Arylthioamides as H_2S-donors: L-Cysteine-activated releasing properties and vascular effects in vitro and in vivo

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Chemistry

Melting points were determined using a Reichert Kofler hot-stage apparatus and are uncorrected. Infrared spectra were recorded with a Nicolet/Avatar 360 FT-IR spectrometer in Nujol mulls. Routine nuclear magnetic resonance spectra were recorded in DMSO-d_6 solution on a Varian Gemini 200 spectrometer operating at 200 MHz. Evaporation was performed in vacuo (rotary evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminum sheets (60 F-254). Combustion analyses on target compounds were performed by our Analytical Laboratory in Pisa. All compounds showed ≥95% purity.

General procedure for the synthesis of 2-substituted-4-hydroxybenzothiamides 1-4, 4-aminobenzothiamide 7, and 2,5-disubstituted-4-methoxybenzothioamides 13, 14. A solution of P_4S_{10} (0.978 g, 44.0 mmol) in 10 mL of ethanol was allowed to stir at room temperature for 1 h. Then, the appropriate commercially available benzonitrile (22.0 mmol) was added and the reaction mixture was stirred at 70 °C for 10 h. After cooling, the organic solvent was evaporated under reduced pressure, and the crude products were purified by recrystrallization from toluene (compound **1**) or by flash chromatography (compounds **2-4**, **7**, **13**, **14**, ether petrol 60-80 °C/ AcOEt=6/4).

*4-Hydroxybenzothioamide **1**.* Yield 78 %; mp 200-202 °C (toluene), lit. ref. [1]: mp 206-207 °C. Anal. Calcd. for C_7H_7NOS: C, 54.88; H, 4.61; N, 9.14; S, 20.93. Found: C, 54.96; H, 4.51; N, 9.29; S, 20.80.

*2-Chloro-4-hydroxybenzothioamide **2**.* Yield 77 %; mp 138-140 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 6.69-6.77 (m, 2H, H-3, H-5); 7.26 (d, 1H, *J*=8.2 Hz, H-6); 9.43 (bs exch., 1H, SH); 9.99 (bs exch., 1H, OH); 10.13 (bs exch., 1H, NH). Anal. Calcd. for C_7H_6ClNOS: C, 44.80; H, 3.22; N, 7.46; S, 17.09. Found: C, 44.96; H, 3.31; N, 7.39; S, 18.90.

2-Fluoro-4-hydroxybenzothioamide ***3*****.** Yield 72 %; mp 275-277 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 6.51-6.66 (m, 2H, H-3, H-5); 7.62-7.71 (m, 1H, H-6); 9.19 (bs exch., 1H, SH); 9.90 (bs exch., 1H, OH); 10.42 (bs exch., 1H, NH). Anal. Calcd. for C_7H_6FNOS: C, 49.11; H, 3.53; N, 8.18; S, 18.73. Found: C, 49.25; H, 3.61; N, 8.09; S, 18.61.

2-(Trifluoromethyl)-4-hydroxybenzothioamide ***4.*** Yield 77 %; mp 167-169 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 6.99-7.03 (m, 2H, H-3, H-5); 7.24 (d, 1H, *J*=7.8 Hz, H-6); 9.51 (bs exch., 1H, SH); 10.02 (bs exch., 1H, OH); 10.26 (bs exch., 1H, NH). Anal. Calcd. for $C_8H_6F_3NOS$: C, 43.44; H, 2.73; N, 6.33; S, 14.50. Found: C, 43.56; H, 2.81; N, 6.29; S, 14.37.

4-Aminobenzothioamide ***7.*** Yield 80 %; mp 175-177 °C, lit ref. [2]: mp 188 °C. Anal. Calcd. for $C_7H_8N_2S$: C, 55.23; H, 5.30; N, 18.40; S, 21.07. Found: C, 55.06; H, 5.39; N, 18.29; S, 20.90.

4-Methoxy-2-nitrobenzothioamide ***8***. Yield 80%; mp 140-143 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 3.86 (s, 3H, OCH_3); 7.26 (dd, 1H, *J*=8.6, 2.4 Hz, H-5); 7.39- 7.49 (m, 2H, H-3, H-6); 9.79 (bs exch., 1H, SH); 10.1 (bs exch., 1H, NH). Anal. Calcd. for $C_8H_8N_2O_3S$: C, 45.28; H, 3.80; N, 13.20; S, 15.11. Found: C, 45.68; H, 3.40; N, 13.40; S, 15.24.

2,5-Dimethyl-4-methoxybenzothioamide ***9***. Yield 73%; mp 87-90 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.07 (s, 3H, 5-CH_3); 2.29 (s, 3H, 2-CH_3); 3.76 (s, 3H, OCH_3); 6.72 (s, 1H, H-3); 7.04 (s, 1H, H-6); 9.23 (bs exch., 1H, SH); 9.81 (bs exch., 1H, NH). Anal. Calcd. for $C_{10}H_{13}NOS$: C, 61.50; H, 6.71; N, 7.17; S, 16.42. Found: C, 61.30; H, 6.91; N, 7.37; S, 16.22.

General procedure for the synthesis of 2,5-substituted-4-hydroxybenzothioamides 5, 6. A stirred suspension of the appropriate 4-methoxybenzothioamides **13**, **14** (0.0010 mol) in 15 ml of dry dichloromethane was cooled at -10 °C and BBr_3 (1.26 ml, 0.0072 mol) was added dropwise. The mixture was left under stirring for 30 min at -10 °C, and then at room temperature for 1h under nitrogen atmosphere. The solution was ice-cooled again and methanol (10 ml) was added to hydrolyze the excess of BBr_3. The solvent was evaporated at reduced pressure, and the solid

precipitate was washed few times with methanol. The residue obtained was finally purified by flash chromatography (AcOEt/ petroleum ether 60-80°C = 4/6 as eluent).

4-Hydroxy-2-nitrobenzothioamide ***5***. Yield 70 %; mp 146-148 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 7.03 (dd, 1H, *J*=8.0, 2.4 Hz, H-5); 7.24-7.32 (m, 2H, H-3, H-6); 9.68 (bs exch., 1H, SH); 9.98 (s exch., 1H, OH); 10.62 (bs exch., 1H, NH). Anal. Calcd. for $C_7H_6N_2O_3S$: C, 42.42; H, 3.05; N, 14.13; S, 16.18. Found: C, 42.52; H, 3.27; N, 14.33; S, 16.33.

4-Hydroxy-2,5-dimethyl-benzothioamide ***6***. Yield 68%; mp 143-145 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.04 (s, 3H, 5-CH_3); 2.20 (s, 3H, 2-CH_3); 6.52 (s, 1H, H-3); 6.99 (s, 1H, H-6); 9.13 (bs exch., 1H, SH); 9.42 (s exch., 1H, OH); 9.71 (bs exch., 1H, NH). Anal. Calcd. for $C_9H_{11}NOS$: C, 59.64; H, 6.12; N, 7.73; S, 17.69. Found: C, 59.79; H, 6.32; N, 7.83; S, 17.83.

General procedure for the synthesis of arylcarbothioamides 8-10, 12. A suspension containing 1.0 mmol of the appropriate commercially available arylcarboxamide and Lawesson's reagent (0.490 g, 1.20 mmol) in 10 mL of dry THF was stirred at room temperature for 1 h. The solvent was evaporated under reduce pressure and the residue was partitioned between 5% aqueous $NaHCO_3$ and AcOEt. The organic solvent was dried over $MgSO_4$ and eliminated under vacuum. The crude products were further purified by flash chromatography using the appropriate eluting mixture.

4-Pyridinecarbothioamide ***8.*** Yield 72 %; mp 188-190 °C (AcOEt as eluent for flash chromatography). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 7.72 (d, 2H, *J*=4.6 Hz, H-2, H-6); 8.66 (d, 1H, *J*=4.6 Hz, H-3, H-5); 9.81 (bs exch., 1H, SH); 10.24 (bs exch., 1H, NH). Anal. Calcd. for $C_6H_7N_2S$: C, 52.15; H, 4.38; N, 20.27; S, 23.20. Found: C, 52.25; H, 4.41; N, 20.18; S, 23.08.

Pyrazine-2-carbothioamide ***9***. Yield 72%; mp 198-200 °C (AcOEt/ petroleum ether 60-80 °C=5/5 as eluent for flash chromatography). ^{1}H NMR (200 MHz, DMSO-d_6, δppm): 8.66 (s, 1H, H-6); 8.84 (s, 1H, H-5); 9.56 (s, 1H, H-3); 10.03 (bs exch., 1H, SH); 10.35 (bs exch., 1H, NH). Anal. Calcd. $C_5H_5N_3S$: C, 43.15; H, 3.62; N, 30.19; S, 23.04. Found: C, 43.35; H, 3.42; N, 30.39; S, 23.17.

*Furan-2-carbothioamide **10***. Yield 71%; mp 128-130 °C (AcOEt/ petroleum ether 60-80 °C=6/4 as eluent for flash chromatography); lit ref [3]: mp 103-105 °C. Anal. Calcd. for C_5H_5NOS: C, 47.23; H, 3.96; N, 11.01; S, 25.22. Found: C, 47.53; H, 3.76; N, 11.21; S, 25.37.

Indol-2-carbothioamide **12**. Yield 70 %; mp 216-218 °C (AcOEt/ petroleum ether 60-80 °C = 5/5 as eluent for flash chromatography). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 6.99-7.06 (m, 1H, H-4); 7.16-7.23 (m, 2H, H-5, H-6); 7.50-7.62 (m, 2H, H-3, H-7); 9.42 (bs exch., 1H, SH); 9.60 (bs exch., 1H, NH); 11.25 (bs exch., 1H, NH). Anal. Calcd. for $C_9H_8N_2S$: C, 61.34; H, 4.58; N, 15.90; S, 17.40. Found: C, 61.14; H, 4.88; N, 15.70; S, 17.53.

Thiophen-2-carbothioamide 11. 2-Thiophenecarboxamide (1.00 g, 7.86 mmol) and Lawesson's reagent (3.18 g, 7.86 mmol) were added to 10 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 **11**. Yield 65%, mp 102-103 °C; lit ref [4]: mp 106-107 °C ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 7.27 (dd, 1H, *J*=5.0, 3.8 Hz, H-3); 7.56 (dd, 1H, *J*=3.8, 1.1 Hz, H-2); 7.68 (dd, 1H, *J*=5.0, 1.1 Hz, H-4). Anal. Calcd. for $C_5H_5NS_2$: C, 41.93; H, 3.52; N, 9.78; S, 44.77. Found: C, 41.81; H, 3.50; N, 9.59; S,44.64.

Pharmacology

Amperometric Determination of H_2S

The characterization of the potential H_2S-generating properties of the tested compounds has been carried out by amperometric approaches, through an Apollo-4000 Free Radical Analyzer (WPI) detector and H_2S-selective minielectrodes. Following the manifacturer's instructions, a "PBS buffer 10x" was prepared ($NaH_2PO_4xH_2O$ 1.28 g, $Na_2HPO_4x12H_2O$ 5.97 g, NaCl 43.88 g in 500 mL H_2O) and stocked at 4 °C. Immediately before the experiments, the "PBS buffer 10x" 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 AB, until the recovery of a stable baseline. Then, 100 µL of a DMSO solution of the tested H_2S-releasing compounds was added, at the final concentration of 1 mM. The eventual generation of H_2S was observed for 15 min. When required by the experimental protocol, L-Cysteine (4 mM) or reduced glutathione (GSH, 4 mM) were 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, which were previously obtained by the use of increasing concentrations of NaHS (1 µM-1 mM) at pH 4.0. The curves relative to the progressive increase of H_2S vs time, following the incubation of the tested compounds, were analysed by the equation

$$C_t = C_{max} - (C_{max} \cdot e^{-k \cdot t})$$

where C_t is the instant concentration at time t, C_{max} is the highest concentration achieved at the steady state. The constant k is $0.693/t_{1/2}$, where $t_{1/2}$ is the time required to reach a concentration = ½ C_{max}. The values of C_{max} and $t_{1/2}$ were calculated by a computer fitting procedure (software: GraphPad Prism 4.0) and expressed as mean ± standard error; at least 5 different curves were performed for each compound. For C_{max} levels < 2 µM, the $t_{1/2}$ was not calculated. ANOVA and Student *t* test were selected as statistical analysis, $P < 0.05$ was considered representative of significant statistical differences.

Evaluation of the membrane hyperpolarizing effects on HASMCs

Human aortic smooth muscle cell (HASMC, Invitrogen) were cultured in Medium 231 (Invitrogen) supplemented with Smooth Muscle Growth Supplement (SMGS, Invitrogen) 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. HASMC were cultured up to about 90% confluence and 24h before the experiment cells were seeded onto a 96-well black plate, clear bottom pre-coated with

gelatine 1% (from porcine skin, Sigma Aldrich), at density of $72x10^3$ per well. After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 1 hour with an appropriate buffer (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, $CaCl_2x2H_2O$ 2 mM, $MgCl_2x6H_2O$, Glucose 5 mM, pH 7.4, at room temperature) containing the bisoxonol dye bis-(1,3-dibutylbarbituric acid) DiBac4(3) at 2.5 μM (Sigma Aldrich). This membrane potential-sensitive dye DiBac4(3) allows us a non-electrophysiological measurement of cell membrane potential; in fact, this lipophylic and negatively-charged oxonol dye shuffles between cellular and extracellular fluids in a membrane potential-dependent manner (following the Nernst equation), thus allowing to assess changes in membrane potential by means of spectrofluorimetric 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 spectrofluorimetric recording is carried out at excitation and emission wavelengths of 488 and 520 nm, respectively (Multiwells reader, Enspire, Perkin-Elmer). Levcromakalim (LCRK), a well-known activator of K_{ATP} channels, was used as reference hyperpolarizing drug. After the assessment of base-line fluorescence, LCRK, the H_2S-donors (100 μM-1 mM) or the vehicle (0.5% solution of DMSO in the above buffer) were added, to evaluate their influence on membrane potential. The concentration 1 mM of NaHS was selected because it caused maximal vasorelaxing effects in the functional assay on rat aortic rings. Preliminary experiments showed that NaHS 1 mM was devoid of significant toxic effects on HASMCs, while higher concentrations caused a reduction in cell viability (data not shown). The relative-fluorescence decrease, linked to hyperpolarizing effects, was calculated as:

$$(F_t-F_0)/F_0$$

where F_0 is the basal fluorescence before the addition of the tested compounds, and F_t is the fluorescence at time t after their administration. The change in fluorescence where expressed as a %

of the maximal one induced by LCRK. Data were expressed as mean ± standard error; six different experiments were performed, each carried out in six replicates.

Evaluation of the functional effects on rat aortic rings

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

To determine a possible vasoactive 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 light ether anaesthesia and bled. The aortae were immediately excised and freed of extraneous tissues. The endothelial layer was removed by gently rubbing the intimal surface of the vessels 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; $CaCl_2$ 1.80; $MgSO_4x7H_2O$ 1.05; NaH_2PO_4 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 means of an isometric transducer (Grass FTO3), connected with a preamplifier (Buxco Electronics) and with a software of data acquisition (BIOPAC Systems Inc., MP 100). After an equilibration period of 60 minutes, 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.

45 min after the confirmation of the endothelium removal, the vessels were contracted by 60 mM KCl, i.e. a depolarizing stimulus able to evoke an almost full contraction of vascular smooth muscle. After the contraction achieved a stable plateau, the preparations were submitted to a wash-

out and to a further equilibration time (45 min). Then, the H_2S-releasing compounds (or the corresponding vehicle) were incubated for 20 minutes, at the concentration 1 mM. At the end of the incubation time, 3-fold increasing concentrations of noradrenaline (NA, 1 nM – 1 μM) were added cumulatively. After completing the above concentration-response curve for NA, the preparations were again submitted to a wash-out and to a further equilibration time (45 min). Then, 3-fold increasing concentrations of noradrenaline (NA, 1 nM – 1 μM) were cumulatively added again. In all cases, the viability of the vessel and the "reversibility" of the effects of H_2S-releasing compounds was demonstrated. The concentration-response curves relative to the vasoconstriction induced by NA were analyzed by the Hill equation. The NA efficacy was evaluated as maximal contractile response, expressed as a percentage (%) of the contractile tone previously induced by 60 mM KCl. The parameter of potency was expressed as pEC_{50}, calculated as negative Logarithm of the molar concentration of NA, evoking a half-maximal response. The parameters of efficacy and potency of NA were expressed as mean ± standard error, for 6-10 experiments.

Evaluation of the H_2S-release with rat aortic rings

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

In order to evaluate the H_2S-release in Tyrode with rat aortic rings, the amperometric approach was used. Five mm thoracic aortic rings of male normotensive Wistar rats (250-350 g) were excised as described above, immersed in 1ml of Tyrode previously gassed with Clioxicarb at room temperature in a Four-Port Closed Chamber (NOCHM-4, WPI), suitable for use with WPI'S H_2S-electrodes. The chambers consist of a close fitting cap through which the electrode can be inserted reducing the surface area of the solution exposed to air in order to guarantee the accuracy of

measurements, a significant reduction of the "noise" and a great improvement of detection sensitivity

The H_2S-selective electrode was equilibrated in Tyrode, until the recovery of a stable baseline. Then, 10µL of a DMSO solution of the tested compounds was added at the final concentration of 1mM. The generation of H_2S was observed for 15 min. The relationship between the amperometric currents (recorded in pA) and the corresponding concentrations of H_2S was determined by opportune calibration curves as described above. The curves relative to the progressive increase of H_2S vs time were analysed by the equation above reported.

Evaluation of the effects on the blood pressure

The experimental protocol was performed as already described, in agreement with the guidelines of the European Community Council Directive 86-609. The animals were anesthetised with sodium pentobarbital (60 mg/Kg). After the administration of the anaesthetic drug, the animal tails were exposed for 15-20 min to irradiation with an I.R. lamp to cause vasodilation of the tail-vessels. Then, four basal values of the systolic blood pressure (SBP) were sphygmomanometrically recorded with the "tail-cuff" method by a BP recorder (Ugo Basile 58500, Italy) at 5-min intervals. The mean of these four measurements was taken as basal SBP. Then, the animals were treated with an oral administration (by gavage) of NaHS 0.1 mg/Kg, compound **1** (at a dose equimolar to NaHS) or the corresponding vehicle. After 20 min, the SBP values were recorded four times at intervals of 5 min (during this period, a maintenance dose of 10 mg/Kg sodium pentobarbital was administered, when required). The mean of these four measurements was taken as SBP, after the drug-administration. At the end of the experiments, the rats were euthanized by an overdose of sodium pentobarbital. In each experiment, the basal value of systolic pressure was calculated, as a mean of the four measurements preceding the administration of the tested compounds. Then, the SBP recorded after

the administration of the compounds was expressed as a % of the basal SBP. Data are expressed as mean ± SEM. Each experimental group was composed of 6 animals.

Statistic analysis

Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism 4.0). ANOVA and Student t test were selected as statistical analyses; $P<0.05$ was considered representative of significant statistical differences.

Substances

NaHS (Sigma-Aldrich), diallyl disulfide and GYY4137 were dissolved 100mM in dimethylsulfoxide (DMSO) and further diluted in Tyrode solution. KCl (Carlo Erba) was dissolved 2.5 M in Tyrode solution. (±) Noradrenaline (+)-bitartrate (Sigma-Aldrich) was dissolved (1 mM) and further diluted in Tyrode solution. Stock solutions (100 mM in EtOH 95%) of Acetylcholine chloride (Sigma-Aldrich) were further diluted in Tyrode solution. All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments have demonstrated ineffectiveness of the administration of the vehicles used under these experimental conditions.

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