

Hydrogen Sulfide Releasing Capacity of Natural Isothiocyanates: Is It a Reliable Explanation for the Multiple Biological Effects of Brassicaceae?

Authors

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Key words

- hydrogen sulfide
- H₂S-donors
- Brassicaceae
- natural isothiocyanates
- glucosinolates

Abstract

Hydrogen sulfide is an endogenous pleiotropic gasotransmitter, which mediates important physiological effects in the human body. Accordingly, an impaired production of endogenous hydrogen sulfide contributes to the pathogenesis of important disorders. To date, exogenous compounds, acting as hydrogen sulfide-releasing agents, are viewed as promising pharmacotherapeutic agents. In a recent report, the hydrogen sulfide-releasing properties of some synthetic aryl isothiocyanate derivatives have been reported, indicating that the isothiocyanate function can be viewed as a suitable slow hydrogen sulfide-releasing moiety, endowed with the pharmacological potential typical of this gasotransmitter. Many isothiocyanate derivatives (deriving from a myrosinase-mediated transformation of glucosinolates) are well-known secondary metabolites of

plants belonging to the family Brassicaceae, a large botanical family comprising many edible species. The phytotherapeutic and nutraceutic usefulness of Brassicaceae in the prevention of important human diseases, such as cancer, neurodegenerative processes and cardiovascular diseases has been widely discussed in the scientific literature. Although these effects have been largely attributed to isothiocyanates, the exact mechanism of action is still unknown. In this experimental work, we aimed to investigate the possible hydrogen sulfide-releasing capacity of some important natural isothiocyanates, studying it *in vitro* by amperometric detection. Some of the tested natural isothiocyanates exhibited significant hydrogen sulfide release, leading us to hypothesize that hydrogen sulfide may be, at least in part, a relevant player accounting for several biological effects of Brassicaceae.

Introduction

Hydrogen sulfide (H₂S) is presently recognized as a pleiotropic endogenous gasotransmitter. At physiological concentrations, it is pivotally involved in the homeostatic regulation of the cardiovascular, respiratory, gastroenteric and nervous systems. Moreover, H₂S plays a significant modulatory role in inflammatory processes. Consistently, H₂S-releasing compounds are currently viewed as promising drugs for several diseases [1, 2]. Ideal H₂S-donors should generate H₂S at a relatively slow rate [1–3]. This pharmacological feature is exhibited by early examples of original synthetic H₂S-releasing agents, such as the phosphinodithioate derivative GYY4137 (morpholin-4-ium-4-methoxyphenyl-morpholino-phosphinodithioate), as well as aminothioliol [4] and aryl thioamide [5] derivatives. Even some examples of natural H₂S-releasing derivatives are recog-

nized. For instance, organic polysulfides of Alliaceae, such as diallyl disulfide (DADS), are H₂S-donors, and such a property is thought to account for the most important biological and pharmacological effects of garlic [6].

In a recent paper of ours, a novel H₂S-releasing chemical moiety has been described: in that work, some synthetic aryl isothiocyanates have been demonstrated to act as H₂S-donor agents and to exhibit significant effects on vascular smooth muscle such as vasodilation related with the activation of vascular voltage-gated potassium channels [7], clearly attributable to the release of H₂S [8].

Notably, many isothiocyanates are naturally occurring compounds. They derive from a myrosinase-mediated transformation of glucosinolates, which are well-known secondary metabolites of plants mainly belonging to Brassicaceae, a large botanical family comprising many edible species,

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such as *Brassica oleracea* L. (var. *italica*, *gemmifera*, *botrytis*, *capitata*, and *acephala*), *Brassica nigra* (L.) Koch, *Sinapis alba* L. etc. Presently, glucosinolates, along with the corresponding isothiocyanates, are receiving greatest scientific interest for their numerous biological and pharmacological effects and useful applications in many aspects of human health [9]. It is worthy to note that there is an intriguing overlapping between many biological effects attributed to some Brassicaceae and those exhibited by the gasotransmitter H_2S .

Nevertheless, the possibly existing H_2S -releasing potential of this class of natural compounds has never been described.

In this paper, some important isothiocyanates, naturally occurring in many species of Brassicaceae, have been selected: allyl isothiocyanate (AITC, highly present in black mustard, *B. nigra*, 4-hydroxybenzyl isothiocyanate (HBITC, highly present in white mustard, *S. alba*), benzyl isothiocyanate (BITC, highly present in garden cress, *Lepidium sativum* L.) and erucin (ERU, present in different species such as broccoli, *B. oleracea* L., and rocket, *Eruca vesicaria* L.) (● Fig. 1). Moreover, the glucosinolate sinigrin (SIN, precursor of AITC) has been also selected for comparison purposes. The possible H_2S -releasing activity of the selected compounds has been investigated by a reliable amperometric approach which was already used for analogous studies [5,8].

Results and Discussion

The H_2S -releasing capacity is a pharmacologically relevant feature, already attributed to naturally occurring compounds, such as the diallyl polysulfides of garlic, but never reported for natural isothiocyanates. Indeed, in previous works of ours, the incubation of the reference drug DADS led to a negligible formation of H_2S in the absence of L-cysteine; but, in the presence of this amino acid, the incubation of DADS was followed by a relatively slow formation of H_2S , with C_{max} of about $20 \mu M$ (● Table 1). As concerns the isothiocyanates tested in the present work, ERU and BITC exhibited the weakest (albeit significant) H_2S -releasing properties. In particular, ERU and BITC behaved as very poor H_2S -donors both in the absence and in the presence of L-cysteine (● Table 1; ● Fig. 2 and 3).

AITC led to the formation of appreciable amounts of H_2S , but only in the presence of L-cysteine. Indeed, in the absence of L-cysteine, the incubation of AITC led to the formation of low concentrations of H_2S ; in contrast, the presence of L-cysteine caused a significant improvement of the H_2S -releasing properties of AITC (● Table 1; ● Fig. 2 and 3). Such a L-cysteine-dependent release of H_2S , probably due to the nucleophilic behaviour of the amino acid, has been already observed for well-known H_2S -donors, such as GYY4137, DADS, and many synthetic thioamides and isothiocyanates [5,8]. In contrast, the allyl glucosinolate SIN was devoid of significant H_2S -releasing effects in both the experimental conditions (absence or presence of L-cysteine) (● Table 1; ● Fig. 2 and 3). This finding indicates that the isothiocyanate moiety is an obligatory requirement.

Among the few selected secondary metabolites of Brassicaceae, BITC was the most effective H_2S -donor, exhibiting significant and remarkable H_2S -release. In particular, in the absence of the L-cysteine, the incubation of HBITC was followed by a slow generation of appreciable levels of H_2S (about $10 \mu M$). The co-presence of the amino acid led to a marked increase in the H_2S -release from HBITC (● Table 1; ● Fig. 4). The H_2S -releasing properties of BITC, recorded in the presence of L-cysteine, were equiva-

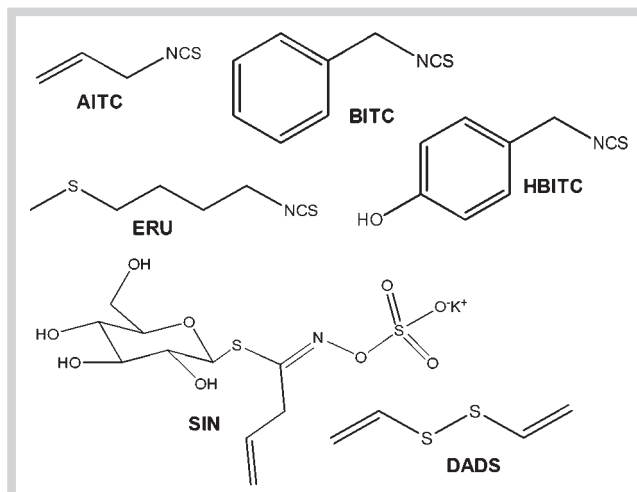


Fig. 1 Chemical structures of the tested compounds.

Table 1 C_{max} parameters of the hydrogen sulfide-donor compounds. The table summarizes C_{max} parameters relative to the H_2S -releasing effects exhibited by the tested compounds after their incubation in AB at physiological pH and temperature, in the absence (AB) or in the presence (AB+L-cys) of L-cysteine (4 mM); the results for DADS come from reference [5]. Data are expressed as means \pm SEM.

Compound	AB C_{max} (μM)	AB + L-Cys C_{max} (μM)
DADS	< 0.5	18.9 ± 4.8
AITC	0.5 ± 0.2	6.2 ± 1.3
BITC	< 0.5	1.9 ± 0.4
ERU	0.5 ± 0.2	1.9 ± 0.1
HBITC	11.0 ± 0.8	17.9 ± 1.5
SIN	< 0.5	< 0.5

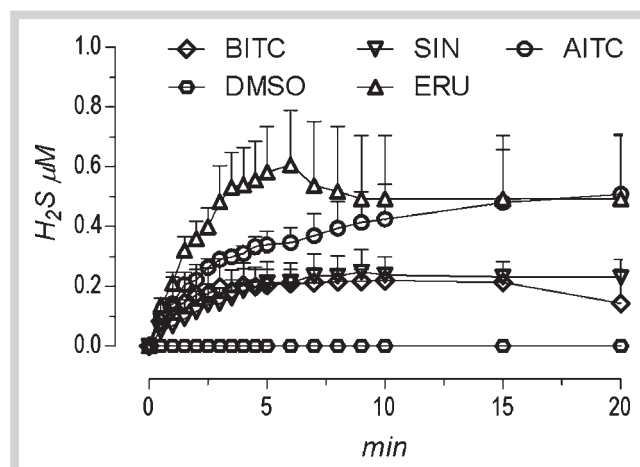


Fig. 2 Hydrogen sulfide-release from allyl isothiocyanate, erucin, benzyl isothiocyanate, and sinigrin in the absence of L-cysteine. The curves describe the increase of the H_2S concentration with respect to time, following the incubation of AITC, ERU, BITC, and SIN, in the absence of L-cysteine. H_2S was recorded by amperometry; the vertical bars indicate SEM.

lent to those exhibited by DADS in previous studies [5]; but, unlike DADS (which showed weaker H_2S -release in the absence of

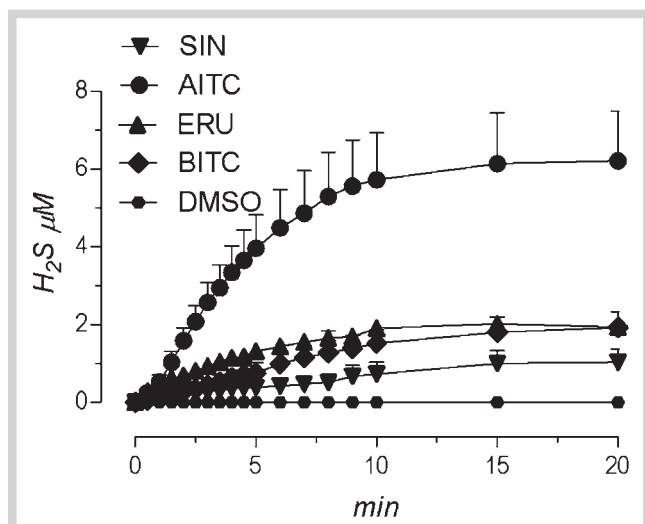


Fig. 3 Hydrogen sulfide-release from allyl isothiocyanate, erucin, benzyl isothiocyanate, and sinigrin in the presence of L-cysteine. The curves describe the increase of the H₂S concentration with respect to time, following the incubation of AITC, ERU, BITC, and SIN, in the presence of L-cysteine. H₂S was recorded by amperometry; the vertical bars indicate SEM.

L-cysteine), BITC behaved as a remarkable H₂S-donor even in the absence of this amino acid.

The nutraceutical and phytotherapeutic usefulness of Brassicaceae in the prevention of important human diseases, such as cancer, neurodegenerative processes, and cardiovascular diseases, has been widely discussed in the scientific literature [9]. Although these effects are reasonably attributed to isothiocyanates (mainly formed from the corresponding glucosinolates by the enzymatic activity of the plant enzyme myrosinase, but also by intestinal bacterial microflora in mammals [10]), the exact mechanism of action is still unknown.

This is the first experimental report demonstrating that some natural isothiocyanates behave as slow H₂S-releasing agent. Only in the last decade, H₂S has been recognized as an important pleiotropic gasotransmitter and has been defined as “the new nitric oxide” [11], since it is endowed with pivotal regulatory roles in almost all physiological systems in the human body and particularly in the cardiovascular one. Therefore, the H₂S-releasing properties of some natural isothiocyanates, as described in this paper, give insights for understanding the biological and pharmacological properties and the mechanism of action of many species of Brassicaceae. Moreover, on the basis of the growing scientific knowledge on the pathophysiological roles of H₂S, this finding may help to assess and identify the pharmaceutical and nutraceutical potential of naturally occurring glucosinolates/isothiocyanates.

Materials and Methods

Amperometric determination of H₂S and statistical analysis

The characterization of the potential H₂S-generating properties of the tested compounds has been carried out by an amperometric approach, through the Apollo-4000 free radical analyzer (WPI) detector and H₂S-selective mini-electrodes. The experiments have been carried out at room temperature. Following

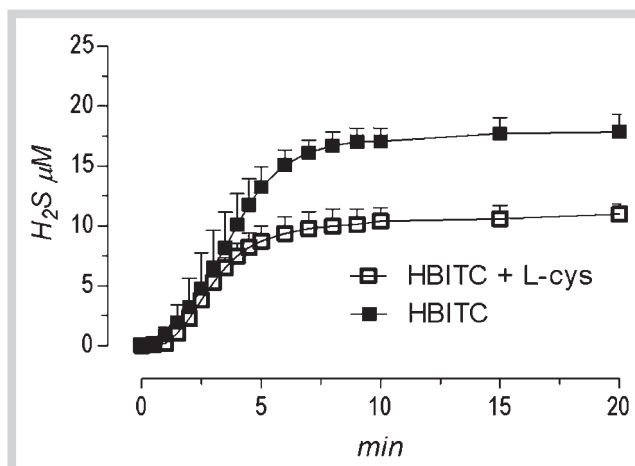


Fig. 4 Hydrogen sulfide-release from 4-hydroxybenzyl isothiocyanate. The curves describe the increase of the H₂S concentration with respect to time, following the incubation of HBITC, either in the absence (white squares) or in the presence of L-cysteine (4 mM) (black squares). H₂S was recorded by amperometry; the vertical bars indicate SEM.

the manufacturer's instructions, a “PBS buffer 10x” was prepared (NaH₂PO₄·H₂O 1.28 g, Na₂HPO₄·12H₂O 5.97 g, NaCl 43.88 g in 500 mL H₂O) and stocked at 4 °C. Immediately before the experiments, the “PBS buffer 10x” was diluted using distilled water (1 : 10) to obtain the assay buffer (AB) and the pH adjusted to 7.4. The H₂S-selective mini-electrode was equilibrated in 10 mL of the AB, until the recovery of a stable baseline. Then, 100 µL of a DMSO solution of all the tested H₂S-releasing compounds was added (the final concentration of the tested H₂S-donors was 1 mM; the final concentration of DMSO in the AB was 1%). The eventual generation of H₂S was observed for 20 min. Preliminary experiments demonstrated that DMSO 1% did not produce any interference on the amperometric recording. When required by the experimental protocol, L-cysteine (4 mM) was added before the addition of the H₂S-donors. The correct relationship between the amperometric currents (recorded in pA) and the corresponding concentrations of H₂S was determined by suitable calibration curves, which were previously obtained by the use of increasing concentrations of NaHS (1 µM, 3 µM, 5 µM, 10 µM) at pH 4.0. The curves relative to the progressive increase of H₂S 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 in the recording time. The constant k is $0.693/t_{hc}$, where t_{hc} (time for half concentration) is the time required to reach a concentration of $1/2 C_{\max}$. The values of C_{\max} and t_{hc} were calculated by a computer fitting procedure (software: GraphPad Prism 5.0) and expressed as mean \pm standard error with at least 5 different curves performed for each compound. ANOVA and Student's t test was selected as statistical analysis; $p < 0.05$ was considered representative of significant statistical differences [8].

Substances

AITC, SIN, BITC and DADS have been purchased from Sigma-Aldrich; ERU and HBITC were purchased from Santa Cruz Biotecology. The purity of the tested compounds was: AITC >97.0%, SIN >99.0%, BITC >98.0%, ERU >98.0%, HBITC >98.0%, and DADS >80.0%. All the tested compounds were dissolved at 100 mM in DMSO (Carlo Erba) and further diluted in aqueous solution. The solutions were prepared immediately before the experiments.

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Conflict of Interest

The authors declare no conflict of interest.

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