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Article

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Iminothioethers as hydrogen sulfide donors: from the gasotransmitter release to the vascular effects.

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KEYWORDS: hydrogen sulfide, vascular smooth muscle, iminothioethers, thioamides, H_2S-donors, vasorelaxation, gasotransmitter.

ABSTRACT: The gasotransmitter hydrogen sulfide (H_2S) is an important tuner of the cardiovascular homeostasis and its deficiency is etiologically associated with a number of cardiovascular diseases. Therefore, the research of original moieties able to release H_2S represents a timely issue for drug discovery. In this work, we developed a collection of iminothioethers (ITEs), exhibiting H_2S-releasing properties and producing vasorelaxing effects on rat aortic rings. Derivatives **4** and **11**, selected as representative of slow and fast rate H_2S-donors respectively, produced a complete recovery of the basal coronary flow, reverting the AngII-induced effects in isolated rat hearts. In addition, studies on human aortic smooth muscle

cells (HASMCs) demonstrated membrane hyperpolarizing effects, well related with intracellular generation of H_2S. Taken together, the results obtained support ITEs **4** and **11** as new pharmacological tools, as well as effective and innovative H_2S-donors for cardiovascular drug discovery.

INTRODUCTION:

Hydrogen sulfide (H_2S) is presently recognized as a fundamental mediator, which controls the homeostasis of many biological systems in the mammalian body.[1,2] This gasotransmitter is biosynthetized by specific enzymes, such as cystathionine-beta-synthase (CBS), cystathionine-gamma-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) starting from the aminoacid L-Cysteine. Among its numerous roles, H_2S is a key regulator of the cardiovascular system, where it is mainly produced by CSE. H_2S acts as a vasodilator[3] through several mechanisms of action often involving the modulation of ion channels or phosphodiesterase (PDE) in vascular smooth muscle.[4-8] The deletion of CSE gene in experimental animals is associated with a significant reduction of endogenous H_2S in blood, and in vascular and myocardial tissues; such a reduction leads to the impairment of endothelium-mediated vasorelaxation and increase in blood pressure.[9] These data clearly indicate that vascular H_2S is a key factor in the regulation of blood pressure and the defective production of endogenous H_2S is likely to be one of the most important etiopathogenetic factor in several forms of hypertension.[10] The roles played by endogenous H_2S in the regulation of the cardiovascular homeostasis pave the way to appealing therapeutic purposes, based on effective and rational pharmacological modulation of the H_2S pathway.[11] Indeed, the administration of exogenous H_2S has been proven to exert significant anti-hypertensive effects in several experimental models of hypertension,[12,13] indicating that "druggable" H_2S-releasing agents can actually be viewed as promising tools to

obtain novel cardiovascular drugs.[14,15] The poor posological control and the high probability of toxic effects strongly exclude the use of gaseous H_2S. Some sulfide salts, such as sodium hydrogen sulfide (NaHS) and calcium sulfide (CaS)[16] are H_2S-generating agents widely used for experimental purposes, but the rapid formation of H_2S (due to the protonation of hydrosulfide and sulfide anions, respectively, at physiological pH) seems to be poorly appropriate for clinical uses. Ideal H_2S-donor drugs should produce H_2S with relatively slow and constant rates. Accordingly, the search of novel H_2S-releasing chemical moieties suitable for the development of clinically effective H_2S-donors is strongly required.

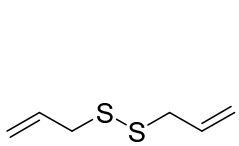
An interesting H_2S-donor feature has been early recognized in natural organosulfur derivatives, such as the polysulfides of *Alliaceae* (for example, diallyl disulfide **1** (DADS), Chart 1).[17] More recently, H_2S-releasing properties have been recognized also in another important class of natural sulfur compounds: the isothiocyanates typical of *Brassicaceae*.[18] Synthetic H_2S-releasing agents are also known; among them, the 4-methoxyphenyl(morpholino)phosphinodithioatemorpholinium salt **2** (GYY4137, Chart 1) is one of the most widely used in pharmacological studies.[19] As well, H_2S-releasing dithiolethiones and thioamides (TAs) are largely used, especially for the synthesis of multitarget drugs.[20-22] Satisfactory H_2S-releasing features of aminothiol and aryl isothiocyanate derivatives have been also reported.[23,24]

Very recently, original examples of "smart" H_2S-donors, able to generate the gasotransmitter based on specific mechanisms of release, which may be useful in specific biological targets, have been described. Among these, molecules exhibiting esterase-mediated production of H_2S[25], pH-controlled mechanisms[26] or initial release of intermediates such as carbonyl sulfide[27] have been reported.

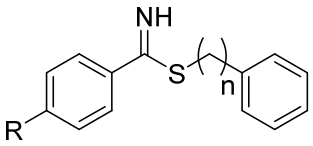
In this context, we recently studied a small library of arylthioamides that exhibited satisfactory properties, including stability in water and relatively slow H_2S generation, triggered by the presence of organic thiols.[28] Slight structural modifications, such as the insertion of small substituents in the benzene ring or the replacement of the benzene ring with heterocycles, afforded different rates of H_2S release, even comparable to or even higher than that of **2**.[28] In addition, a compound from this series produced typical vascular effects of H_2S, both in *in vitro* and *in vivo* experiments, including: (i) inhibition of the norepinephrine-induced vasoconstriction in isolated rat aortic rings; (ii) membrane hyperpolarization in human vascular smooth muscle cells; (iii) reduction of the systolic blood pressure after oral administration.[28]

Despite the huge amount of data on the potential pharmacological usefulness of H_2S donors and H_2S-hybrids, to date, there is poor heterogeneity of H_2S-releasing moieties. Thus, the development of original H_2S-donors characterized by varying physicochemical, biological and pharmacological features represents a very timely issue for drug discovery.

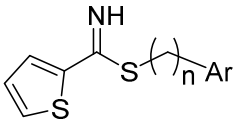
In this paper, we report the synthesis and the pharmacological evaluation of some iminothioether derivatives (ITEs **3-11**, Chart 1), with the aim to investigate such a novel and original chemical moiety as a H_2S-releasing functional group with potential pharmaceutical interest. In addition, a small collection of closely analogous TAs (**12-15**, Chart 1) were synthetized and investigated.



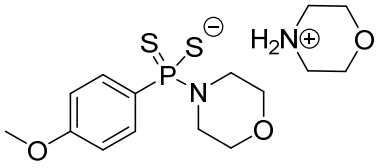
1: DADS



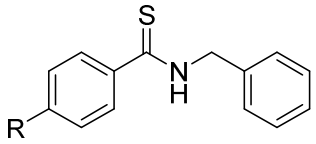
3 n = 1, R = H
4 n = 1, R = OCH_3
5 n = 1, R = OH
6 n = 0, R = H
7 n = 0, R = OCH_3
8 n = 0, R = OH



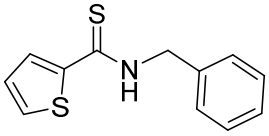
9 n = 1, Ar = C_6H_5
10 n = 1, Ar = naphth-2-yl
11 n = 0, Ar = C_6H_5



2: GYY4137



12 R = H
13 R = OCH_3
14 R = OH



15

Chart 1. Chemical structures of reference H_2S-donors (**1**, **2**) and of the novel synthetized compounds **3-15**.

CHEMISTRY

As reported in **Scheme 1A**, the synthetic procedure for the preparation of the target benzimidothioate derivatives **3-5** started from the commercially available benzamide **18** or 4-methoxybenzamide **19**, obtained through a condensation between the appropriate benzoyl chloride (**16** or **17**) and NH_4OH in the presence of triethylamine. Compounds **18** and **19** were allowed to react with Lawesson's reagent in dry THF solution, to give products **20** and **21**

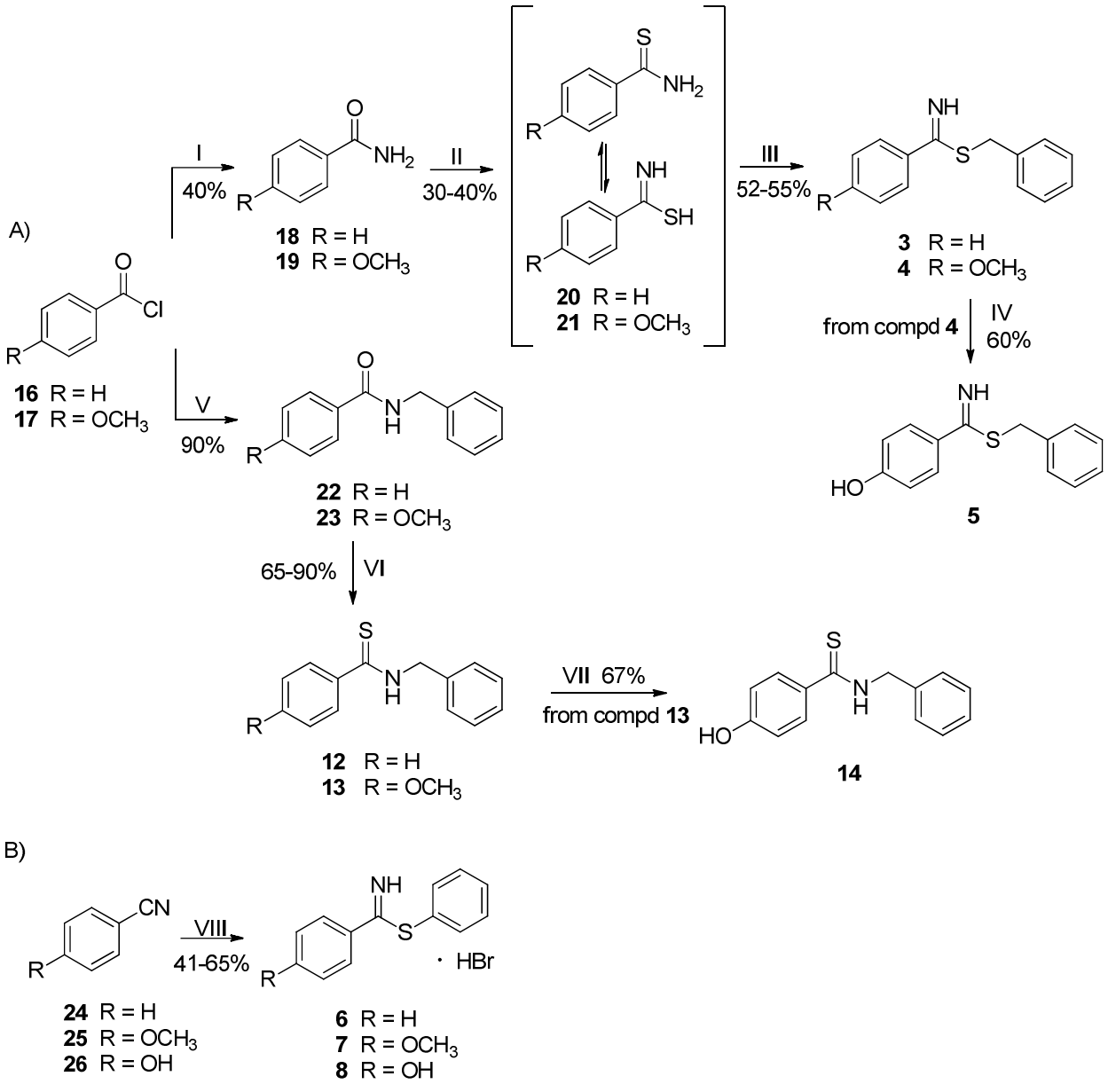
. Compounds **3** and **4** were then obtained by alkylation of compounds **20** and **21**, respectively, with benzylbromide in refluxing $CHCl_3$. Finally, compound **4** was demethylated by treatment with BBr_3 in nitrogen atmosphere, to obtain compound **5** (Scheme 1A).

The preparation of the target *N*-benzylbenzothioamide derivatives **12-14** started from the commercially available *N*-benzylbenzamide **22**, or *N*-benzyl-4-methoxybenzamide **23** obtained

through a condensation between 4-methoxybenzoyl chloride and benzylamine in the presence of triethylamine. Compounds **22** and **23** were allowed to react with Lawesson's reagent in dry THF solution to give products **12** and **13**, respectively. Compound **13** was finally demethylated by treatment with BBr_3, under nitrogen atmosphere, to obtain compound **14** (Scheme 1A).

The arylimidothioate derivatives **6-8** were prepared by treatment of the appropriate 4-substituted benzonitrile **24-26** with thiophenol under an atmosphere of HBr, as previously reported by Baati et al.[29](Scheme 1B).

Scheme 1. Synthesis of imithioether **3-8** and thioamide **12-14** derivatives.



Reagents and conditions. I: NH_4OH, NEt_3, dry toluene, 24 h, r.t.; II: Lawesson's reagent, dry THF, 12 h, r.t.; III: benzylbromide, NaH, $CHCl_3$, 12 h, reflux; IV: BBr_3, dry CH_2Cl_2, 24 h, r.t.;

V: benzylamine, NEt_3, dry toluene, 24 h, r.t.; VI: Lawesson's reagent, dry THF, 12 h, r.t.; VII: BBr_3,dry CH_2Cl_2, 24 h, r.t.; VIII: thiophenol, Et_2O, HBr, 0 °C, 0.5 h.

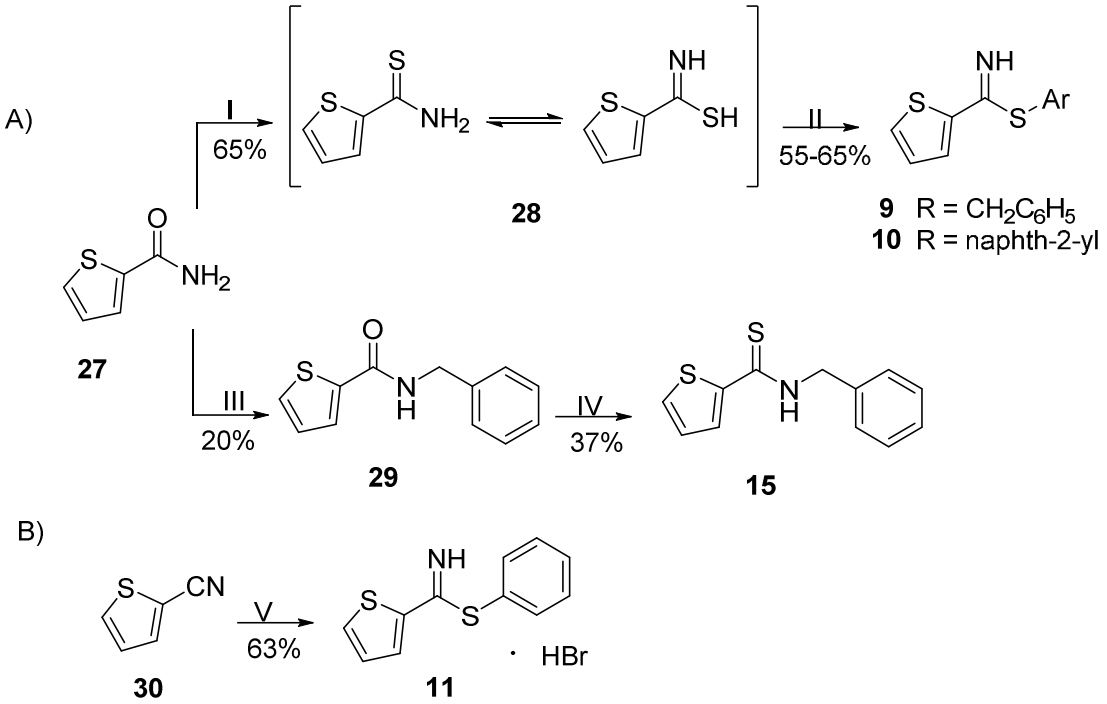
The experimental procedures for the preparation of compounds **9-11**, **15** are outlined in **Scheme 2**.

The thiophene derivatives **9**, **10** and **15** were synthesized as reported in Scheme 2A. Reaction of 2-thiophenecarboxamide **27** with the Lawesson's reagent in chlorobenzene yielded the corresponding thioamide **28**, which was then condensed with benzylbromide or naphth-2-ylmethylbromide to give the target products **9** and **10**, respectively. The thioamide **15** was obtained by two sequential reactions of 2-thiophenecarboxamide **27** with benzyl bromide, in the presence of NaH, and then with Lawesson's reagent (Scheme 2A)**.**

The phenyl thiophene-2-carbimidothioate **11** was prepared starting from the 2-thiophene-nitrile **30** by the same procedure applied for compounds **6-8**.[29](Scheme 2B).

All the target compounds **3-15** were finally purified by flash chromatography, when necessary (see Experimental section).

Scheme 2. Synthesis of imithioether **9-11** and thioamide **15** derivatives.



Reagents and conditions: I: Lawesson's reagent, C_6H_5Cl, 12 h, 130 °C; II: appropriate arylbromide, $CHCl_3$, 12 h, reflux; III: benzyl bromide, NaH, DMF, 1 h, r.t.; IV: Lawesson's reagent, C_6H_5Cl, 12 h, 130 °C; V: thiophenol, Et_2O, HBr, 0 °C, 0.5 h.

RESULTS AND DISCUSSION

Evaluation of H_2S-release by the amperometric assay.

The investigation of the H_2S-releasing properties of the novel synthesized compounds was carried out *in vitro* by an amperometric assay, by means of a H_2S-selective minielectrode, to have a real-time determination of the H_2S-release and thus to perform a qualitative/quantitative

description of the process. Table 1 lists the parameters of Cmax (the highest concentration achieved in the recording time) and $t_{1/2}$ (the time required to reach a concentration = ½ Cmax) from the tested compounds (incubated at the concentration 1mM), recorded in the absence (-L-Cys) or in the presence (+L-Cys) of an excess of L-Cysteine (4 mM). Data of reference H_2S-donors **1** and **2** were also reported for comparison purposes.

In general, all the compounds (ITEs and TAs) showed very poor H_2S-release in the absence of L-Cys, consistently with the H_2S-releasing profile exhibited by the reference H_2S-donors (**1** and **2**), in previous experiments.[28] In particular, in the absence of L-Cys, the H_2S-generation from the ITE **10** and TA **13** was almost negligible (under the levels of determination), while all the other compounds and the reference H_2S-donors exhibited low but evident release of H_2S, although in some cases it was under the level of accurate quantification (compounds **11**, **14**, **15**, **1** and **2**).

The pre-incubation with an excess of L-Cys (4 mM) improved the H_2S-release from almost all the synthesized molecules, as well as from **1** and **2**. In particular, the maximal concentrations of H_2S (Cmax), generated from the tested compounds upon incubation (for 30 min) in the presence of L-Cys, ranked from 0.31 μM (**15**) to 19.0 μM (**11**); the Cmax of **1** was 19.4 μM. In the presence of L-Cys, H_2S-release from compound **13** was low but evident (under the level of accurate quantification), while no detectable release of H_2S was recorded for **10** (Table 1).

Looking at the L-Cys mediated effects, almost all the compounds showed progressive and time-related "slow" H_2S-releasing profiles, with $t_{1/2}$ values ranging from 4.4 and 11.9 min. The H_2S-releasing profile of **4** is shown in Figure 1, as representative example; in previously published data, **1** exhibited a relatively faster, but clearly time-related, H_2S-releasing process, with $t_{1/2}$ value of 1.5 min.[28] Compound **11** exhibited a profile of relatively "rapid" donor ($t_{1/2}$ = 0.28 min,

Table 1), reaching the peak concentration (19.0 μM) in about 1 min, followed by a progressive decrease of the H_2S concentration (Figure 1).

Taken together, these data indicate that, in the presence of organic thiols (L-Cys), most of the TA and ITE compounds behaved as H_2S-donors, albeit with different features both in the quantitative and in the kinetic aspects that can be related to their chemical structure. Specifically, the "rank order" for quantitative H_2S release within the ITEs series was: **11** >**4** ≥**5** >**9** >**3** >**10**. From a structure-activity relationship point of view, it could be observed that the insertion of a substituent (OCH_3, OH) on the phenyl ring featuring the iminothioether function of **3** determines a slight improvement in the H_2S-releasing properties in compounds **4** and **5**. The replacement of the same phenyl ring of **3** with a thienyl moiety in **9** did not produce significant effects on Cmax values. On the contrary, the direct comparison between **11** and **9** indicates that the S-phenyl substitution is highly effective in increasing the quantitative H_2S release and its rate; in addition, this effect seems to be due to the presence of the phenyl substituent on the S atom, rather than the thiophene ring, as compound **9** exhibited almost equivalent levels of H_2S release if compared with phenyl derivatives **3-5**. Replacement of the benzyl group in compound **9** with a naphth-2-ylmethyl moiety gives compound **10** that is devoid of any H_2S-releasing activity, probably due to chemical-physical issues connected to its high lipophilicity.

Finally, the H_2S release from TA compounds was significant, but quite low (Table 1). The comparison between the ITEs **3-5**, **9** and the corresponding analogous **12-15**, indicates that the former functional group leads to a general quantitative improvement in H_2S release, suggesting ITE as a novel original H_2S-donor group useful in the future development of innovative cardiovascular drugs.

Noteworthy, it is widely accepted that the biological activity of H_2S-donors is not directly related with the mere quantitative aspects of the release: indeed, even small amounts of H_2S generated in a long-lasting manner, can evoke effects better than those evoked by fast and transient generation of large amounts of the gasotransmitter. This has been described for slow H_2S-donors, such as **2**[19] or some aryl-isothiocyanates,[24] which caused vasorelaxing effects with potency values higher than sodium hydrosulfide, a widely used salt that instantaneously generates H_2S at physiological pH. As well, the effects of H_2S donors on inflammatory processes are strongly influenced by the rate of the H_2S release, and again slow H_2S-donors exhibited more favorable profiles of activity.[19]

Therefore, ideal H_2S donors for many clinical uses should produce H_2S with slow releasing rates, ensuring low and long-lasting concentration.[14] In this perspective, the L-Cys-induced H_2S-release from almost all the compounds tested in this study exhibited quite "slow" rate, comparable and even longer than those exhibited by well-known donors, such as **1** and **2** (see Table 1), when tested in the same experimental conditions.[28] Only compound **11** exhibited a short $t_{1/2}$ value (< 1 min), generating an early peak of H_2S concentration, followed by a progressive decrease (Figure 1).

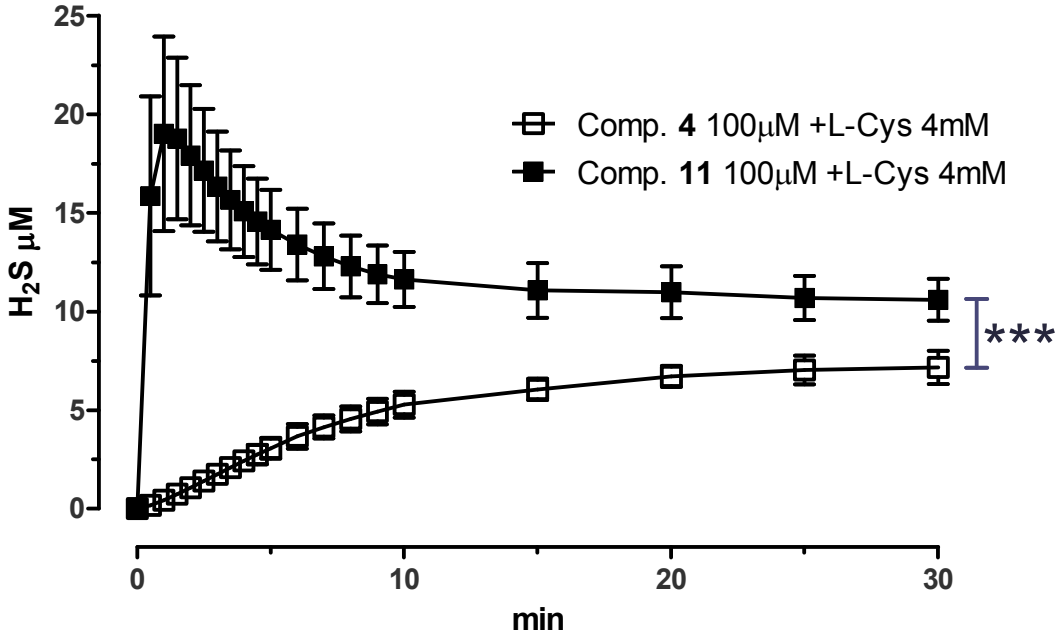


Figure 1. Amperometric recordings of the H_2S-release from **4** and **11** in the presence of L-Cys (+ L-Cys). The curves describe the increase of the H_2S concentration with respect to time, following the incubation of tested compounds, highlighting a slow and gradual increase of the H_2S production after **4** administration and, conversely, a rapid and massive H_2S production followed by a rapid decrease after **11** administration; the vertical bars indicate SEM**.** Two-way ANOVA showed extremely significant difference between the two curves ($P < 0.001$).

Functional evaluation of the vasorelaxant effects on rat aortic rings.

The vasorelaxing effects of ITEs and TAs were tested on pre-contracted rat aortic rings. Almost all the tested compounds showed full or almost full vasorelaxant efficacy (Emax) on endothelium-denuded rat aortic rings pre-contracted with 25 mM KCl, with potency indexes (pIC_{50}) ranging between 3.20 and 3.89 (Table 1). The vasorelaxing effects were also exhibited by compounds **13**. This compound released well detectable, but very low (under the level of

accurate quantification), H_2S concentrations in the amperometric assay. Therefore, it cannot be excluded that the vasorelaxing activity can be attributable even to other mechanisms different from H_2S release. Consistent with the data previously obtained, compound **10**, which lacks of any detectable H_2S-releasing activity, showed very poor vasorelaxant activity and a non-calculable potency index, strongly suggesting that the vasorelaxing effect of the other compounds is actually mediated by H_2S.

N	H_2S-release				Vasorelaxing Effect	
	-L-Cys		+L-Cys 4mM		Emax	pIC_{50}
	Cmax (μM)	$t_{1/2}$(min)	Cmax (μM)	$t_{1/2}$(min)		
1	< 0.30	N.C.	19.4 ± 5.5	1.5 ± 0.3	41.7 ± 1.7	N.C.
2	< 0.30	N.C.	10.3 ± 2.6	2.5 ± 0.8	60.2 ± 3.7	3.68 ± 0.01
3	2.30 ± 0.40	3.61 ± 0.38	3.20 ± 0.50	5.23 ± 0.58	86.5 ± 3.6	3.37 ± 0.02
4	1.18 ± 0.30	17.35 ± 2.90	7.20 ± 0.80	5.73 ± 0.52	89.9 ± 3.3	3.59 ± 0.02
5	0.50 ± 0.10	5.28 ± 0.71	6.80 ± 0.80	5.10 ± 0.62	82.1 ± 3.4	3.31 ± 0.01
6	N.T.[a]	-	N.T.[a]	-	N.T.[a]	-
7	N.T.[a]	-	N.T.[a]	-	N.T.[a]	-
8	N.T.[a]	-	N.T.[a]	-	N.T.[a]	-
9	1.90 ± 0.1	26.91 ± 4.18	4.60 ± 0.90	6.66 ± 0.73	89.9 ± 2.1	3.58 ± 0.03
10	N.D.	N.D.	N.D.	N.D.	20.4 ± 3.3	N.C.
11	< 0.30	10.24 ± 2.11	19.0 ± 4.90	0.28 ± 0.13	99.7 ± 0.3	3.20 ± 0.01
12	0.70 ± 0.10	8.70 ± 1.20	1.90 ± 0.40	11.86 ± 1.35	92.7 ± 0.4	3.79 ± 0.03
13	N.D.	N.D.	< 0.30	N.C.	96.8 ± 0.6	3.89 ± 0.01
14	< 0.30	1.25 ± 0.23	1.10 ± 0.10	4.43 ± 0.30	88.2 ± 0.4	3.46 ± 0.02
15	< 0.30	4.3 ± 0.71	0.31 ± 0.09	7.53 ± 0.70	91.7 ± 0.7	3.62 ± 0.03

Table 1. Parameters of Cmax and $t_{1/2}$, emerging from the amperometric detection of H_2S-release from the tested compounds (incubated at the concentration 1mM) in the absence (-L-Cys) or in the presence (+L-Cys) of an excess of L-Cys (4 mM), and parameters of Emax (maximal

vasorelaxing effect evoked by the tested compounds 1mM) and pIC_{50} of the vasorelaxing effects recorded on pre-contracted rat aortic rings. In the amperometric detection, the lower limit of reliable quantitative determination of H_2S was 0.3 μM. Data are expressed as means ± standard error. N.T.= Not tested (chemically unstable in experimental conditions); N.D. = not detectable; N.C. = not calculable. All the synthetized compounds, except compound **10**, exhibited vasorelaxing efficacy parameters significantly higher ($P<0.01$) than **1** and **2**.

Functional evaluation of the effects on coronary flow.

On the basis of the results emerging from the amperometric assay and the functional data on the aortic rings, two iminothioether derivatives, **4** and **11**, were selected as representative for further pharmacological investigation on the basis of the following issues: (i) **4** exhibited high vasorelaxant potency, appreciable quantitative H_2S-release and a slow releasing rate; (ii) **11** showed lower vasorelaxant potency, but it generated the highest concentration of H_2S, with a quite fast rate. The two selected compounds were then evaluated in Langendorff-perfused rat hearts. As expected, the perfusion with Angiotensin II (AngII, 0.1 μM) caused a significant reduction (by about 25%) of the coronary flow (CF) in isolated rat hearts when compared to the basal CF (basal flow = 10.45 ± 0.64 ml/min/g). The "add-on" perfusion with the ITE **4** (300 μM) produced extremely significant effects in the coronary bed, leading to an intense increase of the coronary flow, up to 165%, i.e. higher than the basal one. (Figure 2). In contrast, perfusion with **11** (300 μM) led to an apparent increase of the coronary flow, but this effect was not significantly different than that induced by the vehicle (Figure 2).

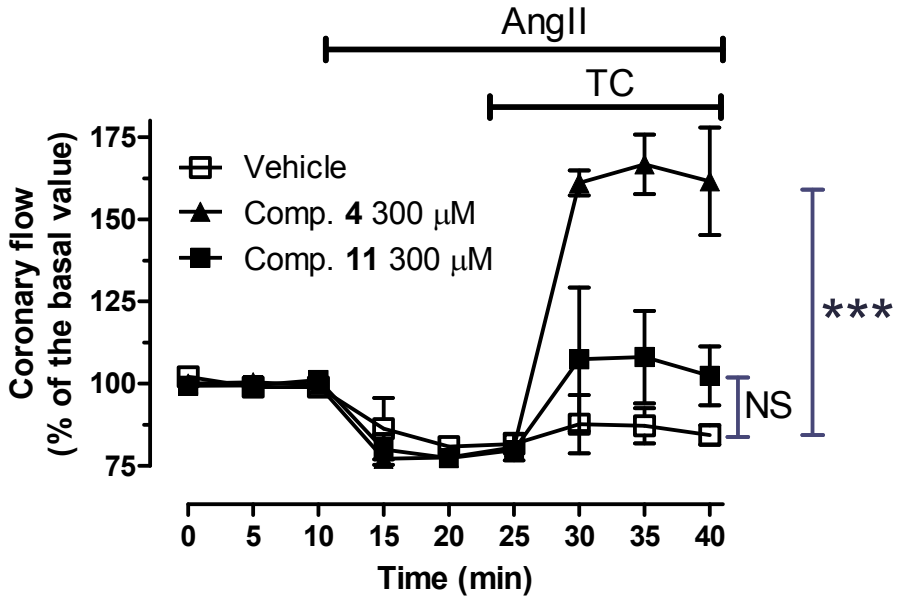


Figure 2. Changes (in %) of CF, induced by perfusion of AngII, followed by the add-on perfusion of vehicle, **4** or **11**. After the equilibration time, three measurements of the basal flow were carried out at 5 min intervals, starting from min 0. Immediately after the recording of the third basal value, AngII was perfused from min 10; the perfusion with AngII was maintained until the end of the experiment (upper bar). Starting from min 25, the tested compound (TC) or the vehicle were perfused (lower bar), together with AngII. Data are expressed as a % of the mean basal coronary flow, and are expressed as means± standard error, from hearts of 6-9 animals. NS = the differences between the curves are not statistically significant; *** the differences between the curves are extremely significant ($P < 0.001$).

Evaluation of membrane hyperpolarization of human aortic smooth muscle cells (HASMCs).

Among the heterogeneous mechanisms of action accounting for the vasorelaxant activity of H_2S, the activation of ATP-sensitive potassium (K_{ATP}) channels[5,30] and of vascular Kv7 potassium

channels,[4] with membrane hyperpolarization of vascular smooth muscle cells, seem to play a relevant role. In this view, it was thought interesting to evaluate the effects of **4** and **11** on the membrane potential of cultured human vascular smooth muscle cells (HASMCs), taking 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one **31** (NS1619), a well-known potassium channel activator, as reference hyperpolarizing agent[31].

Both ITEs **4** and **11** (100 μM - 1 mM) caused a significant and concentration-related membrane hyperpolarization of HASMCs. In particular, the hyperpolarizing response evoked by the highest tested concentration of **11** (1mM) was significantly lower (36 ± 5 %) than that evoked by the reference **31** (Figure 3). Actually, in previous works, we could observe that even the fast H_2S-donor, sodium hydrosulfide, evokes moderate hyperpolarizing effects.[4,24] Compound **4** evoked strong membrane hyperpolarization, exhibiting high level of efficacy (149 ± 2%) and significantly overcoming **31** (Figure 3).

The effects observed on the coronary flow, in rat isolated hearts, and on the membrane potential of HASMCs witness again that a "slow" and moderate H_2S-release seems to be preferable to a "fast" H_2S-release for the vascular effects.

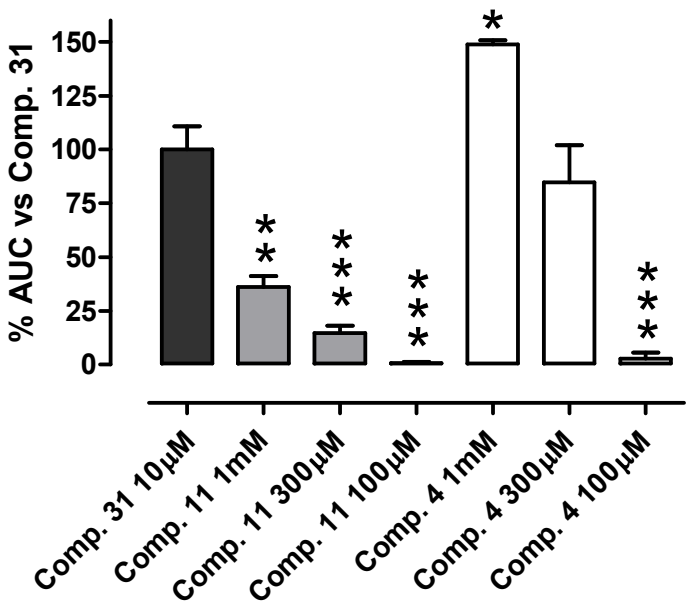


Figure 3. The graph shows the hyperpolarizing effect of tested compounds on sarcolemmal membrane of HASMCs. Data are expressed as mean ± standard error. Six different experiments were performed, each in six replicates. Asterisks indicate significant difference from the effect evoked by **31** (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

Evaluation of H_2S-release in HASMCs.

The amperometric technique well defines the kinetics (i.e., the rate) of the L-Cys-dependent H_2S-release of compounds **3-5** , **9-15**, suggesting that many of these may act as "smart" donors: they are expected to be relatively stable in water, but they behave as H_2S-generating agents in biological environments (for example, the cell cytosol), where they can interact with endogenous organic thiols (L-Cys, glutathione, etc). However, this assay was carried out only in buffer aqueous solution in the absence and in the presence of L-Cys. Therefore, a further evaluation was performed in order to demonstrate that the H_2S-release actually occurs in cells, without adding exogenous thiols. In particular, the H_2S generation was detected in HASMCs by

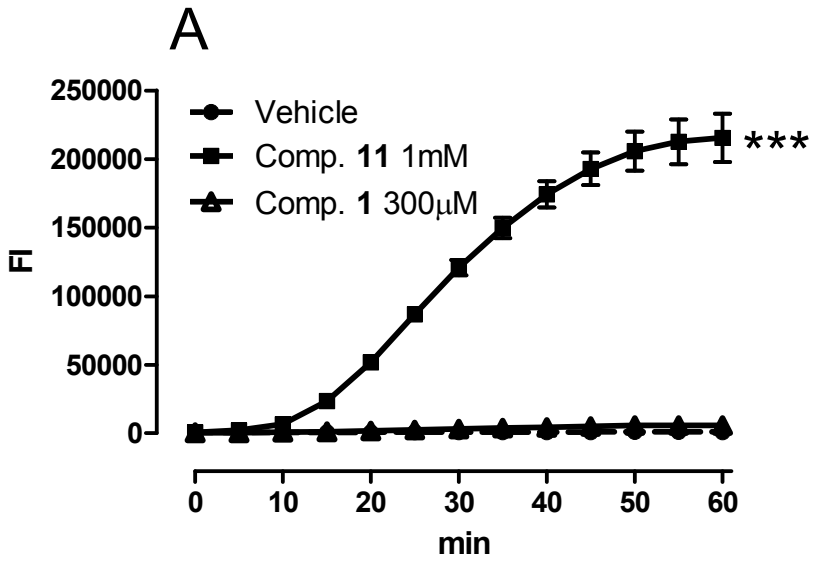
spectrofluorometric measurements using the dye 3'-methoxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl 2-(pyridin-2-yldisulfanyl)benzoate (Washington State Probe-1,WSP-1), which specifically and irreversibly interacts with H_2S.[32] The fluorescence produced by this interaction was quantitatively recorded by a spectrofluorometric approach, and also observed by fluorescence microscopy.

Spectrofluorometric measurements showed that the addition of the vehicle did not cause any significant increase of fluorescence. In contrast, the addition of 1mM **11** to HASMCs, pre-loaded with the fluorescent dye WSP1, led to a massive time-dependent increase of fluorescence (FI, fluorescence index), indicating an extremely significant generation of H_2S ($P<0.01$ vs vehicle). The maximal value of FI, recorded after 1 h of incubation, was about 50-fold higher than that evoked by 300 μM reference **1** (Figure 4A). After 1 h of incubation, the fluorescence increase reached an apparently stable "steady state", suggesting that the H_2S-releasing process is completed, and thus confirming the profile of "fast" donor for **11**.

In contrast, the addition of 1mM **4** to WSP1-preloaded HASMCs led to a significant ($P<0.01$ vs vehicle) but moderate time-dependent increase of FI. The maximal level of FI recorded after 1 h of incubation was significantly lower ($P < 0.01$) than that evoked by **11** (1 mM) and was almost completely comparable to that evoked by **1** (300 μM). In addition, after 1 h of incubation, the fluorescence increase did not yet reach a "steady state" and was still in progress, indicating that the H_2S-releasing process is not complete, thus confirming the profile of "slow" donor for **4** (Figure 4B).

Fluorescence microscopy allowed us to observe a clear increase of WSP-1-evoked fluorescence inside the HASMCs treated with **4**, indicating an intracellular localization of the H_2S release from this ITE (Figure 5). **1** showed a similar feature (Figure 5). In contrast, a significant cell loss

was observed in HASMCs treated with **11** (data not shown), suggesting that ITE may have caused cell damage and consequent vulnerability in the experimental procedures used in the microscopy approach, which are more "invasive" if compared with the spectrofluorometric technique.



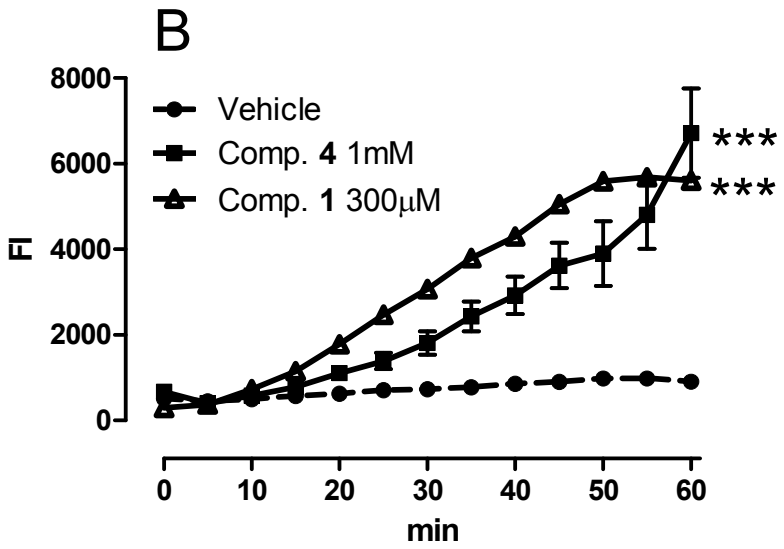


Figure 4. The graphs show the WSP-1 fluorescence increase evoked by the administration of vehicle, **11** and **1** (A), **4** and **1** (B) on HASMCs. Data were expressed as mean ±standard error. Three different experiments were carried out, each in triplicate. *** = significantly different from the vehicle ($P < 0.01$).

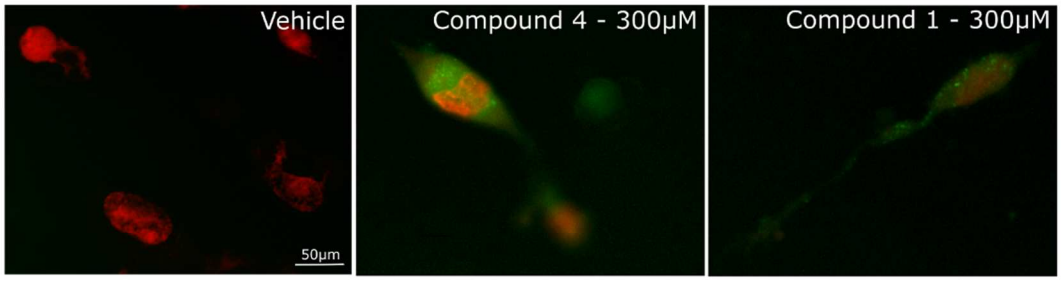


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Figure 5. Fluorescence microscopy images, showing the fluorescence evoked in HASMCs (pre-loaded with WSP-1 dye), after the administration of vehicle, compound **4** (300μM) and **1** (300 μM). The green fluorescence indicates a significant activation of the fluorophore, due to the generation of H_2S and its interaction with WSP-1. Propidium iodide was used to identify the nuclei, in red.

Evaluation of cGMP in HASMCs.

Intracellular contents of cGMP in HASMCs were determined by ELISA assay. In basal conditions no detectable levels of cGMP could be observed both in vehicle treated cells and also in HASMCs treated with the selected H_2S-donors. In contrast, in sodium nitroprusside (SNP) pre-treated cells treated with the vehicle (DMSO 0.1%) well-detectable and significant concentration of cGMP was detected (0.446 ± 0.005 pmol/ml). The administration of compound **4** to SNP-pretreated cells, led to small but significant increase in cGMP concentration (0.489 ±

0.014 pmol/ml). Finally, compound **11** determined in SNP_pretreated cells a larger and significant increase of cGMP intracellular concentration (0.667 ± 0.021 pmol/ml).

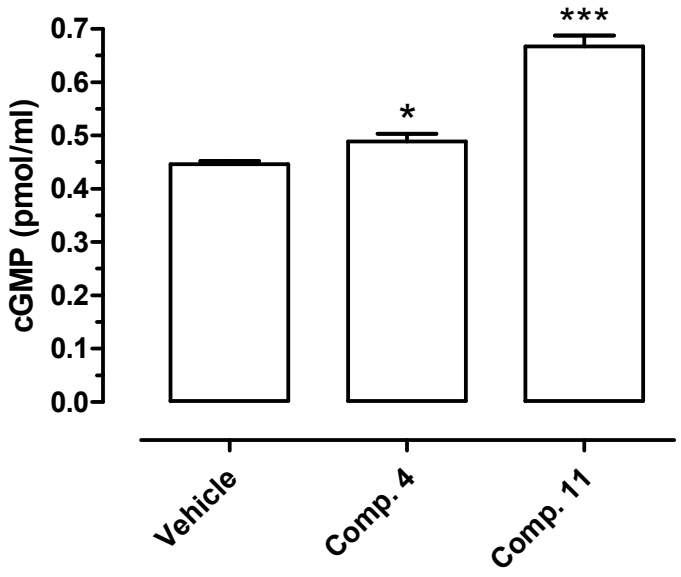

Figure 6. Effects of selected H_2S-donors on intracellular increase of cGMP. HASMC were pre-treated with SNP (1mM) and then incubated with vehicle (DMSO 0.1%), compound **4** or compound **11** (300 μM), for 20 min. Data are expressed as mean ± SEM. The asterisks indicate significant differences vs vehicle (* = $P < 0.05$; *** = $P < 0.001$).

Effects of selected H_2S-donors on blood pressure.

Normotensive Wistar rats showed basal systolic pressure (Psys) of 137 ± 2 mmHg. Intraperitoneal administration of L-NAME (100 mg/Kg) caused a significant increase of Psys 158 ± 2 mmHg. The administration of vehicle (DMSO 0.33ml/Kg i.p.) did not cause any significant change of Psys in rats with L-NAME-induced hypertension. In contrast, the reference H_2S-donor **1** (133 μmol/Kg) caused a significant ($P < 0.01$) decrease of Psys (-39 ± 5%).

Compound **4** (133 μmol/Kg) exhibited a similar pharmacological behavior. In particular, it promoted a significant ($P < 0.01$) decrease of Psys (-36 ± 4%) in L-NAME-induced hypertensive animals. Compound **11** (133 μmol/Kg) did not influence the Psys.

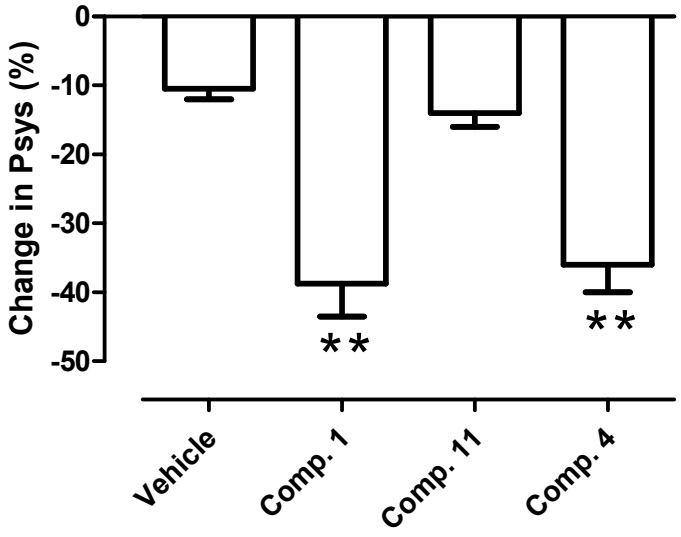

Figure 7. Changes in Psys (expressed as a % of the L-NAME-induced hypertensive Psys), following the i.p. administration of compounds **4** and **11**, the reference H_2S-donor **1** or the corresponding vehicle. The asterisk indicates significant difference vs vehicle (** = $P < 0.01$).

CONCLUSIONS

In this work, the ITE group was investigated as potential new H_2S-releasing moiety, in view of its similarity with TA (a consolidated H_2S-donor moiety). Thus, a small collection of ITE derivatives were synthesized and pharmacologically characterized.

In the amperometric assay, many of the ITE derivatives behaved as smart H_2S-donors (quite stable in water, but able to generate H_2S in the presence of L-Cys, mimicking the endogenous thiols), and showed a general increase in H_2S release with respect to their TA analogues. Thus, the H_2S-donor profile of ITEs can be considered as an original and innovative finding of this work. In addition, almost all the TAs and ITEs exhibited full vasorelaxing effects when tested on pre-contracted rat aortic rings, with pIC_{50} values that significantly correlate with $t_{1/2}$ values.

On the basis of the H_2S-releasing profile emerging from the amperometric assay (i.e., a "cell-free" experimental model), two iminothioether derivatives, **4** and **11**, were selected for further pharmacological investigation, as representative of slow and fast rate H_2S-donors, respectively.

To demonstrate the ability of ITEs to release H_2S in a cell-based experimental model, without the adding of exogenous thiols, the H_2S generation from the compounds in HASMCs was investigated by means of a spettrofluorometric approach. The incubation of the two derivatives on WSP-1-preloaded HASMCs confirmed the profile of "fast" and "slow" donor, respectively for **4** and **11**. In particular, the incubation of **11** led to a massive increase of FI, indicating a dramatically efficient production of H_2S inside cells. In contrast, compound **4** led to a moderate and more gradual increase of fluorescence, related with the intracellular production of H_2S. Fluorescence microscopy confirmed the intracellular localization of the H_2S-generation for **11**.

As concerns the functional pharmacological effects, the "add-on" perfusion in isolated hearts with **11** showed an apparent trend to increase the coronary flow, however, this effect did not reach the level of statistical significance. In contrast, in the same experiments, **4** exhibited stronger effects, evoking an intense and extremely significant increase of coronary flow, up to 165%, i.e. higher than the basal one.

The possible involvement of membrane hyperpolarizing effects in the H_2S-induced vasorelaxation by **4** and **11** was also investigated. The results confirmed that the slow H_2S releasing compound **4** is able to promote strong and concentration-dependent hyperpolarizing responses, exhibiting high level of efficacy and significantly overcoming the reference hyperpolarizing drug **31**. While, the fast H_2S-donor **11** showed lower hyperpolarizing effects.

Beside the membrane hyperpolarizing effects, even the inhibition of phosphodiesterases (PDEs) and consequent rise of intracellular cGMP are recognized as relevant mechanisms of action accounting for the vasorelaxing effects of H_2S[6,33]. Thus, the effects of compounds **4** and **11** on the intracellular levels of cGMP have been also investigated. In HASMC, the guanylate cyclase activation was triggered by the nitric oxide donor SNP and the concentration of cGMP was significantly increased by the tested compounds, suggesting a probable involvement of H_2S-mediated inhibition of PDE. In particular, compound **11** evoked the higher effect seeming to indicate that a more rapid release would be preferable for this specific effects and further stressing that both the different kinetics of release and the H_2S concentration may influence the overall pharmacodynamic profiles of H_2S-donors.

Finally, the in vivo anti-hypertensive effects of **4** and **11** were evaluated in an experimental model of L-NAME-induced hypertension. In this experimental protocol, the slow H_2S-donor **4** evoked marked hypotensive activity, fully comparable with those shown by the reference drug **1**. In contrast, the fast donor **11** did not promote any significant decrease of blood pressure.

Taken together, the data reported in this work demonstrated that ITE can be considered as a satisfactory new H_2S-releasing moiety and that ITE-based compounds, endowed with appropriate H_2S-releasing profile, may represent an original and promising class of "smart" H_2S-donors for the development of cardiovascular drugs.

EXPERIMENTAL SECTION

Chemistry

General Material and Methods. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references; coupling constants *J* are reported in hertz. ^{1}H NMR and ^{13}C NMR spectra of all compounds were obtained with a Varian Gemini 200 MHz or a Bruker TopSpin 3.2 400 MHz spectrometer. ^{13}C NMR spectra were fully decoupled. The following abbreviations are used: singlet (s), doublet (d), triplet (t), double−doublet (dd), and multiplet (m). Chromatographic separation was performed on silica gel columns by flash (Kieselgel 40, 0.040−0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063−0.200 mm; Merck) chromatography. The ≥95% purity of the tested compounds was determined by HPLC, using a Shimadzu LC-20AD SP liquid chromatograph equipped with a DDA Detector at 196 nm (column C18 (250 mm x 4.6 mm, 5 μm, Shim-pack)); the mobile phase, delivered at isocratic flow, consisted of methanol (70–80%) and water (20–30%) and a flow rate of 1.0 mL/min. Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F_{254}) sheets that were visualized under a UV lamp. The microwave-assisted procedures were carried out with a CEM Discover LabMate microwave. Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was always used as the drying agent. Commercially available chemicals were purchased from Sigma-Aldrich.

Benzyl benzimidothioate (3). A solution of benzylbromide (0.17 mL, 1.40 mmol) in 1.0 mL of $CHCl_3$ was added dropwise to a stirred solution of benzothioamide **20** (0.200 g, 1.40 mmol) in 3.0 mL of the same solvent. The reaction mixture was heated to reflux for 12 h (TLC analysis). Then the solvent was concentrated under reduced pressure. Then diethylether was added until the

formation of a precipitate, which was collected by vacuum filtration and then washed with cold ether. Yield: 55%; mp = 168-170 °C; ^{1}H NMR (DMSO-d_6, ppm): 4.75 (s, 2H); 7.36-7.46 (m, 3H); 7.52-7.54 (m, 2H); 7.63-7.67 (m, 2H); 7.79-7.83 (m, 1H); 7.89-7.91 (m, 2H). ^{13}C NMR (DMSO-d_6, ppm): 37.34; 128.87; 128.95; 129.53; 129.92; 130.01; 131.44; 133.38; 135.75; 186.98.[34]

Benzyl 4-methoxybenzimidothioate (4). Compound **4** was obtained from compound **21** (0.234 g, 1.40 mmol) and benzylbromide (0.17 mL, 1.40 mmol) following the same procedure described for **3** . The crude product was collected by vacuum filtration and then washed with cold ether. Yield: 52 %; mp = 195-197 °C; ^{1}H NMR (DMSO-d_6, ppm): 3.89 (s, 3H); 4.70 (s, 2H); 7.18 (d, 2H, *J* = 8.8 Hz); 7.36-7.45 (m, 3H); 7.51-7.54 (m, 2H); 7.95 (d, 2H, *J* = 8.8Hz); 11.69 (bs, exch. D_2O, 1H). ^{13}C NMR (DMSO-d_6, ppm): 37.14; 56.59; 115.55; 122.80; 128.93; 129.52; 129.92; 131.62; 133.45; 165.91; 185.46.[34]

Benzyl 4-hydroxybenzimidothioate (5). To a stirred suspension of benzyl-4-methoxybenzimidothioate **4** (0.270 g, 1.00 mmol) in 10.0 mL of dry dichloromethane, cooled at -10 °C, a solution of BBr_3 (1.26 mL, 7.31 mmol) in 1.0 mL of the same solvent, was added dropwise. The mixture was left under stirring at room temperature for 24 h under nitrogen atmosphere (TLC analysis). The solvent was evaporated at reduced pressure, and the solid precipitate was washed several times with methanol. The product was finally purified by flash chromatography eluting with petroleum ether 60-80 °C/AcOEt (7/3). Yield: 60%; mp = 133-136 °C; ^{1}H NMR (DMSO-d_6, ppm): 4.32 (s, 2H); 6.82 (d, 2H, *J*= 8.2 Hz); 7.28-7.44 (m, 5H); 7.65 (d, 2H, *J*= 8.4 Hz); 11.09 (bs, exch. D_2O, 1H); 11.52 (bs, exch. D_2O, 1H). ^{13}C NMR (DMSO-d_6, ppm): 33.96; 115.78; 127.69; 128.01; 128.98; 129.30; 129.42; 129.90; 160.58; 168.09.

Phenyl benzimidothioate hydrobromide (6). A solution of benzonitrile **24** (0.94 g, 9.16 mmol) and thiophenol (0.93 ml, 9.16 mmol) in Et_2O (1mL) was kept under an atmosphere of HBr in an ice-bath. A precipitate separated very quickly. After stirring for 0.5 h at r.t., the white precipitate was filtered and washed with Et_2O. The crude compound was sufficiently pure to be used without further purification. Yield: 41%; white hygroscopic solid. ^{1}H NMR (DMSO-d_6, ppm): 7.71-7.62 (m, 5H); 7.78-7.83 (m, 3H); 7.95-7.97 (m, 2H). ^{13}C NMR (DMSO-d_6, ppm): 168.33, 136.23, 134.72, 131.66, 129.95, 128.66, 128.06, 127.91, 127.66.

4-Methoxyphenyl benzimidothioate hydrobromide (7). Compound **7** was obtained from 4-methoxybenzonitrile **25** (1.22 g, 9.16 mmol) and thiophenol (0.93 mL, 9.16 mmol) in Et_2O (1mL) following the same procedure described for **6**. Yield: 55%; mp = 198-200 °C. ^{1}H NMR (DMSO-d_6, ppm): 3.85 (s, 3H); 7.21 (d, 2H, *J* = 9.0 *Hz*); 7.63-7.70 (m, 3H); 7.76-7.78 (m, 2H); 8.00 (d, 2H, *J* = 9.0 *Hz*). ^{13}C NMR (DMSO-d_6, ppm): 165.78; 135.98; 132.68; 131.78; 131.43; 123.73; 122.56; 115.42; 56.59.

4-Hydroxyphenyl benzimidothioatehydrobromide (8). Compound **8** was obtained from 4-hydroxybenzonitrile **26** (1.09 g, 9.16 mmol) and thiophenol (0.93 mL, 9.16 mmol) in Et_2O (1mL) following the same procedure described for **6**.Yield: 65%; mp = 204-206 °C. ^{1}H NMR (DMSO-d_6, ppm): 7.01 (d, 2H, *J* = 7.8 *Hz*); 7.63- 7.72 (m, 3H); 7.76- 7.78 (m, 2H); 7.93 (d, 2H, *J* = 7.8 *Hz*); 11.09 (bs, exch. D_2O, 1H). ^{13}C NMR (DMSO-d_6, ppm): 165.46; 135.98; 132.64; 132.21; 131.42; 123.69; 120.58; 116.81.

Benzyl thiophene-2-carbimidothioate (9). Benzyl bromide (0.360 g, 2.10 mmol) was added to a solution of the compound **28** (0.300 g, 2.10 mmol) in $CHCl_3$ (10.0 mL). The resulting mixture was refluxed for 12 h. After cooling to r.t. the reaction mixture was added with Et_2O (5 mL). The white precipitate was filtered and washed with Et_2O. The crude product was sufficiently pure to

be used without further purification. Yield: 65%; mp = 180-183 °C. ^{1}H NMR ($CDCl_3$, ppm): 5.07 (s, 2H); 7.28-7.30 (m, 1H); 7.33-7.38 (m, 3H); 7.47-7.49 (m, 2H); 7.83 (d, 1H, *J*=4.8 Hz); 8.76 (d, 1H, *J*=3.6 Hz). ^{13}C NMR ($CDCl_3$, ppm): 176.95, 137.83, 137.04, 132.48, 131.78, 130.39, 129.76, 129.27, 128.91, 40.04.

Naphth-2-ylmethyl thiophene-2-carbimidothioate (10). Compound **10** was obtained from compound **28** (0.300 g, 2.10 mmol) and 2-(bromomethyl)naphthalene (0.460 g, 2.10 mmol) following the same procedure described for **9**. The crude product was sufficiently pure to be used without further purification. Yield: 55%; mp = 200-201 °C; lit. ref. n.[35]: mp = 198-199 °C.^{13}C NMR ($CDCl_3$, ppm): 176.97, 137.89, 137.12, 133.42, 133.29, 132.58, 130.46, 129.40, 129.32, 129.08, 128.11, 127.88, 126.90, 126.82, 126.81, 40.48.

Phenyl thiophene-2-carbimidothioate hydrobromide (11). Compound **11** was obtained from 2-thiophenecarbonitrile **30** (1.00 g, 9.16 mmol) and thiophenol (0.93 mL, 9.16 mmol) in Et_2O (1mL) following the same procedure described for **6**. Yield: 63%; mp = 220-221°C. ^{1}H NMR ($CDCl_3$, ppm): 7.36 (dd, 1H, *J*=5.2,4.8 Hz); 7.63-7.66 (m, 4H); 7.70-7.73 (m, 1H); 7.98 (dd, 1H, *J* = 5.2, 1.2 Hz); 8.10 (br s, 1H, NH); 8.99 (dd, 1H, *J*= 4.8, 1.2 Hz). ^{13}C NMR ($CDCl_3$, ppm): 178.05, 139.25, 139.18, 136.05, 133.82, 132.03, 130.85, 130.72, 121.22.

***N*-Benzylbenzothioamide (12)** . A mixture of *N*-benzylbenzamide **22** (0.300 g, 1.40 mmol) and Lawesson's reagent (0.679 g, 1.70 mmol) in 15.0 mL of dry THF was stirred at room temperature for 12 h (TLC analysis). Then, the organic solvent was evaporated to dryness. The resulting solid was washed with a 5% solution of $NaHCO_3$ and then extracted with AcOEt . The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The product was finally purified by flash chromatography (petroleum ether 60-80 °C/AcOEt=6/4 as eluent).Yield: 90%; mp = 78-80 °C; lit. ref. n.[36]: mp = 84-85 °C.

***N*-Benzyl-4-methoxybenzothioamide (13)**. Compound **13** was obtained from compound **23** (0.300 g, 1.20 mmol) and Lawesson's reagent (0.604 g, 1.50 mmol) following the same procedure described for **12**. The product was finally purified by flash chromatography (petroleum ether 60-80 °C/ AcOEt = 6/4 as eluent). Yield: 65%; mp = 90-93 °C; lit. ref. n.[36]: mp = 97-98 °C.

***N*-Benzyl-4-hydroxybenzothioamide (14)**. Compound **14** was obtained from compound **13** (0.180 g, 0.70 mmol) and BBr_3 (0.83 mL, 5.00 mmol) following the same procedure described for **5**. The product was finally purified by flash chromatography eluting with petroleum ether 60-80 °C/AcOEt (7/3). Yield: 67%; mp = 82-84 °C; ^{1}H NMR (DMSO-d_6, ppm): 4.98 (d, 2H, *J*= 6.0 Hz); 6.79 (d, 2H, *J* = 8.6 Hz); 7.25-7.37 (m, 5H); 7.78 (d, 2H, *J*= 8.6Hz); 10.02 (bs, exch. D_2O, 1H); 10.45 (t, exch. D_2O, 1H, *J* = 5.6 Hz). ^{13}C NMR (DMSO-d_6, ppm): 49.33; 114.96; 127.42; 128.00; 128.74; 129.90; 132.09; 138.28; 160.73; 197.12.

***N*-Benzylthiophene-2-carbothioamide (15).** *N*-Benzylthiophene-2-carboxamide **29** (0.310 g, 1.43 mmol) and Lawesson's reagent (0.580 g, 1.43 mmol) were added to 5 mL of chlorobenzene. The solution was heated to 130 °C for 4 h. The solvent was removed under vacuum and the crude product was purified by flash column chromatography eluting with hexane/AcOEt (8:2) to give **15**. Yield: 37%; mp = 87-88 °C; lit. ref. n.[37]: mp = 85-86 °C.

4-Methoxybenzamide (19). A solution of NH_4OH (0.07 mL, 1.93mmol) in 3.0 mL of dry toluene was added dropwise to a stirred solution, cooled at 0°C, of 4-methoxybenzoylchloride **17** (0.300 g, 1.72 mmol) in 3.0 mL of the same solvent, followed by addition of a solution of triethylamine (0.28 mL, 2.0 mmol). The reaction mixture was allowed to room temperature, stirred for 24 h (TLC analysis). The precipitate formed was collected by vacuum filtration and

washed with a 5% solution of $NaHCO_3$ to afford 0.103 g of pure **19**. Yield: 40%; mp = 164-167 °C; lit. ref. n.[38]: mp = 166-168 °C.

Benzothioamide (20). Compound **20** was obtained from compound **18** (0.300 g, 2.40 mmol) and Lawesson's reagent (1.165 g, 2.80 mmol) following the same procedure described for **9**. The product was finally purified by flash chromatography (petroleum ether 60-80 °C/ AcOEt = 6/4 as eluent).Yield: 40%; mp = 110-112 °C; lit. ref. n.[39]: mp = 114-116 °C.

4-Methoxybenzothioamide (21). Compound **21** was obtained from compound **19** (0.600 g, 3.90 mmol) and Lawesson's reagent (1.928 g, 4.80 mmol) following the same procedure described for **9** . The product was finally purified by flash chromatography (petroleum ether 60-80 °C/ AcOEt = 6/4 as eluent).Yield: 30%; mp = 139-141 °C; lit. ref. n.[39]: mp = 144-146 °C.

***N*-Benzyl-4-methoxybenzamide (23)**. A solution of benzylamine (0.14 mL, 1.31mmol) in 3.0 mL of dry toluene was added dropwise to a stirred solution, cooled at 0 °C, of 4-methoxybenzoylchloride **17** (0.200 g, 1.20 mmol) in 3.0 mL of the same solvent, followed by addition of triethylamine (0.20 mL, 1.42 mmol). The reaction mixture was allowed to room temperature, stirred for 24 h (TLC analysis). The precipitate formed was collected by vacuum filtration and washed with a 5% solution of $NaHCO_3$ to afford 0.260 g of **23**. Yield: 90%; mp = 120-122 °C; lit. ref. n.[38] :mp = 124-126 °C.

Thiophene-2-carbothioamide (28). 2-Thiophenecarboxamide **27** (1.00 g, 7.86 mmol) and Lawesson's reagent (3.18 g, 7.86 mmol) were added to 10.0 mL of chlorobenzene. The solution was heated to 130 °C for 12 h. The solvent was removed under vacuum and the crude product was purified by flash column chromatography eluting with hexane/AcOEt (7:3) to give **28**. Yield: 65 %; mp = 102-103 °C; lit. ref. n.[28]: mp = 104-105 °C.

***N*-Benzylthiophene-2-carboxamide (29).** To a stirred solution of NaH (0.540 g, 23.59 mmol, 60% dispersion in mineral oil) in dry DMF (10mL) and under N_2 atmosphere, was added the 2-thiophenecarboxamide **27** (1.00 g, 7.86 mmol). After 30 min at room temperature, the reaction mixture was cooled to 0 °C and a solution of benzyl bromide (1.61 g, 9.44 mmol) in DMF (2 mL) was added. The mixture was stirred at room temperature for 1 h. Then water was added and the aqueous phase was extracted with AcOEt. The combined organic phases were washed with ice and NaCl, dried, filtered, and concentrated. The residue was purified by flash column chromatography eluting with hexane/AcOEt (8:2) to afford **29**. Yield: 20%; mp = 114-115 °C; lit. ref. n.[40]: mp = 118-119 °C.

Determination of H_2S by amperometry. The H_2S-generating properties of the tested compounds have been evaluated by amperometric approach, through an Apollo-4000 Free Radical Analyzer (WPI) detector and H_2S-selective mini-electrodes. The experiments were carried out at room temperature. Following the manufacturer's instructions, a "PBS buffer 10×" was prepared ($NaH_2PO_4 \cdot H_2O$ 1.28 g, $Na_2HPO_4 \cdot 12H_2O$ 5.97 g, NaCl 43.88 g in 500 ml H_2O) and stocked at 4 °C. Immediately before the experiments, the "PBS buffer 10×" was diluted in distilled water (1:10), to obtain the assay buffer (AB); pH was adjusted to 7.4. The H_2S-selective minielectrode was equilibrated in 10 ml of the AB, until the recovery of a stable baseline. Then, 100 µl of a dimethyl sulfoxide (DMSO) solution of the tested compounds was added (final concentration of the H_2S-donors 100 µM; final concentration of DMSO in the AB 1%). The generation of $H_2S$was observed for 30 min. When required by the experimental protocol, 4 mM L-Cysteine was added, before the H_2S-donors.The correct relationship between the amperometric currents (recorded in pA) and the corresponding concentrations of H_2S was determined by

opportune calibration curves with increasing concentrations of NaHS (1 μM, 3 μM, 5 μM, 10 μM) at pH 4.0.The lower limit of reliable quantitative determination was 0.3 μM. The curves relative to the progressive increase of H_2S vs time, following the incubation of the tested compounds, were analyzed by the equation:

$$Ct=Cmax-(Cmax\cdot e^{-k\cdot t})$$

where Ct is the concentration at time t, and Cmax is the highest concentration achieved in the recording time. The constant k is $0.693/t_{1/2}$, where $t_{1/2}$ is the time required to reach a concentration = ½ Cmax. At least 5 different experiments were performed for each compound.

Animal procedures. All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86–609 and in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki, EU Directive 2010/63/EU for animal experiments). The experiments were authorized by the Ethical Committee of the University of Pisa (Protocol number 0037321/2013).

Evaluation of the functional effects on rat aortic rings. To determine a possible vasodilator mechanism of action, the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g). Rats were sacrificed by cervical dislocation under overdose of sodium pentobarbital and bled. Heart and aorta were immediately excised and freed of extraneous tissues. The endothelial layer was removed by gently rubbing the intimal surface of the aortae with a hypodermic needle. Five mm wide aortic rings were suspended, under a preload of 2 g, in 20 ml organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2\cdot 2H_2O$ 1.80; $MgSO_4\cdot 7H_2O$ 1.05; $NaH_2PO_4\cdot H_2O$ 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37 °C and continuously gassed with Clioxicarb, a mixture of

O_2 (95%)and CO_2 (5%). Changes in tension were recorded by anisometric transducer (Grass FTO3), connected with a preamplifier (Buxco Electronics) and with a software for data acquisition (BIOPAC Systems Inc., MP 100). After an equilibration period of 60 min, the endothelial removal was confirmed by the administration of acetylcholine (ACh, 10 μM) to KCl (25 mM)-precontracted vascular rings. A relaxation <10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation ≥10% (i.e. significant presence of the endothelium), were discarded. Then, 45 min after the confirmation of the endothelium removal, the aortic preparations were re-contracted by 25 mM KCl and when the contraction reached a stable plateau, the tested H_2S-donors were added cumulatively (1 μM–1 mM). Preliminary experiments showed that the KCl (25 mM)-induced contractions remained in a stable tonic state for at least 40 min. The vasorelaxing efficacy (Emax) was defined as maximal vasorelaxing response achieved with the highest concentration (1 mM) of the tested compounds, and was expressed as a percentage of the contractile tone induced by KCl. The parameter of potency was expressed as pIC_{50}, calculated as negative Logarithm of the molar concentration evoking a half-reduction of the KCl-induced contraction.

Data were obtained from aortae of 6–9 animals/group. ANOVA and Student t test were selected as statistical analysis, $P < 0.05$ was considered representative of significant statistical differences.

Effects of H_2S-donors on angiotensin II-reduced Coronary Flow (CF). The heart was mounted on a Langendorff apparatus, perfusion was carried out at constant pressure (70-80 mmHg). The heart rate (HR) and left ventricular developed pressure (LVDP) were continuously monitored in order to discard hearts showing severe arrhythmia or unstable LVDP and HR

values. Coronary flow (CF) was volumetrically measured at 5 min intervals and expressed as ml/min, normalized by the heart weight (g). After a 20 min equilibration period, the effects of the selected H_2S-donors on angiotensin II-reduced CF were assessed: 0.1μM angiotensin II (AngII) was administered through the perfusion. Once obtained a stable coronary spasm (evaluated as a reduction of the CF), compounds **11** or **4** (300 μM) were administered for 20 min (in the constant presence of 0.1 μM AngII). Preliminary experiments demonstrated that 0.1 μM AngII caused a rapid decrease of the CF, which reached and maintained a stable level for at least 1h. Changes in CF, were expressed as percentage of the basal CF. Experiments were carried out in hearts from 6-9 animals/group.

Effects of H_2S-donors on blood pressure *in vivo.*

The effects of the selected H_2S-donors and of the reference drug compound **1** on blood pressure were tested on an experimental model of hypertension, induced by the administration of L-N^G-nitroarginine methyl ester (L-NAME), inhibitor of nitric oxide synthase[41]. Male 12-weeks-old normotensive Wistar rats (250 g) were anaesthetized with sodium thiopental 60 mg/Kg. After the administration of the anaesthetic drug, the animal tails were exposed to a 15 min of irradiation with an IR lamp to determine a vasodilation of the tail-vessel, permitting an easier recording of the basal systolic blood pressure with the "tail-cuff" method by a BP recorder (Ugo Basile 58500). Basal level of systolic blood pressure (Psys) was recorded for 20 min, at 5 min intervals. Then, the rats received an i.p. injection of 100 mg/Kg L-NAME, and the Psys increase was further monitored for 20 min at 5 min intervals. Thereafter, **1** (133 μmol/Kg), or equimolar doses of the tested compounds (**4** and **11)**, or the corresponding vehicle (DMSO, 0.33 ml/Kg), were

administered i.p. to different groups, each composed of six rats. Starting from the administration of the tested compounds, the Psys values were recorded, for 30 at 5 min intervals.

Basal Psys was expressed as a mean of the four measurements carried out in each rat before the administration of L-NAME. L-NAME-induced hypertensive Psys was expressed as a mean of the four measurements carried out in each rat after the administration of L-NAME.

Change in systolic blood pressure, recorded after the drug administration, was expressed as percentage of the L-NAME-increased Psys and calculated as mean value of the six recordings carried out after the drug administration. Blood pressure measurements were carried out in 6 animals/group.

Evaluation of the membrane hyperpolarizing effects on HASMCs. The membrane hyperpolarizing effects were evaluated on human aortic smooth muscle cell (HASMC, Life Technologies) by spectrofluorometric methods, as already described.[42] Briefly, HASMCs were cultured in Medium 231 (Life technologies) supplemented with Smooth Muscle Growth Supplement (SMGS, Life Technologies) and 1% of 100 units/ml penicillin and 100 mg/ml streptomycin (Sigma Aldrich) in tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. HASMCs were cultured up to about 90% confluence and 24 h before the experiment cells were seeded onto a 96-well black plate, clear bottom pre-coated with gelatine1% (from porcine skin, Sigma Aldrich), at density of 72×10^3 per well. After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 1 h in the buffer standard (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, $CaCl_2 \cdot 2H_2O$ 2 mM, $MgCl_2 \cdot 6H_2O$1 mM, Glucose 5 mM, pH 7.4, at room temperature) containing the bisoxonol dye bis-(1,3-dibutylbarbituric acid) DiBac4(3) (Sigma Aldrich) 2.5 μM. This membrane potential-sensitive dye DiBac4(3) allowed us a non-

electrophysiological measurement of cell membrane potential;[5] in fact, this lipophilic and negatively-charged oxonol dye shuffles between cellular and extracellular fluids in a membrane potential-dependent manner (following the Nernst laws), thus allowing to assess changes in membrane potential by means of spectrofluorometric recording. In particular, an increase of fluorescence, corresponding to an inward flow of the dye, reflects a membrane depolarization; in contrast, a decrease in fluorescence, due to an outward flow of the dye, is linked to membrane hyperpolarization. The spectrofluorometric recording is carried out at excitation and emission wavelengths of 488 and 520 nm, respectively (Multiwells reader, Enspire, PerkinElmer). **31** (10 μM), a well-known activator of BKCa channels, was used as reference hyperpolarizing drug. After the assessment of base-line fluorescence, the tested compounds were added and the trends of fluorescence was followed for 40 min. The relative fluorescence decrease, linked to hyperpolarizing effects, was recorded every 2.5 min and was calculated as:

$$(Ft-F0)/F0$$

where F0 is the basal fluorescence before the addition of the tested compounds, and Ft is the fluorescence at time t after their administration. The area under curve (AUC) was calculated and the changes in fluorescence were expressed as % of that induced by **31** 10μM. Six different experiments were performed, each carried out in six replicates.

Evaluation of H_2S release on HASMCs. HASMCs were seeded (30000/per well) in a culture slide pre-coated with gelatine1% (from porcine skin, Sigma Aldrich)and after 24h were pre-loaded with a 100μM solution of the fluorescent dye WSP-1 (Washington State Probe-1,3'-methoxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl-2(pyridin-2-yldisulfanyl) benzoate, Cayman Chemical). In particular, WSP-1 was first incubated with HASMC for 30 min (allowing

cells to up-load the dye), then the supernatant was removed and replaced with a solution of tested compounds in buffer standard. **1** 300 μM was used, on the basis of previous set-up experiments, as a reference H_2S-donor. When WSP-1 reacts with H_2S, it releases a fluorophore detectable with a spectrofluorometer at λ=465-515 nm. The increasing of fluorescence (expressed as fluorescence index=FI) was monitored for 1h, by means of an EnSpire (Perkin-Elmer) spectrofluorometer. Six different experiments were performed, each carried out in six replicates. Cell fluorescence was also evaluated by a fluorescence microscope (Nikon): after the tested compounds incubation (1h), cells were washed and fixed with Bouin solution for 10 min, then cells were washed again 2 times and propidium iodide was added to highlight nuclei. Then, the culture slide was examined at fluorescence microscope.

Determination of cGMP increase on HASMC. Confluent human aortic smooth muscle cells (HASMCs) were first washed with Dulbecco's phosphate buffered saline (DPBS), and then incubated in Hanks' balanced salt solution (HBSS) with or without sodium nitroprusside (SNP) 1mM for 2 hours. Then, cells were treated with the tested compounds 300 μM or with vehicle for 20 min. After the treatment, cells were washed with Hanks' balanced salt solution and cGMP was extracted using 0.1N HCl. cGMP content was measured in the extracts using a commercially available cGMP ELISA kit following the manufacturer's instructions (Cayman Chemical). Three different experiments were performed, each carried out in three replicates.

Statistical analysis. All the experimental data were analyzed by a computer fitting procedure (software: GraphPad Prism4.0) and expressed as mean ±standard error. ANOVA and Student t

test were selected as statistical analysis. When required, the Bonferroni post hoc test has been used. $P < 0.05$ was considered representative of significant statistical differences.

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Author Contributions

E. Barresi, G. Nesi, V. Citi, E. Piragine and I. Piano performed the research, S. Rapposelli, S. Taliani and V. Calderone designed the research, analyzed the data and wrote the manuscript, L. Testai contributed to preliminary studies, F. Da Settimo, C. Gargini and M.C. Breschi revised and wrote the manuscript, A. Martelli contributed to preliminary studies, designed and performed the research, analyzed the data and wrote the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information Availability: Molecular formula strings.

ABBREVIATIONS

AB, assay buffer; Ach, acetylcholine; AngII, angiotensin II; AUC, area under curve; CBS, cystathionine-beta-synthase; CF, coronary flow; Cmax, the highest concentration achieved in the recording time; CSE, cystathionine-gamma-lyase; DADS, diallyl disulfide; DiBac4(3), bisoxonol dye bis-(1,3-dibutylbarbituric acid); DMSO, dimethyl sulfoxide; Emax, vasorelaxing efficacy; FI, fluorescence index; HASMCs, human aortic smooth muscle cells; HR, heart rate; ITEs, iminothioethers; K_{ATP}, ATP-sensitive potassium channels; L-Cys, L-Cysteine; LVDP, left ventricular developed pressure; 3MST, 3-mercaptopyruvate sulfurtransferase; pIC_{50}, potency index; PDE, phosphodiesterase; SMGS, Smooth Muscle Growth Supplement; $t_{1/2}$, the time required to reach a concentration = ½ Cmax; TAs, thioamide compounds; TC, tested compounds; WSP-1: Washington State Probe-1.

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