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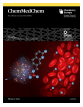

Synthesis and Vasorelaxant Activity of Nitrate–Coumarin Derivatives

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Due to the need for new chemical entities for cardiovascular diseases, we have synthesized a new series of nitrate–coumarins and evaluated their vasorelaxant activity in contraction-relaxation studies using rat aorta rings precontracted with phenylephrine or by depolarization with a high concentration of potassium chloride. Four of the new compounds were able to relax smooth vascular muscle with a similar profile and potency to glyceryl trinitrate (IC_{50} = 12.73 nM) and sodium nitroprusside (IC_{50} = 4.32 nM). Coumarin-7-yl-methyl nitrate (**4**), the best compound within the series, was able to relax smooth vascular muscle in the low nanomolar range (IC_{50} = 1.92 nM). The mechanisms of action have been explored, being the activation of sGC and the opening of K^+ channels involved. Our studies indicate that the new nitrate derivatives are reversible and not deleterious for aortic rings, suggesting that these compounds have a potential interest for the development of new and highly efficient vasodilator drugs.

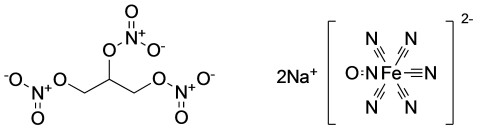
Introduction

Endothelial nitric oxide (NO) is a key compound in the regulation of cardiovascular homeostasis, with outstanding vasodilator properties.[1] It is a gaseous free radical synthesized by the endothelial nitric oxide synthase (eNOS) from L-arginine. NO acts as an endogenous activator of soluble guanylate cyclase (sGC), since it has a high affinity for the heme group, which forms part of the structure of the sGC. The activation of sGC allows the formation of cyclic GMP (cGMP), which acts as a second messenger inducing vasodilation and other effects.[1,2]

NO can also be produced from a chemical donor, with organic nitrates being the most widely used. These drugs can generate NO *in vivo*, regardless of the functional state of the vascular endothelium, so they are used to treat different diseases, including ischemia, heart failure and arterial hypertension. Therefore, the search for new NO donor drugs continues to be an alternative of interest for the treatment of cardiovascular diseases.[3]

The most representative example of nitrovasodilators is nitroglycerin (glyceryl trinitrate, GTN), an organic nitrate ester (R–O–NO_2, Figure 1) introduced into the clinic more than a century ago.[4] Since then, the list of nitrovasodilators used in therapy has been increasing. Other organic nitrate esters used in therapeutics are isosorbide mononitrate, isosorbide dinitrate or pentaerythrityl tetranitrate. Although it has been accepted for years that the generation of NO is involved, there are still many controversies about the mechanism of the vasodilator action of these molecules.[5,6] As for sodium nitroprusside (SNP), a S–nitrosothiol composed of ferrous iron complexed with nitric oxide (NO) and five cyanide ions (Figure 1), used in therapeutics as a vasodilator in hypertensive crisis and cardiovascular emergencies. SNP ability to produce NO depends on the oxidation of sulfhydryl residues (SH) present in sGC.[7,8]

Despite these drugs are widely used in clinical practice, they rapidly induce tolerance and have adverse effects that represent a significant limitation.[5] Therefore, the search for new nitrovasodilators continues to be of interest to increase the available therapeutic arsenal.[3]



Nitroglycerin, GTN — Sodium nitroprusside, SNP

Figure 1. Chemical structures of nitroglycerin (GTN) and sodium nitroprusside (SNP), two of the most representative examples of nitrovasodilators.

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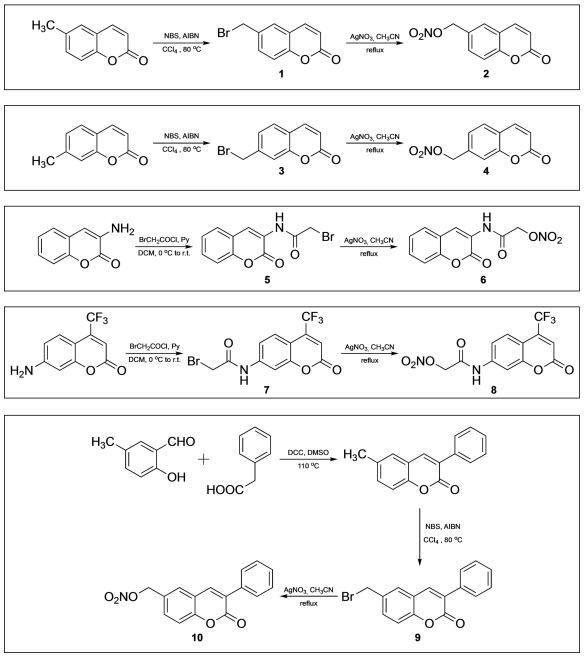
Coumarins represent an important group of secondary metabolites widely distributed in the plant kingdom. Due to their large number of pharmacological and biochemical properties, they constitute an important group of pharmacological agents with potential therapeutic application. Several studies, including those carried out by our group, have shown that a pharmacomodulation of the coumarin structure can give rise to different pharmacological activities: monoamine oxidase inhibition,[9,10] acetyl- and butyrylcholinesterase inhibition,[11,12] antioxidant,[13,14] anti-inflammatory,[15,16] antithrombotic,[17,18] inhibition of β-amyloid aggregation[19] and vasorelaxant.[20–24] The vasorelaxant effects begin to be observed at concentrations in the micromolar range, reaching the higher effects at concentrations in the millimolar range.[20–24]

In the present work, we intend to evaluate the vascular relaxant activity of newly synthesized nitrate–coumarins, obtained with the aim of seeking a possible synergy between the effect of coumarins and a possible NO donor effect. Our experiments also provided new data on the structure-activity relationship of the new series of compounds. In order to explore, for the first time, the potential of nitrate–coumarins, we have measured the vasorelaxant activity of the new compounds in rat aorta rings pre-contracted with phenylephrine or by depolarization with a high concentration of potassium chloride (KCl), using NPS and GTN as reference compounds.

Results and Discussion

The nitrate derivatives **2**, **4**, **6**, **8** and **10** were efficiently synthesized according to the protocol outlined in Scheme 1,



Scheme 1. Reactions and conditions for obtaining the nitrate derivatives **2**, **4**, **6**, **8** and **10**.

starting from the commercially available 6/7-methyl- or 3/7-aminocoumarins, or the previously prepared 6-methyl-3-phenylcoumarin, trough the bromomethyl intermediates **1**, **3**, **5**, **7** and **9**.[25,26] The 6-methyl-3-phenylcoumarin has been previously prepared via Perkin reaction, following the conditions included in the scheme and detailed in reference.[27] All the synthetic details are described in the Supporting Information.

Starting from the 6-methylcoumarin, 7-methylcoumarin or 6-methyl-3-phenylcoumarin, a bromination reaction in the presence of *N*-bromosuccinimide (NBS) and azobisisobutyronitrile (AIBN, catalyst), in carbon tetrachloride (CCl_4), under reflux for 18 hours, was performed to give compounds **1**, **3** and **9**, in yields between 58 and 68%. Starting from the 3-aminocoumarin or 7-amino-4-trifluormethylcoumarin, an acylation reaction in the presence of 2-bromoacetyl chloride, using pyridine in dichloromethane, from 0 °C to room temperature, overnight, afforded the desired coumarins **5** and **7**, in yields of 76 and 83%, respectively. For both reactions, the obtained mixtures were purified by flash chromatography, using a mixture of *n*-hexane and ethyl acetate (9:1), as detailed in the Supporting Information. The nitrate derivatives **2**, **4**, **6**, **8** and **10** were finally obtained starting with the readily synthetized bromomethylcoumarins, in the presence of a silver nitrate ($AgNO_3$) solution, in acetonitrile (CH_3CN), at reflux for 2 hours. The obtained solids were recrystallized from ethanol (EtOH) to give the desired coumarins **2**, **4**, **6**, **8** and **10**, in yields between 70–75%. The substitution patterns of these molecules were selected considering the versatility of their nature, size and volume and position, directing related to their steric hindrance. For this, the possibility of introducing a linker (amide group) between the coumarin scaffold and the nitrate, was also considered. In this way we obtained a small family of chemically versatile nitrate derivatives to perform a preliminary screening on the potential of these molecules as vasodilators.

In the contraction-relaxation studies, isolated aortic rings did not show spontaneous contractile activity. Phenylephrine (1 μM) or KCl (60 mM) caused a sustained contraction of the rings and the maximum tension reached has been 1.91 ± 0.71 g (n = 39) and 1.82 ± 0.66 g (n = 36), respectively. In both cases, this contractile effect was maintained without significant changes in tension in the control rings for at least 90 minutes.

The cumulative addition of increasing concentrations (0.1 nM–1 μM) of GTN, SNP or the new compounds (except **8**) caused a potent concentration-dependent vasorelaxant effect in the absence of endothelium (Table 1, Figures 2 and 3), indicating that their effect occurs by direct action on vascular smooth muscle.

In general, the new nitrate–coumarin derivatives, except **8**, behave in a similar way to GTN, relaxing with high potency and intensity the aortic rings pre-contracted with phenylephrine, and exerting a potent but less intense effect on the rings pre-contracted with KCl. On the other hand, SNP does not show such a wide difference between the relaxations induced against both vasoconstrictor agents (Figure 2). The differences between the IC_{50} values for each compound against phenylephrine indicate that the order of potency of the studied compounds is **4** > SNP > **2** > GNT > **10** > **6** > **8** (Table 1).

Table 1. IC_{50} values (nM) for the vasorelaxation induced in rat aorta rings pre-contracted with phenylephrine (1 μM) or extracellular high-KCl (60 mM).[a]

	Phenylephrine		KCl	
	Control	ODQ	Control	ODQ
GTN	12.73 ± 1.26	*	*	*
SNP	4.32 ± 0.14	40.46 ± 3.22	5.76 ± 0.86	*
2	5.86 ± 0.87	*	*	*
4	1.92 ± 1.10	*	*	*
6	132.27 ± 12.21	*	*	*
8	> 1000	*	*	*
10	24.86 ± 1.85	*	933.25 ± 18.26	*

[a] GTN: glyceryl trinitrate (nitroglycerin), SNP: sodium nitroprusside. * IC_{50} not calculated (50% relaxation is not reached in the curve). Each value represents the mean ± SEM from, at least four experiments.

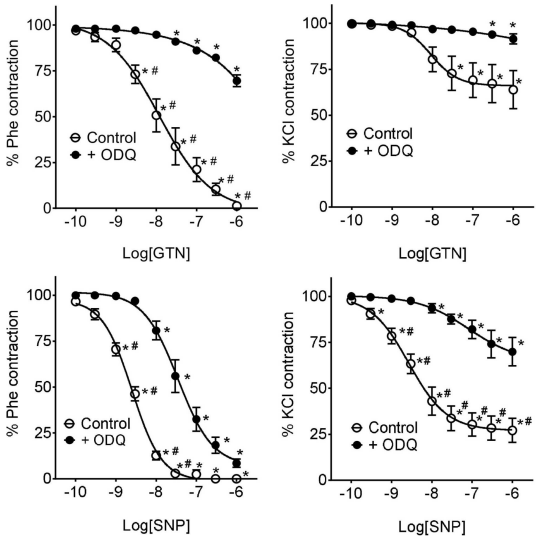


Figure 2. Vasorelaxant effect induced by cumulative additions of glyceryl trinitrate (GTN) or sodium nitroprusside (SNP) in isolated rat aortic rings without endothelium precontracted with phenylephrine (Phe, 1 μM) or KCl (60 mM) in the absence (control) or presence of ODQ (5 μM). Each point represents the mean ± s.e.m. (represented by the vertical lines) of, at least, 4 experiments. *$P < 0.05$ with respect to maximal contraction, #$P < 0.05$ vs. ODQ-pretreated rings.

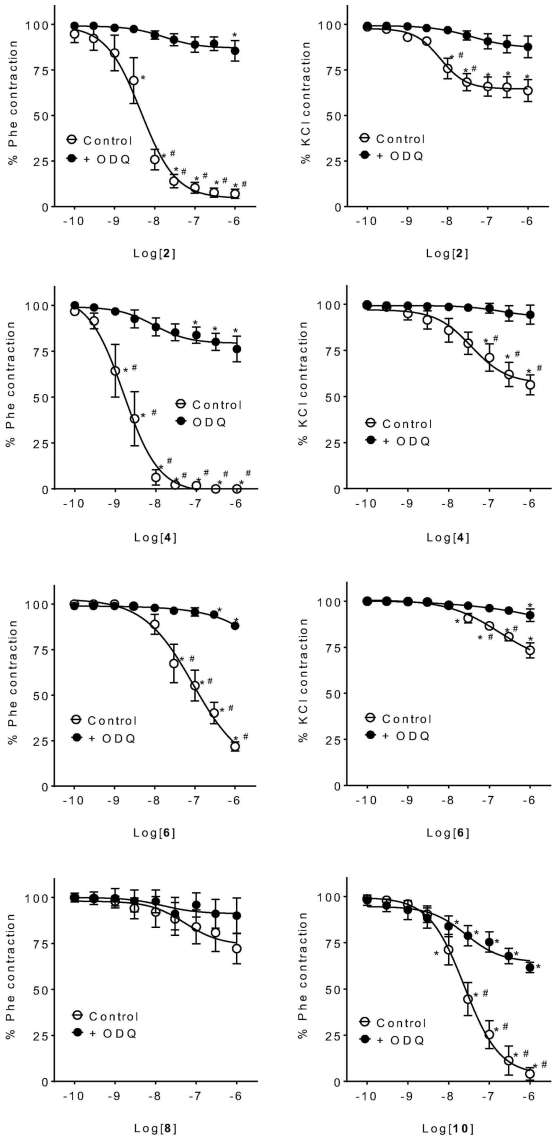


Figure 3. Vasorelaxant effect induced by cumulative additions of the compounds **2**, **4**, **6**, **8** and **10** in isolated rat aortic rings without endothelium, precontracted with phenylephrine (Phe, 1 μM) or KCl (60 mM) (b) in the absence (control) or in the presence ODQ (5 μM). Each point represents the mean ± s.e.m. (represented by the vertical lines) of, at least, 4 experiments. *$P < 0.05$ with respect to maximal contraction, #$P < 0.05$ vs. ODQ-pretreated rings.

Some of the new compounds without a nitrate group in their structures (compounds **1** and **7**) also caused a vasorelaxant effect, but only at concentrations higher than 10 μM (data not shown). Other studies also have shown that, in rat aortic smooth muscle, the vasorelaxant effect of coumarins usually appears in a micromolar range.[20–22,24] However, it cannot be completely ruled out that the presence of the coumarin group in the molecule somehow facilitates the vasodilator activity observed here. Even if the coumarin scaffold *per se* does not seem to be involved in the potent and intense effect of the new nitrate derivatives, the position of the substitution patterns on the scaffold seems to play an important role. Also, the presence of other substituents beside the nitrate, as the case of the trifluoromethyl, may affect the activity. As observed, moving the substituent from position 6 to 7 (compounds **2** and **4**) has led to a 3-fold increase in the activity. Also, moving the substituent from position 3 to 7, and adding a trifluoromethyl at position 4 (compounds **6** and **8**), has led to a 7-fold decrease in the activity. Also, the introduction of the amide linkers between the coumarin and the methyl nitrate substituent, has led to an important decrease in the potency of the design molecules. Therefore, structural modifications may result in differences in the generation of NO and/or the ability of the compounds to reach the biological target.

The fact of having a nitrate group in the molecules suggests that the mechanism of action of the new nitrate–coumarin derivatives could be related to that described for GTN and other classic nitrovasodilators, such as isosorbide dinitrate or nicor-

andil, which have been considered to act as NO generators *in vivo*, after being metabolized.[28] However, none of the metabolic pathways described for GTN satisfactorily explains the kinetics or pharmacological behavior of this drug. In fact, the production of NO induced by GTN is higher than that which would correspond to its stoichiometric conversion to vasoactive compounds and occurs considerably faster. Several studies have shown that maximal GTN-induced vasodilation occurs 1–3 minutes after administration,[29] while NO production is considerably slower (> 60 minutes).[30] Consequently, other physiological mechanisms have been proposed, that would be rapidly activated by GTN, and that would amplify its effect, such as the possible direct activation of eNOS, leading to faster vasodilation.[31] However, this would not be an explanatory mechanism for the effects observed here, since these experiments were performed in aortic rings lacking endothelium and, therefore, there is no eNOS in our preparations.

Our hypothesis that the new nitrate–coumarin derivatives may act as NO donors is supported by the experiments in the presence of 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a selective sGC inhibitor that acts by binding to the NO-binding heme group in this enzyme.[32,33] A 15 minutes preincubation with ODQ (5 μM) drastically reduced the vasodilator effect of the new nitrate–coumarin derivatives and GTN and, to a lower extent, also significantly inhibited SNP-induced vasorelaxation. These results coincide with others previously described in the literature, which had shown a different sensitivity of GTN and SNP to the effect of ODQ, this effect being less affected since its activation of sGC depends to a lower extent on the activation of the heme site of sGC.[33] Also, ODQ may interfere with SNP bioactivation by inhibiting one or more enzymes of the cytochrome P-450 system.[32] Thus, the activation of sGC induced by the new nitrate–coumarin derivatives would be mediated by the production of NO, which binds to the heme region of the enzyme, but would not depend on the oxidation of sulfhydryl residues (SH) as in the case of the SNP.[33] From our experiments, the possibility that the new nitrate–coumarin derivatives could act by directly stimulating sGC, independently of NO generation, as is the case with heme-dependent direct activators (YC-1, BAY 41-2272, BAY 41-8543, CFM-1571 and A-350619) or independent of the heme group (BAY 58-2667 and HMR-1766) cannot be ruled out.[34]

The lack of an important effect of the new compounds or GTN on KCl-contracted rings suggests that their mechanism of action can be due, in part, to the opening of K^+ channels in the plasma membrane since, with high extracellular K^+ concentrations, the activation of the K^+ channels is ineffective when it comes to causing a hyperpolarization and, in consequence, vasodilation. In this sense, cromakalim, and other K^+ channel activators, do not inhibit contractions in an extracellular medium with K^+ concentrations higher than 30 mM.[35]

The possibility of an increase in the intracellular concentration of cGMP induced by the studied compounds justifies the fact that part of their vasodilator effects may be due to the activation of K^+ channels, as discussed above. In fact, activation of cGMP-dependent protein kinases (PKGs) by cGMP leads, among other effects, to activation of calcium (Ca^{2+})-activated K^+ channels, which hyperpolarizes the membrane *via* transient K^+ efflux, thereby reducing Ca^{2+} influx across the membrane voltage-operated Ca^{2+} channels[7] and causing vasodilation.

The experiments carried out to verify the reversibility of the effects of the new nitrate–coumarin derivatives showed high recovery percentages of the contractile response induced by phenylephrine or KCl after a washout period of 90 minutes (Figure 4). These values are similar to those observed with GTN and NPS, and indicate that the effect of the new compounds is reversible and not deleterious for aortic rings.

Conclusion

The synthetized nitrate–coumarin derivatives show a potent and intense vasodilator activity on isolated rat aortic rings deprived of endothelium. The effect seems to be related to the nitrate group and the coumarin structure does not seem to be significantly involved in the effect. However, the position of the substitution pattern on this scaffold seems to be relevant for the activity. The inhibition of the vasodilator effect by ODQ indicates that it is largely due to the activation of sGC. Moreover, the higher effect of the new compounds on rings pre-contracted with phenylephrine than on those pre-contracted with KCl suggests that the opening of K^+ channels can be also involved in their mechanism of action. The new nitrate–coumarins can have the same mechanism of action as GTN. In contact with biological tissues, the compounds may generate NO, responsible for activating the heme group of sGC, increasing the formation of cGMP and inducing vasodilation. Finally, our studies indicate that the effect of the new nitrate derivatives is reversible and not deleterious for aortic rings.

Experimental Section

Synthesis of final nitrate–coumarins (2, 4, 6, 8 and 10). A dropwise solution of silver nitrate ($AgNO_3$, 2.34 mmol) in acetonitrile (2 mL)

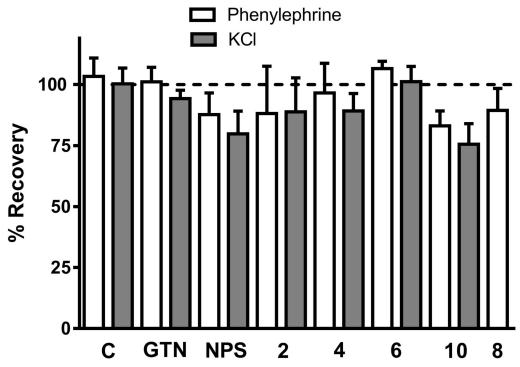


Figure 4. Percentages of recovery of the contraction induced by phenylephrine (1 μM) or KCl (60 mM) in rat aortic rings without endothelium after the administration of the tested compounds and a washout period of 90 min, in comparison with their own effect before the treatments. No significant differences have been found.

was added to a solution of the corresponding bromomethylcoumarin (1.67 mmol) in acetonitrile (3 mL). The resulting mixture was stirred at reflux temperature for 2 hours, and after cooling to room temperature, the solution was filtered. The filtrate's solvent was evaporated and the obtained solid was recrystallized from ethanol.

Contraction-relaxation studies in rat isolated thoracic aortic rings. Briefly, aortic rings taken out of male Wistar Kyoto rats and stripped of endothelium were properly set in an multi chamber organ bath containing 10 mL of Krebs bicarbonate solution thermoregulated at 37 °C and bubbled with carbogen. Isometric tension was recorded using a calibrated computerized system. The rings were then equilibrated at a resting tension of 2 g for at least 1 hour, during which the physiological solution was replaced every 10 minutes. Afterwards, a stable contraction was induced by the addition of phenylephrine (1 μM) and the endothelial integrity was determined by the relaxation in response to acetylcholine (1 μM). The preparations were then washed for 1 hour. Subsequently, the endothelium-denuded aortic rings were treated with phenylephrine (1 μM) or KCl (60 mM). After obtaining a stable contraction, cumulative concentrations of the new compounds, GTN or SNP, were added to the tissue bath and the response was monitored. In a subset of experiments, 15 minutes before the addition of phenylephrine or KCl, the tissues were incubated with ODQ (5 μM). Finally, all the preparations were washed for 90 min and the responses to phenylephrine or KCl were evaluated again in order to assess their functionality.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Nitrate–coumarins · vasorelaxation · nitroglycerin · sodium nitroprusside · nitric oxide

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