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# NOSH-Aspirin: A Novel Nitric Oxide–Hydrogen Sulfide-Releasing Hybrid: A New Class of Anti-inflammatory Pharmaceuticals

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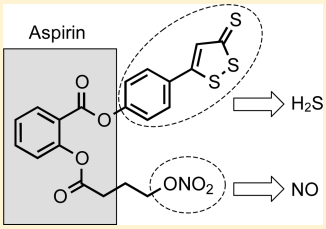
Supporting Information

**ABSTRACT:** A series of new hybrids of aspirin (ASA), bearing both nitric oxide (NO) and hydrogen sulfide ($H_2S$)-releasing moieties were synthesized and designated as NOSH compounds (**1**–**4**). NOSH-**1** (4-(3-thioxo-3H-1,2-dithiol-5-yl) phenyl 2-((4-(nitrooxy)-butanoyl)oxy) benzoate); NOSH-**2** (4-(nitrooxy)butyl (2-((4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)carbonyl)phenyl)); NOSH-**3** (4-carbamothioylphenyl 2-((4-(nitrooxy)butanoyl)-oxy)benzoate); and NOSH-**4** (4-(nitrooxy)butyl 2-(5-((*R* )-1,2-dithiolan-3-yl)pentanoyloxy)-benzoate). The cell growth inhibitory properties of compounds **1**–**4** were evaluated in eleven different human cancer cell lines of six different tissue origins. These cell lines are of adenomatous (colon, pancreatic, lung, prostate), epithelial (breast), and lymphocytic (leukemia) origin. All NOSH compounds were extremely effective in inhibiting the growth of these cell lines. NOSH-**1** was the most potent, with an $IC_{50}$ of 48 ± 3 nM in HT-29 colon cancer cells. This is the first NSAID-based compound with such potency. This compound was also devoid of any cellular toxicity, as determined by LDH release. NOSH-**1** was comparable to aspirin in its anti-inflammatory properties, using the carrageenan rat paw edema model.




**KEYWORDS:** *Nitric oxide, hydrogen sulfide, aspirin, anti-inflammatory, anticancer*

Nonsteroidal anti-inflammatory drugs (NSAIDs), in general, and aspirin, in particular, are recognized as the prototypical chemopreventive agents against many forms of cancers.[1] However, long-term use of NSAIDs may lead to serious side effects, including gastrointestinal and renal.[1] The search for "better NSAIDs" has led to the development of selective cyclooxygenase-2 inhibitors (Coxibs) and nitric oxide-releasing NSAIDs (NO-NSAIDs). Several large-scale clinical trials have shown that long-term use of coxibs is associated with an increased risk of adverse myocardial events.[2]

The development of NO-NSAIDs was based on the observation that NO has some of the same properties as prostaglandins within the gastric mucosa. Therefore, coupling an NO-releasing moiety to an NSAID might deliver NO to the site of NSAID-induced damage, thereby decreasing gastric toxicity. Animal and human studies have shown that many NO-NSAIDs are indeed safer to the GI mucosa than the parent NSAID.[3,4]

Recently, a new class of NSAIDs possessing a hydrogen sulfide ($H_2S$)-releasing moiety (HS-NSAIDs) have been described in the literature.[5−9] We have shown that these compounds can be useful in controlling cancer.[10−12] However, NO-NSAIDs and HS-NSAIDs have several drawbacks, limiting their development as pharmaceuticals. For example, HS-NSAIDs have relatively high $IC_{50}$s for cell growth inhibition. Some NO-NSAIDs can form quinone methide intermediates, questioning the role of NO in their biological activity.[13−15] Others yet have high $IC_{50}$s for cell growth inhibition.[16] Therefore, we postulated that a new hybrid that incorporated the active parts of each compound might be more potent than either one alone. Our hypothesis has proved to be correct. Here we describe the synthesis of four NOSH (nitric oxide-, hydrogen sulfide-releasing) compounds that release both $H_2S$ and NO (Figure 1). One of the compounds, NOSH-**1**, has

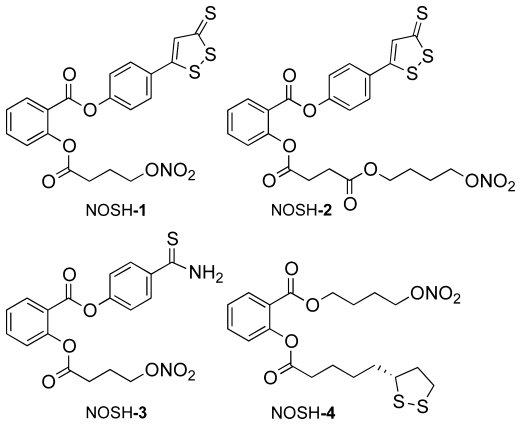



**Figure 1.** Chemical structures of NOSH compounds.

$IC_{50}$s for cell growth inhibition in the low nanomolar range and shows strong anti-inflammatory properties.

The NOSH compounds reported here were developed by using aspirin as a scaffold to which NO and $H_2S$ releasing

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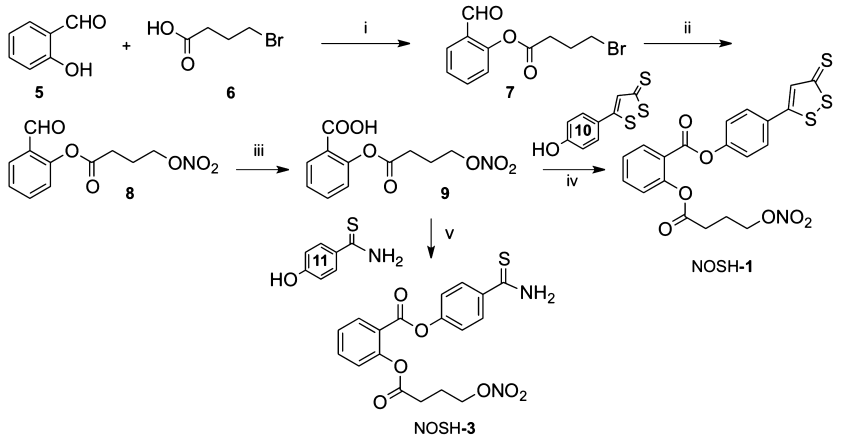
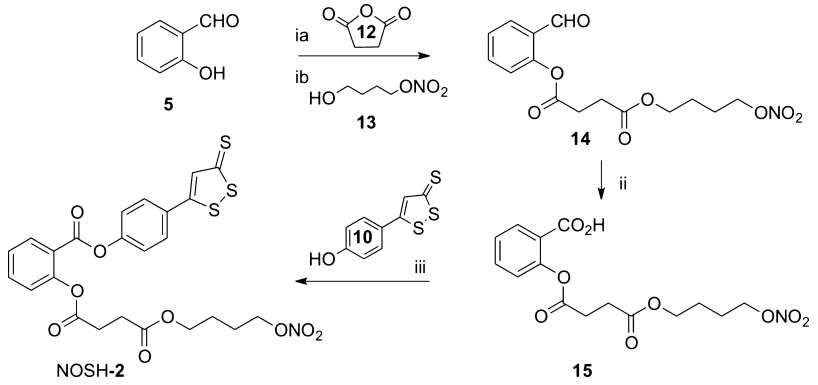
**Scheme 1. Synthesis of NOSH-1 and NOSH-3**[a]




[a]Conditions: (i) DCC/DMAP, DCM, rt, 6 h, (ii) $AgNO_3$, $CH_3CN$, rt, 12 h, (iii) $KMNO_4$, acetone, 0 °C to rt, 3 h, (iv) ADT-OH (**10**), DCC/DMAP, DCM, rt, 6 h, (v) TBZ (**11**), DCC/DMAP, DCM, rt, 6 h.

**Scheme 2. Synthesis of NOSH-2**[a]




[a]Conditions: (ia) succinic anhydride (**12**), DMAP, DCM, rt, 12 h, (ib) 4-hydroxybutyl nitrate (**13**), DCC, rt, 6 h, (ii) $KMnO_4$, acetone, 0 °C to rt, 3 h, (iii) ADT-OH (**10**), DCC/DMAP, DCM, rt, 6 h.

moieties were coupled with one of the 1, 2 positions. We used nitrate (-$ONO_2$) for NO release and attached it to the aspirin through an aliphatic spacer, while one of the following $H_2S$-releasing moieties, 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH), or 4-hydroxy benzothiazamide (TBZ) or lipoic acid were directly coupled to aspirin (NOSH-**1**−**4**, Figure 1). Salicylaldehyde was used as the starting material for NOSH-**1**−**3**, and aspirin was used for NOSH-**4**.

Salicylaldehyde (**5**) coupled with 4-bromobutyric acid (**6**) in the presence of DCC/DMAP was used to yield compound **7**. The bromo moiety in compound **7** was then substituted with nitrate using $AgNO_3$ to give compound **8**. Then the aldehyde group of compound **8** was oxidized to its corresponding carboxylic group in the presence of $KMnO_4$ to yield compound **9**.[17] This was then used as the precursor for preparation of NOSH-**1** and -**3** using either 5-(4-hydroxyphenyl)-3H-1, 2-dithiole-3-thione (ADT-OH, **10**) or 4-hydroxythiobenzamide (TBZ, **11**), respectively (Scheme 1).

For preparation of compound NOSH-**2**, salicyladehyde (**5**), succinic anhydride (**12**), and a catalytic amount of DMAP in methylene chloride were treated for 24 h, at room temperature, to prepare the succinic acid linked intermediate. To this intermediate *in situ* were added hydroxybutyl nitrate (**13**) and DCC to afford compound **14**. This was further oxidized by $KMnO_4$ to its corresponding aromatic carboxylic acid (**15**), which was coupled to ADT-OH (**10**) in the presence of DCC/DMAP in methylene chloride to give NOSH-**2** (Scheme 2).

NOSH-**4** was synthesized by using lipoic acid as $H_2S$-releasing donor. We used aspirin as the starting material and coupled it with compound **13** in the presence of DCC/DMAP to give **16**.[18] This then underwent deacetylation by $K_2CO_3$ in THF/MeOH (1:1) to produce compound **17**.[19] This was then coupled with (*R*)-lipoic acid (**18**) in the presence of DCC/DMAP to produce NOSH-**4** (Scheme 3).

We investigated the effects of NOSH-**1**−**4** and ASA on the growth properties of eleven different cancer cell lines of six different histological subtypes. The cell lines were that of colon (HT-29, COX-1 and COX-2 positive; HCT 15, COX null; and SW480, COX-1 positive, low levels of endogenous COX-2), breast (MCF7, [ER(+)]; MDA MB-231 and SKBR3, [ER(−)]), T-cell leukemia (Jurkat), pancreas (BxPC3, both COX-1 and COX-2 positive; MIAPaCa-2, COX-null), prostate (LNCaP), and lung (A549). All four NOSH compounds were extremely effective in inhibiting the growth of these cell lines (Table 1). NOSH-**1** was very potent, and its $IC_{50}$ for cell growth inhibition ranged from 48 to 280 nM. The corresponding $IC_{50}$ values for NOSH-**2**, -**3**, and -**4** were 70−120, 4300−7500, and 240−800 nM, respectively. The growth inhibition by NOSH-**1**−**4** versus traditional ASA was very high in the cell lines studied. In a fold comparison study of the $IC_{50}$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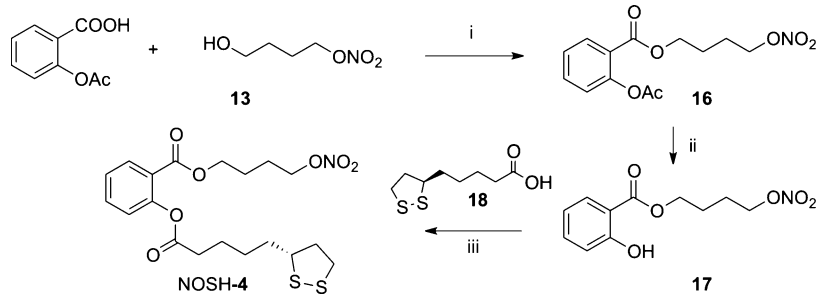
**Scheme 3. Synthesis of NOSH-4**[a]




[a]Conditions: (i) 4-hydroxybutyl nitrate (**13**), DCC/DMAP, DCM, rt, 6 h, (ii) $K_2CO_3$, THF/MeOH (1:1), 15 min, rt, (iii) (*R*)-lipoic acid (**18**), DCC/DMAP, DCM, rt, 6 h.

values (ASA/NOSH-**1**−**4**), NOSH-**1** was at least 100,000-fold more potent than ASA in HT-29 colon cancer cells. The increases in potency for NOSH-**2**, -**3**, and -**4** in the same cell line were >60,000-fold, >600-fold, and >16,000-fold, respectively. In general, NOSH-**1** was the most potent in all cell lines. Cyclooxygenase (COX) represents the best-known mechanistic target of NSAIDs. An interesting aspect of growth inhibition also emerges with respect to COX expression in the cell lines examined. NOSH-**1**−**4** showed similar effects on two colon cancer cell lines, HT-29 (expresses COX-1 and COX-2) and HCT 15 (no COX expression),[20] and on two pancreatic cancer cell lines, BxPC-3 (expresses COXs) and MIA PaCa-2 (no COX expression),[21] suggesting a COX-independent effect.

This high degree of potency raised the question as to how toxic this compound was to the cells. To assess this, we used lactate dehydrogenase (LDH) release as a measure of cellular toxicity. Cells were treated with several concentrations of NOSH-**1** for 2−24 h and compared to untreated controls. Although the cytotoxicity caused by NOSH-**1** was both dose- and time-dependent, this was minimal (Figure 2). At 4-times its $IC_{50}$, LDH release was less than 10% at 24 h. LDH release for shorter durations of treatment (2 h, 4 h, 6 h, and 8 h) ranged between 0.5 and 4% at its $IC_{50}$ and between 1 and 5% at 4-times its $IC_{50}$. This demonstrates a remarkable degree of safety for a compound that is so potent.

The most common use for NSAIDs (including aspirin) is the treatment of inflammatory conditions. Therefore, we wanted to compare the COX-dependent anti-inflammatory activity of ASA to that of NOSH-**1**. This was done by using the rat paw edema model, as described in the Supporting Information. After inducing inflammation in rat's paw with carrageenan, animals receiving vehicle showed a fast time-dependent increase in paw volume ($\Delta V$ = 1.1 mL) after 2−3 h, which decreased gradually every hour thereafter until the end of the experiment (6 h) (Figure 3A). In contrast, animals receiving ASA showed a weak inflammatory response ($\Delta V$ = 0.4 mL) at 1 h, decreasing to about $\Delta V$ = 0.35 mL over the next 2 h and then decreasing to about $\Delta V$ = 0.35 mL after 6 h. The anti-inflammatory effect registered in animals treated with NOSH-**1** was dose-dependent. Rats treated with low dose NOSH-**1** (0.21 mmol/kg) showed a change in paw volume $\Delta V$ = 0.5 mL after 1 h which increased to $\Delta V$ = 0.6 mL by 3 h and then came down to about $\Delta V$ = 0.4 mL over the next 3 h. Rats treated with high dose NOSH-**1** (0.52 mmol/kg), a dose which was slightly less than that of ASA (0.56 mmol/kg), showed a plateaued change in paw volume of $\Delta V$ = 0.45 mL after 1−2 h, which then deceased steadily over the next 4 h to $\Delta V$ = 0.35 mL, a change that was comparable to that of ASA (Figure 3A).

Prostaglandins ($PGE_2$) are the main product of cyclooxygenase-mediated arachidonic acid metabolism.[1] Comparison of $PGE_2$ content of paw exudates from control, ASA-treated, and NOSH-**1**-treated animals showed a clear and significant COX inhibition by aspirin and NOSH-**1**. Figure 3B shows that aspirin (0.21 mmol/kg) caused a considerable decrease in $PGE_2$ levels (12 ± 3 pg/mg protein) compared with the control group (82 ± 2 pg/mg). Treatment with NOSH-**1** reduced $PGE_2$ levels to 42 ± 3 and 21 ± 4 pg/mg at 0.21 and 0.52 mmmol/kg, respectively. We further evaluated the effect of NOSH-**1** on COX expression in paw exudates. Figure 3C shows that COX-1 was constitutively expressed in the controls; this was induced by carrageenan and inhibited to the same extent by NOSH-**1** regardless of the dose. On the other hand, COX-2, which produces inflammatory $PGE_2$, was barely detectable in the controls, was significantly induced by carrageenan, and was dose-dependently inhibited by NOSH-**1**.

We also determined the inhibitory effect of ASA and NOSH-**1** on proinflammatory cytokine tumor necrosis factor-$\alpha$ (TNF-$\alpha$) in plasma obtained from control and NOSH-**1**-treated animals. Administration of ASA (0.56 mmol/kg) increased the TNF-$\alpha$ concentration by about 20-fold (10 ± 1 control and 200 ± 10 pg/mL ASA); however, this rise was considerably lower in the NOSH-**1** (55 ± 2 pg/mL at 0.21 mmol/kg and 40 ± 3 pg/mL at 0.52 mmol/kg) treated animals (Figure 4).

The NOSH compounds were designed to release both NO and $H_2S$. In order to show that indeed this was the case *in vivo*, blood was collected from vehicle-, ASA-, and NOSH-**1**-treated animals at the end of the carrageenan-induced edema studies. Figure 5 shows that indeed both NO and $H_2S$ were dose-dependently significantly higher in NOSH-**1**-treated animals.

In the present study, we described the synthesis of four compounds designed to release both NO and $H_2S$. These NOSH compounds used aspirin as a scaffold and were shown to inhibit the growth of several cancer cell lines arising from a variety of tissue types such as colon, breast, pancreas, lung, prostrate, and T cell leukemia. The compounds described here are the first to show $IC_{50}$ values for cell growth inhibition that are in the nanomolar range and yet are devoid of any cellular toxicity. These NOSH compounds were more potent than ASA, with enhanced potency ranging from at least 650 to greater than 100,000-fold. Of the four NOSH compounds evaluated here, NOSH-**1** was consistently the most potent in all cell lines tested, and in some cases this enhancement was in excess of 150-fold over the others. Our data indicate that the effect of these NOSH compounds may be tissue-type independent since the NOSH-**1**−**4** were effective against adenomatous, epithelial, and lymphocytic cancer cell lines. Here we studied


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**Table 1. $IC_{50}$ nM for Cell Growth Inhibition at 24 h[a]**

| NOSH | colon | | | breast | | | pancreas | | lung | prostate | leukemia |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | HT-29 | HCT15 | SW480 | MDA MB231 | SKBR3 | MCF7 | MIA PaCa2 | BxPC3 | A540 | LNCAP | Jurkat |
| 1 | 48 ± 3 | 50 ± 5 | 60 ± 4 | 100 ± 11 | 75 ± 5 | 280 ± 16 | 47 ± 5 | 57 ± 4 | 50 ± 7 | 88 ± 8 | 100 ± 8 |
| 2 | 80 ± 5 | 90 ± 6 | 97 ± 7 | 85 ± 8 | 88 ± 7 | 70 ± 5 | 102 ± 18 | 100 ± 9 | 120 ± 14 | 100 ± 12 | 90 ± 5 |
| 3 | 7500 ± 355 | 5900 ± 305 | 5300 ± 240 | 6000 ± 220 | 6500 ± 268 | 5700 ± 323 | 4800 ± 322 | 5500 ± 390 | 6500 ± 224 | 4300 ± 212 | 7000 ± 321 |
| 4 | 300 ± 35 | 520 ± 21 | 600 ± 25 | 800 ± 22 | 550 ± 28 | 280 ± 15 | 800 ± 39 | 700 ± 32 | 300 ± 12 | 500 ± 18 | 240 ± 11 |
| ASA | >5,000,000 nM at 24 h in all cell lines | | | | | | | | | | |

[a]Colon, breast, pancreas, lung, prostate, and leukemia cancer cell lines were treated with various concentrations of NOSH-**1**, NOSH-**2**, NOSH-**3**, NOSH-**4**, and aspirin (ASA). Cell viability was determined at 24 h, from which $IC_{50}$ values were calculated. Results are mean ± SEM of at least four different experiments performed in triplicate. $P < 0.001$ for all NOSH compounds compared to ASA in all cell lines.

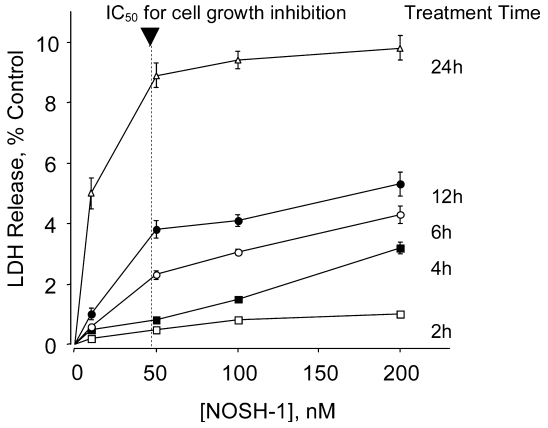


**Figure 2.** Toxicity profile of NOSH-**1** as measured by LDH release in HT-29 colon cancer cells.

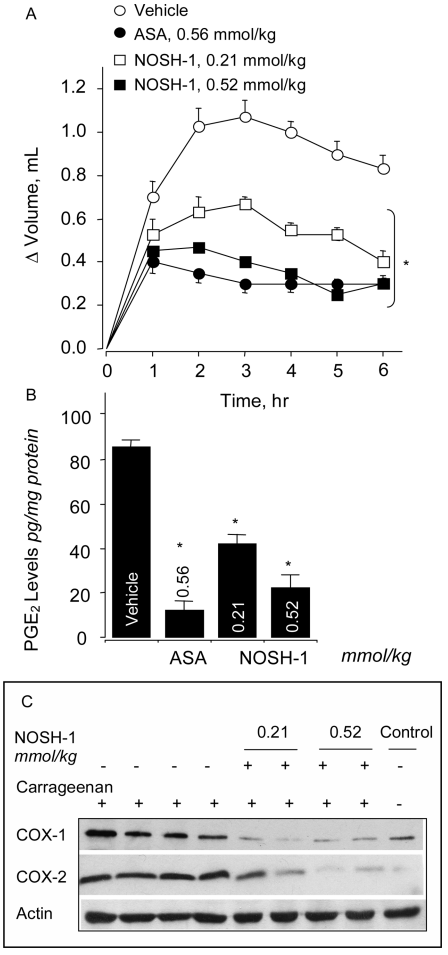


**Figure 3.** Anti-inflammatory properties of NOSH-**1**. Rat paw edema was induced by carrageenan injection. (A) ASA and NOSH-**1** caused a significant reduction in paw volume at all time points. Results are mean ± SEM of four rats in each group; *$P < 0.05$ versus vehicle treated rats at all time points. (B) ASA and NOSH-**1** caused a significant reduction in $PGE_2$ levels in the paw exudate. Results are mean ± SEM for four rats in each group; *$P <$ 0.01versus vehicle. (C) NOSH-**1** inhibits induction of COX-1 and COX-2 by carrageenan. Results show one animal is the control, four are in carrageenan injected, and two are in NOSH-**1** treated at two different doses.


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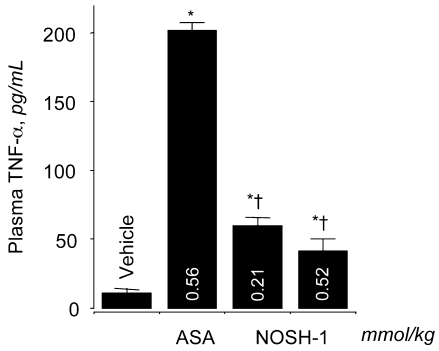


**Figure 4.** Effect of ASA and NOSH-**1** on plasma TNF-$\alpha$. ASA caused a significant rise in plasma TNF-$\alpha$; however, this rise was significantly less in the NOSH-**1** treated rats. Results are mean ± SEM for four rats in each group; *$P$ < 0.01 vs vehicle, †$P$ < 0.01 vs ASA.

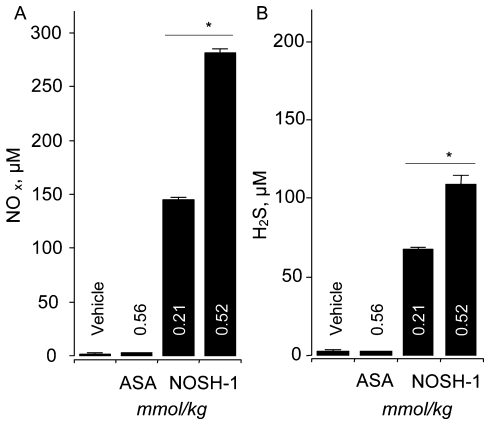


**Figure 5.** NO and $H_2S$ levels *in vivo* after NOSH-**1** administration. The plasma concentration of $NO_x$ and $H_2S$ was quantified as detailed in the Supporting Information. Results are mean ± SEM of four rats in each group. *$P$ < 0.001 versus vehicle and ASA-treated animals.

eleven cell lines originating from six different tissues; therefore, it may be envisaged that our findings are part of a generalized effect, especially since all cell types responded, although in a differential manner. NOSH-**1** also showed strong anti-inflammatory properties that were comparable to that of ASA, as demonstrated by measuring the *in vivo* carrageenan-induced rat paw edema, and direct measurement of cyclooxygenase-dependent production of $PGE_2$.

We are currently studying the molecular targets of these interesting compounds with respect to cell growth inhibition and are evaluating them in various animal models of cancer. Some on the non-Cox targets being investigated include NF-$\kappa$B, reactive oxygen species, the intrinsic apoptosis pathway, and Wnt signaling.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Synthetic experimental details, analytical data of compounds, and biological assay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

NSAIDs, nonsteroidal anti-inflammatory drugs; NO, nitric oxide; $H_2S$, hydrogen sulfide; NOSH, nitric oxide- and hydrogen sulfide-releasing; COX, cyclooxygenase; $PGE_2$, prostaglandin $E_2$; LDH, lactate dehydrogenase

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