the two-tailed Student's *t*-test and ANOVA. A value of $P < 0.05$ was considered as representative of significant differences.

5.2.3.2. Cardiac protocol. The inotropic tension developed by the hearts at the end of the re-perfusion period was measured and expressed as a percentage of the inotropic tension developed by the hearts at the end of the pre-ischemic period. The values of percentage are reported as mean ± SEM.

The statistical comparison of experimental data was performed by the two-tailed Student's *t*-test and ANOVA. A value of $P < 0.05$ was considered as representative of significant differences.

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