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Synthesis and anti-cancer potential of the positional isomers of NOSH-aspirin (NBS-1120) a dual nitric oxide and hydrogen sulfide releasing hybrid

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Abstract

We recently reported the synthesis of NOSH-aspirin, a novel hybrid compound capable of releasing both nitric oxide (NO) and hydrogen sulfide (H₂S). In NOSH-aspirin, the two moieties that release NO and H₂S are covalently linked at the 1, 2 positions of acetyl salicylic acid, i.e. *ortho*-NOSH-aspirin. Here we report on the synthesis of *meta*- and *para*-NOSH-aspirins. We also made a head-to-head evaluation of the effects of these three positional isomers of NOSH-aspirin on colon cancer cell kinetics and induction of reactive oxygen species, which in recent years has emerged as a key event in causing cancer cell regression. Electron donating/withdrawing groups incorporated about the benzoate moiety significantly affected the potency of these compounds with respect to colon cancer cell growth inhibition.

Graphical abstract

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Authorship Contributions

Participated in research design: Kashfi, Chattopadhyay, Kodela,

Conducted experiments: Vannini, Kodela, Chattopadhyay, MacKessack-Leitch, Eschbach

Performed data analysis: Vannini, Kodela, Chattopadhyay, Kashfi

Wrote or contributed to the writing of the manuscript: Vannini, Kodela, Kashfi

Keywords

Nitric oxide; Hydrogen sulfide; NOSH-aspirin; Colon cancer; Cell kinetics; Reactive oxygen species

There is a functional relationship between inflammation and cancer with a considerable body of evidence supporting the notion that tumors can originate at the sites of infection or chronic inflammation.¹ For example, chronic inflammation increase the risk of developing bladder, cervical, gastric, intestinal, esophageal, ovarian, prostate, and thyroid cancers.² These observations provide the underlying rationale for the use of nonsteroidal anti-inflammatory drugs (NSAIDs) as anti-cancer agents. To date, over thirty epidemiological studies, collectively describing results on greater than 1 million subjects, have established NSAIDs as the prototypical chemopreventive agents against many forms of cancer.^{3–7} More recently, it has been reported that daily aspirin use, whether regular strength or low dose, resulted in reductions in cancer incidence and mortality.⁸ It is also important to note that aspirin use has also been shown to prevent distant metastasis.⁹ However, regular NSAID use may lead to serious, life threatening side effects including but not limited to gastrointestinal (GI), renal and cardiovascular (reviewed in¹⁰). In our efforts to improve the safety profile of NSAIDs, we developed NOSH-NSAIDs.^{11, 12} These are a new class of anti-inflammatory agents in which the traditional NSAIDs are coupled to moieties that can release nitric oxide (NO) and hydrogen sulfide (H₂S), two gasotransmitters of physiological relevance.¹³ Our rationale was based on the observations that NO¹⁴ and H₂S¹⁵ have some of the same general properties as prostaglandins (PGs) within gastric mucosa and therefore should enhance the local mucosal defense mechanisms; thereby compensating for the reduced gastric PGs caused by the NSAIDs. Our initial observations have shown that, NOSH-aspirin, NOSH-naproxen, and NOSH-sulindac are devoid of any appreciable GI side effects¹⁶, and have strong anti-inflammatory^{11, 12}, and anti-cancer^{12, 17} properties. Furthermore, NOSH-aspirin was more potent than aspirin to attenuate microglial and astrocytes activation in an *in vitro* model of neuroinflammation¹⁸, and was also shown to have potent analgesic properties.¹⁹ In order to potentially improve on our initial observations with NOSH-aspirin, we explored structure activity relationships of its differentially substituted analogs. Hence, here we report the synthesis and biological activity of the positional isomers of NOSH-ASA, (*o*-, *m*-, and *p*-NOSH-ASA). Electron-donating/withdrawing groups are also incorporated around the benzoate moiety to evaluate their effect on the potency of these compounds (Fig 1).

The positional isomers of NOSH-ASA were synthesized as described in scheme 1. *o*-NOSH-ASA (**1**) was synthesized as previously described.¹¹ For compounds **2–9**, to synthesize the bromo compounds, to a solution of salicylaldehyde (200.0 mg, 0.78 mmol) in dichloromethane, DCC (170.0.0 mg, 0.78 mmol) and DMAP (9.6 mg, 0.08 mmol) were

added at 0°C under an argon atmosphere. Then 4-bromobutyric acid (178.0 mg, 0.78 mmol) was added and the reaction mixture was stirred overnight at room temperature. After completion of the reaction as checked by TLC, water was filtered off, and the residue was extracted using dichloromethane (2 × 75 mL). The organic solvent was then removed under reduced pressure to obtain the crude product, which was then further purified by column chromatography to afford the bromo compounds (**2a–9a**).²⁰

For the synthesis of nitro compounds, we added AgNO₃ (1.32 g, 7.8 mmol) to a solution of the bromo compounds (1.0 g, 3.9 mmol) in CH₃CN (80 mL) and stirred the mixture for 6 h at 70°C. The reaction mixture was then filtered through Celite and concentrated under reduced pressure. The obtained crude residues were then treated with dichloromethane (50 mL) and water (50 mL). After separation, the aqueous layer was extracted two more times with dichloromethane (50 mL). The combined organic layers were dried, filtered, and concentrated under reduced pressure. The crude product thus obtained was purified by silica gel column chromatography to get the nitro compounds (**2b–9b**).²¹

For the synthesis of the aromatic carboxylic acid groups from the corresponding aldehydes, we added a solution of KH₂PO₄ (0.4 g, 2.94 mmol) in water (5 mL) and 30% H₂O₂ (0.25 mL, 2.19 mmol) to a solution of the aromatic aldehyde compound (0.5 g, 2.09 mmol) in acetonitrile (30 mL) followed by drop wise addition of a solution of 80% NaClO₂ (0.3 g, 2.65 mmol) in water (6.0 mL) at 0°C. The mixture was stirred at same temperature for 2 h. The reaction was followed to completion as monitored by TLC after which Na₂SO₃ was added to destroy the excess of H₂O₂. After acidification with 6M HCl, the mixture was diluted with H₂O (100 mL) and extracted twice with DCM (50 mL). The organic layer was dried filtered and concentrated under reduced pressure. The obtained crude product was purified by silica gel column chromatography to yield corresponding acid products (**2c–9c**).²²

For the synthesis of the final NOSH-ASA analogues, to the solution of acid compounds (200.0 mg, 0.78 mmol) in dichloromethane we added DCC (170.0 mg, 0.78 mmol) and DMAP (9.6 mg, 0.08 mmol) at 0°C under an argon atmosphere. To this we then added ADT-OH ((5-(4-hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (178.0 mg, 0.78 mmol) and the whole reaction mixture was stirred overnight at room temperature. After completion of the reaction as checked by TLC, the mixture was filtered off and water was added followed by extraction into dichloromethane (2 × 75 mL). Organic solvent was then removed under reduced pressure to get the crude product which was then further purified by column chromatography to afford the pure final compounds (**2–9**).²³

We evaluated the inhibitory effects of aspirin and compounds **1–9** on cell growth by an MTT assay using HT-29 and HCT 15 human colon cancer cell lines.²⁴ From this data we constructed dose-response curves and hence calculated the IC₅₀s for cell growth inhibition (Table 1). Compounds **1–9** were all significantly more potent than aspirin in inhibiting the growth of both cells lines. In HT-29 cells, for compounds **1**, **2**, and **3** that is for *o*-NOSH-aspirin, *m*-NOSH-aspirin, and *p*-NOSH-aspirin, the IC₅₀s for cell growth inhibition at 24 h were 48 ± 7 nM, 220 ± 80 nM, and 450 ± 125 nM, respectively. Therefore, *o*-NOSH-aspirin (**1**) is the most potent amongst the positional isomers, followed by *m*-NOSH-aspirin (**2**) and

then *p*-NOSH-aspirin (**3**). In HCT 15 cells, for compounds **1**, **2**, and **3**, IC₅₀s at 24 h were 57 ± 5 nM, 110 ± 15 nM, and 380 ± 30 nM, respectively. It appears that the IC₅₀s for each compound is in the same order of magnitude in both cell lines, strongly suggesting that the effect on cell growth inhibition is independent of the cyclooxygenase (COX) status of the cell line as HT-29 cells express both COX-1 and COX-2 whereas HCT 15 cells are COX null.²⁵ For *o*-NOSH-aspirin (**1**), incorporation of OMe or Cl about the benzoate moiety, that is compounds **4** and **5** respectively, increased the IC₅₀s for cell growth inhibition in both cell lines (Table 1). In HT-29 cells, the IC₅₀ for **4** and **5** increased to 200 ± 50 nM and 400 ± 150 nM respectively; and in HCT 15 cells, the IC₅₀ for **4** and **5** increased to 380 ± 150 nM and 170 ± 30 nM, respectively. These data suggest that analogs **4** and **5** are not as potent as **1**, although they still have strong anti-cancer activity. For the *meta* analogs, **2**, **6**, and **7**, incorporation of the OMe group did increase the IC₅₀s in both cell lines from 220 ± 80 nM in HT-29 cells (**2**) and 110 ± 15 nM in HCT 15 cells (**2**) to 430 ± 30 nM (**6**) and 330 ± 30 nM (**6**), respectively. Incorporation of Cl, (**7**) did not affect the IC₅₀ in either of the cell lines, the values being 210 ± 110 nM in HT-29 cells and 140 ± 30 nM in HCT 15 cells (Table 1). For the *para* analogs, **3**, **8**, and **9** in the HT-29 cells, incorporation of OMe or Cl decreased the IC₅₀s for cell growth inhibition. The IC₅₀ for **3** (450 ± 125 nM) went down to 130 ± 30 nM in **8**, and to 60 ± 10 nM in **9**, the latter mirroring the IC₅₀ of **1** (Table 1). Therefore, in HT-29 cells incorporation of Cl appears to enhance the potency of **3**. In HCT 15 cells, there was a downward trend by introducing the OMe group, the IC₅₀ of 380 ± 30 nM for **3** went down to 220 ± 5 nM for **8**. However, incorporation of Cl in **9** had no effect on potency, its IC₅₀ being 330 ± 30 nM. It therefore appears that HT-29 and HCT 15 cells respond differently to the effects of *p*-NOSH-ASA analogs towards cell growth inhibition. Currently, we do not have an explanation for this apparent differential response, which suggests an effect that may be dependent on COX expression, we are further investigating this.

The effects of **1**, **2**, and **3** were determined on colon cancer cell kinetics.²⁶ Two determinants of cellular mass are proliferation and apoptosis. For proliferation, HT-29 cells were treated with different concentrations of **1**, **2**, or **3** for 24h, followed by determination of PCNA antigen expression. *o*-NOSH-aspirin (**1**) reduced PCNA expression in these cells in a dose dependent manner (Fig 2A, upper panel). At 10, 25, 50 and 100 nM, the respective PCNA positive cells were 80 ± 2%, 65 ± 3%, 48 ± 2%, and 20 ± 3% of the vehicle-treated cells. *m*-NOSH-aspirin (**2**) and *p*-NOSH-aspirin (**3**) showed similar results to (**1**) but at much higher concentrations, Fig 2A, middle panel and Fig 2A, lower panel, respectively. Using the Annexin V-FITC staining and flow cytometry, we determined the population of cells undergoing apoptosis. Treatment with (**1**) at 10, 25, 50 and 100 nM resulted in 15 ± 1%, 35 ± 2%, 65 ± 4%, and 78 ± 3% cells in early apoptotic phase, respectively, compared to untreated control (Fig 2C, upper panel). Qualitatively, **2** (50–500 nM) and **3** (100–1000 nM) showed similar results to **1** but at much higher concentrations as indicated, Fig 2C, middle and lower panels, respectively. Compounds **1**, **2**, and **3** also affected the cell cycle progression as measured by DNA content of HT-29-treated cells using flow cytometry. Cells treated with each compound at their respective 1×IC₅₀ and 2×IC₅₀ for cell growth inhibition; that is for **1** at 50 and 100 nM, for **2** at 250 and 500 nM and for **3** at 500 and 1000 nM accumulated progressively in G₀/G₁ phase (Fig 2B, upper, middle, and lower panels respectively). For **1** an arrest at G₀/G₁ phase was evident at 1× and 2× IC₅₀ compared to

control: G₀/G₁ increased from 39.6% to 53.6% and 71.9%, respectively, while the population in S phase was reduced from 31.9% to 26.1% and 18.2%, and G₂/M was reduced from 28.5% to 20.3% and 9.9%, respectively (Fig 2B, upper panel). Compounds **2** and **3** at their respective 1× and 2× IC₅₀ showed similar results to **1** (Fig 2B, middle, and lower panels respectively). Therefore, **1**, **2**, or **3** inhibit proliferation of HT-29 cells by a combined induction of G₀/G₁ arrest and apoptosis.

Compounds **1**, **2**, and **3** induced reactive oxygen species (ROS) levels.²⁷ We used the fluorescent probes 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) a probe for H₂O₂ and other peroxides that also detects greater than ten individual reactive species or dihydroethidium (DHE) which is an intracellular probe that preferentially measures superoxide anion (O₂^{•-}).^{28, 29} HT-29 cells were treated with **1**, **2**, and **3** at their respective IC₅₀s for cell growth inhibition for 1h, stained with DCFDA, and analyzed for levels of intracellular peroxides. Compared to control, treatment with **1**, **2**, and **3** increased the population of cells showing DCFDA-dependent fluorescence, indicating an induction of intracellular peroxides (Fig 3A). For **1**, **2**, and **3**, ROS increased to 16, 15, and 11-fold respectively, compared to basal levels. We also monitored intracellular levels of O₂^{•-} (Fig 3B) which increased to 18, 21, and 10-fold over the basal levels for **1**, **2**, and **3**, respectively.

In summary, the positional isomers of NOSH-aspirin and their analogs (**1–9**) inhibited the growth of two human colon cancer cell lines with moderately low IC₅₀s. The growth inhibitory effects of **1**, **2**, and **3**, was associated with G₁ to S cell cycle arrest, inhibition of proliferation and induction of apoptosis. At 1× IC₅₀, **1**, **2**, and **3**, significantly reduced the proportion of cells in S phase and blocked DNA synthesis inhibiting cell renewal, whereas at 2× IC₅₀, **1**, **2**, and **3**, induced cell death. Thus, the proapoptotic effect of **1**, **2**, and **3**, suggests strong antineoplastic potential. Oxidative stress is produced in cells when there is disturbance in the equilibrium between ROS formation and endogenous antioxidant defense mechanisms. In general, cancer cells have high constitutive oxidative stress levels³⁰ and may be pushed over a threshold to cell death by exogenous ROS. In our study, **1**, **2**, and **3** induced cell cycle changes and apoptosis in HT-29 cells, which was associated with parallel increases in ROS and superoxide anion levels, thus favoring cell death.

In conclusion, **1**, **2**, and **3**, suppressed growth of HT-29 cells by induction of apoptosis and alteration of cell cycle phases. This effect correlated with induction of ROS. Therefore, **1**, **2**, and **3** demonstrate anti-cancer potential against colon cancer and warrant further study.

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20. (2a): 3-formylphenyl 4-bromobutanoate, (Yield-93%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.14 (m, 2H), 2.81 (t, *J* = 7.32 Hz, 2H), 3.54 (t, *J* = 6.35 Hz, 2H), 7.36 (dd, *J* = 8.3, 2.44 Hz, 1H), 7.56 (t, *J* = 7.81 Hz, 1H), 7.61 (bs, 1H), 7.76 (d, *J* = 7.81 Hz, 1H), 9.99 (s, 1H). (3a): 4-formylphenyl 4-bromobutanoate, (Yield-82%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.15 (m, 2H), 2.80 (t, *J* = 7.31 Hz, 2H), 3.53 (t, *J* = 6.35 Hz, 2H), 7.24 (d, *J* = 8.78 Hz, 2H), 8.12 (d, *J* = 8.82 Hz, 2H). (4a): 2-formyl-4-methoxyphenyl 4-bromobutanoate, (Yield-86%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.18 (m, 2H), 2.58 (t, *J* = 6.84 Hz, 2H), 3.48 (t, *J* = 6.35 Hz, 2H), 3.82 (s, 1H), 6.94 (d, *J* = 8.79 Hz, 1H), 7.01 (d, *J* = 2.93, 1H), 7.15 (dd, *J* = 9.28, 2.93 Hz, 1H), 9.86 (s, 1H). (5a): 4-chloro-2-formylphenyl 4-bromobutanoate, (Yield-78%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.23 (m, 2H), 2.82 (t, *J* = 6.83 Hz, 2H), 3.64 (t, *J* = 6.34 Hz, 2H), 7.12 (d, *J* = 8.2 Hz, 1H), 7.63 (dd, *J* = 7.8, 1.95 Hz, 1H), 8.10 (d, *J* = 1.84 Hz, 1H). (6a): 5-formyl-2-methoxyphenyl 4-bromobutanoate, (Yield-89%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.14 (m, 2H), 2.78 (t, *J* = 7.32 Hz, 2H), 3.54 (t, *J* = 6.38 Hz, 2H), 3.93 (s, 3H), 6.94 (d, *J* = 8.2 Hz, 1H), 7.02 (d, *J* = 2.93, 1H), 7.14 (dd, *J* = 8.3, 2.72 Hz, 1H), 9.89 (s, 1H). (7a): 2-chloro-3-formylphenyl 4-bromobutanoate, (Yield-81%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.34 (m, 2H), 2.90 (t, *J* = 6.83 Hz, 2H), 3.57 (t, *J* = 6.35 Hz, 2H), 7.40 (dd, *J* = 8.06, 1.95 Hz, 1H), 7.43 (t, *J* = 7.81 Hz, 1H), 7.85 (dd, *J* = 7.33, 1.96 Hz, 1H), 10.47 (s, 1H). (8a): 4-formyl-2-methoxyphenyl 4-bromobutanoate, (Yield-90%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.17 (m, 2H), 2.56 (t, *J* = 7.32 Hz, 2H), 3.47 (t, *J* = 6.35 Hz, 2H), 3.95 (s, 1H), 7.04 (d, *J* = 8.3 Hz, 1H), 7.41 (bs, 1H), 7.42 (dd, *J* = 7.81, 1.47 Hz, 1H), 7.81 (s, 1H). (9a): 2-chloro-4-formylphenyl 4-bromobutanoate, (Yield-78%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.22 (m, 2H), 2.83 (t, *J* = 6.82 Hz, 2H), 3.64 (t, *J* = 6.32 Hz, 2H), 7.58 (d, *J* = 8.2 Hz, 1H), 7.96 (d, *J* = 8.6 Hz, 1H), 8.12 (d, *J* = 1.42 Hz, 1H), 9.98 (s, 1H).
21. (2b): 3-formylphenyl 4-(nitrooxy) butanoate, (Yield- 72%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.09 (m, 2H), 2.73 (t, *J* = 7.32 Hz, 2H), 4.62 (t, *J* = 6.35 Hz, 2H), 7.42 (d, *J* = 6.84, 1H), 7.60 (t, *J* = 7.81 Hz, 1H), 7.64 (bs, 1H), 7.79 (d, *J* = 7.81 Hz, 1H), 9.98 (s, 1H). (3b): 4-formylphenyl 4-(nitrooxy) butanoate, (Yield-76%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.10 (m, 2H), 2.73 (t, *J* = 7.32 Hz, 2H), 4.62 (t, *J* = 6.34 Hz, 2H), 7.26 (d, *J* = 8.82 Hz, 2H), 8.2 (d, *J* = 8.78 Hz, 2H). (4b): 2-formyl-4-methoxyphenyl 4-(nitrooxy)butanoate, (Yield-78%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.20 (m, 2H), 2.80 (t, *J* = 7.32 Hz, 2H), 3.86 (s, 1H), 4.61 (t, *J* = 6.35 Hz, 2H), 7.09 (d, *J* = 8.79 Hz, 1H), 7.17 (dd, *J* = 8.79, 2.44 Hz, 1H), 7.34 (d, *J* = 2.93, 1H) 10.01 (s, 1H). (5b): 4-chloro-2-formylphenyl 4-(nitrooxy) butanoate, (Yield-68%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.22 (m, 2H), 2.82 (t, *J* = 6.82 Hz, 2H), 4.62 (t, *J* = 6.35 Hz, 2H), 7.12 (d, *J* = 8.2 Hz, 1H), 7.63 (dd, *J* = 7.8, 1.86 Hz, 1H), 8.10 (d, *J* = 1.95 Hz, 1H). (6b): 5-formyl-2-methoxyphenyl 4-(nitrooxy) butanoate, (Yield-76%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.20 (m, 2H), 2.77 (t, *J* = 7.33 Hz, 2H), 3.92 (s, 3H), 4.61 (t, *J* = 6.38 Hz, 2H), 7.10 (d, *J* = 8.30 Hz, 1H), 7.59 (bs, 1H), 7.79 (dd, *J* = 7.32, 0.98 Hz, 1H), 9.87 (s, 1H). (7b): 2-chloro-3-formylphenyl 4-(nitrooxy)butanoate, (Yield-70%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.23 (m, 2H), 2.83 (t, *J* = 6.82 Hz, 2H), 4.63 (t, *J* = 6.25 Hz, 2H), 7.35 (d, *J* = 7.81 Hz, 1H), 7.42 (t, *J* = 7.8 Hz, 1H), 7.42 (s, 1H), 7.97 (d, *J* = 7.82 Hz, 1H). (8b): 4-formyl-2-methoxyphenyl 4-(nitrooxy)butanoate, (Yield-78%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.10 (m, 2H), 2.67 (t, *J* = 6.86 Hz, 2H), 3.79 (s, 1H), 4.50 (t, *J* = 6.35 Hz, 2H), 7.13 (d, *J* = 7.81 Hz, 1H), 7.37 (d, *J* = 8.20, 1H), 7.39 (bs, 1H), 7.84 (s, 1H). (9b): 2-chloro-4-formylphenyl 4-(nitrooxy)butanoate, (Yield-78%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.24 (m, 2H), 2.83 (t, *J* = 6.84 Hz, 2H), 4.62 (t, *J* = 6.34 Hz, 2H), 7.36 (d, *J* = 8.3 Hz, 1H), 7.84 (dd, *J* = 8.3, 1.95 Hz, 1H), 7.99 (d, *J* = 1.95 Hz, 1H), 9.96 (s, 1H).
22. (2c): 3-((4-(nitrooxy) butanoyl) oxy) benzoic acid, (Yield-78%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.22 (m, 2H), 2.78 (t, *J* = 7.33 Hz, 2H), 4.62 (t, *J* = 5.86 Hz, 2H), 7.39 (dd, *J* = 8.3, 1.46 Hz, 1H), 7.85 (bs, 1H), 8.03 (d, *J* = 7.81 Hz, 1H). (3c): 4-((4-(nitrooxy) butanoyl) oxy) benzoic acid, (Yield-73%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.20 (m, 2H), 2.76 (t, *J* = 6.84 Hz, 2H), 4.60 (t, *J* = 5.86 Hz, 2H), 7.23 (d, *J* = 8.79 Hz, 2H), 8.16 (d, *J* = 8.78 Hz, 2H). (4c): 5-methoxy-2-((4-(nitrooxy)butanoyl)oxy)benzoic acid, (Yield-80%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.20 (m, 2H), 2.78 (t, *J* = 7.32 Hz, 2H), 3.88 (s, 1H), 4.62 (t, *J* = 6.35 Hz, 2H), 7.07 (d, *J* = 8.79 Hz, 1H), 7.18 (dd, *J* = 8.78, 2.93 Hz, 1H), 7.62 (d, *J* = 2.93, 1H). (5c): 5-chloro-2-((4-(nitrooxy)butanoyl)oxy)benzoic acid, (Yield-67%). ¹H-NMR (CDCl₃, 500 MHz): δ 2.19 (m, 2H), 2.78 (t, *J* = 6.83 Hz, 2H), 4.60 (t, *J* = 6.35 Hz, 2H), 7.10 (d, *J* = 8.3 Hz, 1H), 7.59 (dd, *J* = 7.8, 1.95

- Hz, 1H), 8.08 (d, $J = 1.95$ Hz, 1H). (6c): 4-methoxy-3-((4-(nitrooxy) butanoyl) oxy) benzoic acid, (Yield-75%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.20 (m, 2H), 2.77 (t, $J = 6.84$ Hz, 2H), 3.91 (s, 3H), 4.62 (t, $J = 6.38$ Hz, 2H), 7.03 (d, $J = 8.79$ Hz, 1H), 7.79 (bs, 1H), 8.03 (d, $J = 8.31$ Hz, 1H). (7c): 2-chloro-3-((4-(nitrooxy)butanoyl)oxy)benzoic acid, (Yield-72%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.24 (m, 2H), 2.82 (t, $J = 6.83$ Hz, 2H), 4.62 (t, $J = 5.86$ Hz, 2H), 7.36 (d, $J = 7.81$ Hz, 1H), 7.40 (t, $J = 7.8$ Hz, 1H), 7.44 (s, 1H), 7.93 (d, $J = 7.82$ Hz, 1H). (8c): 3-methoxy-4-((4-(nitrooxy)butanoyl)oxy)benzoic acid, (Yield-78%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.23 (m, 2H), 2.79 (t, $J = 6.84$ Hz, 2H), 3.92 (s, 1H), 4.64 (t, $J = 6.34$ Hz, 2H), 7.17(d, $J = 8.3$ Hz, 1H), 7.72 (bs, 1H), 7.79 (dd, $J = 8.3$, 1.46 Hz, 1H). (9c): 3-chloro-4-((4-(nitrooxy) butanoyl) oxy) benzoic acid, (Yield-68%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.23 (m, 2H), 2.83 (t, $J = 6.83$ Hz, 2H), 4.62 (t, $J = 6.35$ Hz, 2H), 7.27 (d, $J = 8.3$ Hz, 1H), 8.06 (dd, $J = 8.3$, 1.46 Hz, 1H), 8.34 (d, $J = 1.47$ Hz, 1H).
23. (2): 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 3-((4-(nitrooxy)butanoyl)oxy)benzoate, (Yield-73%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.22 (m, 2H), 2.79 (t, $J = 7.33$ Hz, 2H), 4.62 (t, $J = 6.35$ Hz, 2H), 7.38 (d, $J = 8.79$ Hz, 2H), 7.41 (dd, $J = 8.3$, 1.46 Hz, 1H), 7.45 (s, 1H), 7.58 (t, $J = 7.8$ Hz, 1H), 7.76 (d, $J = 8.78$ Hz, 2H), 7.94 (s, 1H), 8.11 (d, $J = 7.82$ Hz, 1H). EIMS: 477 (M+). (3): 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 4-((4-(nitrooxy)butanoyl)oxy)benzoate, (Yield-65%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.21 (m, 2H), 2.78 (t, $J = 7.33$ Hz, 2H), 4.61 (t, $J = 5.86$ Hz, 2H), 7.28 (d, $J = 8.3$ Hz, 2H), 7.36 (d, $J = 8.3$ Hz, 2H), 7.44 (s, 1H), 7.74 (d, $J = 8.79$ Hz, 2H), 8.26 (d, $J = 8.79$ Hz, 2H). EIMS: 477 (M+). (4): 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 5-methoxy-2-((4-(nitrooxy)butanoyl)oxy)benzoate, (Yield-68%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.14 (m, 2H), 2.74 (t, $J = 7.33$ Hz, 2H), 3.89 (s, 1H), 4.53 (t, $J = 6.35$ Hz, 2H), 7.13 (d, $J = 9.28$ Hz, 1H), 7.22 (dd, $J = 8.79$, 2.93 Hz, 1H), 7.31 (d, $J = 8.3$, 2H), 7.44 (s, 1H), 7.71 (d, $J = 3.62$ Hz, 1H), 7.75 (d, $J = 8.3$ Hz, 2H). (5): 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 5-chloro-2-((4-(nitrooxy)butanoyl)oxy)benzoate, (Yield-62%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.16 (m, 2H), 2.94 (t, $J = 7.32$ Hz, 2H), 4.52 (t, $J = 6.35$ Hz, 2H), 7.18 (d, $J = 8.8$ Hz, 1H), 7.32 (d, $J = 8.8$ Hz, 2H), 7.42 (s, 1H), 7.70 (dd, $J = 8.6$, 2.45 Hz, 1H), 7.78 (d, $J = 8.6$, 2H), 8.22 (bs, 1H). (6): 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 4-methoxy-3-((4-(nitrooxy)butanoyl)oxy)benzoate, (Yield-78%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.21 (m, 2H), 2.79 (t, $J = 7.32$ Hz, 2H), 4.62 (t, $J = 6.38$ Hz, 2H), 7.06 (d, $J = 8.8$ Hz, 1H), 7.36 (d, $J = 8.6$, 2H), 7.43 (s, 1H), 7.74 (d, $J = 8.6$ Hz, 2H), 7.90 (bs, 1H), 7.94 (s, 1H), 8.12 (dd, $J = 8.2$, 1.43 Hz, 1H). (7): 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 2-chloro-3-((4-(nitrooxy)butanoyl)oxy)benzoate, (Yield-68%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.25 (m, 2H), 2.85(t, $J = 7.33$ Hz, 2H), 4.62 (t, $J = 5.86$ Hz, 2H), 7.24 (d, $J = 7.8$ Hz, 1H), 7.41 (d, $J = 8.8$ Hz, 2H), 7.44 (s, 1H), 7.68 (d, $J = 7.8$ Hz, 1H), 7.74 (d, $J = 8.8$ Hz, 2H), 7.99 (dd, $J = 8.8$, 2.92 Hz, 1H). (8): 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 3-methoxy-4-((4-(nitrooxy)butanoyl)oxy)benzoate, (Yield-72%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.22 (m, 2H), 2.81 (t, $J = 7.36$ Hz, 2H), 3.94 (s, 1H), 4.64 (t, $J = 6.42$ Hz, 2H), 7.05 (d, $J = 8.6$ Hz, 1H), 7.38 (d, $J = 8.8$, 2H), 7.46 (s, 1H), 7.67 (d, $J = 1.46$ Hz, 1H), 7.76 (d, $J = 8.8$ Hz, 2H), 7.84 (dd, $J = 8.2$, 1.43 Hz, 1H). (9): 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 3-chloro-4-((4-(nitrooxy)butanoyl)oxy)benzoate, (Yield-75%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 2.26 (m, 2H), 2.86 (t, $J = 7.30$ Hz, 2H), 3.89 (s, 1H) 4.66 (t, $J = 6.32$ Hz, 2H), 7.38 (d, $J = 8.8$ Hz, 2H), 7.44 (s, 1H), 7.62 (d, $J = 8.2$, 1H), 7.78 (d, $J = 8.8$, 2H), 8.16 (dd, $J = 8.8$, 2.42 Hz, 1H), 8.34 (d, $J = 2.42$ Hz, 1H).
24. Cell culture: HT-29 and HCT 15 human colon cancer cell lines were purchased from American Type Culture Collection (Manassas, V., and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 50 U/mL penicillin G and 50 $\mu\text{g/mL}$ streptomycin sulfate (Gibco, BRL) at 37°C in a humidified atmosphere containing 5% CO_2 . All compounds were dissolved in DMSO (100 mM) and diluted to the desired concentrations. The final concentration of DMSO was adjusted to 1% in all media.; Cell growth inhibition assay: A commercially available kit (colorimetric MTT assay kit Roche; Indianapolis).
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26. Effects of **1**, **2**, and **3** on colon cancer cell kinetics. Proliferation: HT-29 cells (1×10^6 cells/mL) were treated for 24 hrs with various concentrations of **1**, **2**, and **3**. PCNA was determined using an ELISA Kit (Calbiochem, La Jolla, CA), in accordance with the manufacturers protocol. For apoptosis, HT-29 cells (0.5×10^6 cells/mL) were treated for 24 hrs with various concentrations of

1, 2, and 3. Cells were washed with and resuspended in 1X Binding Buffer (Annexin V binding buffer, 0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25mM CaCl₂) from BD BioSciences (San Diego, CA). Then 5µL of Annexin V-FITC (final concentration: 0.5 µg/ml) was added followed by Propidium iodide (final concentration 20 µg/mL). The cells were incubated at room temperature for 5–15 min in the dark. Finally, the cells were transferred to FACS tubes and analysed by flow cytometry as described above using Flow Jo software. For cell cycle analysis: HT-29 cells (0.5×10^6) were treated with **1, 2, and 3** for 24 h at their respective IC₅₀ for cell growth inhibition. Cells were harvested by trypsinization and centrifugation. They were then fixed in 70% ethanol for 10 min at –20°C, pelleted (5000 rpm × 10 min at 4°C), resuspended and incubated in PBS containing 1% FBS/0.5% NP-40 on ice for 5 min. Cell were washed again in 500 µL of PBS/1% FBS containing 40 µg/mL propidium iodide (used to stain for DNA) and 200 µg/mL RNase type IIA, and analyzed within 30 min by flow cytometry. Cell cycle phase distributions of control and treated HT-29 cells were obtained using a Coulter Profile XL equipped with a single argon ion laser. For each subset, >10,000 events were analyzed. All parameters were collected in list mode files. Data were analyzed on a Coulter XL Elite Work station using the Software programs MultigraphTM and MulticycleTM. The percentage of cells in G₀/G₁, G₂/M, and S phases was determined from DNA content histograms.

27. Determination of reactive oxygen species (ROS): HT-29 cells (0.3×10^6 cells/well) were plated in 6-well plates for 24 hours after which they were treated with **1, 2, and 3** for 1 hour at their respective IC₅₀s for cell growth inhibition. Cells were then trypsinized, washed once in PBS resuspended and then incubated for 30 min at 37°C in the dark with the oxidation-sensitive fluorescent probes 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, 10 µM) or dihydroethidium (DHE, 5 µM) (Molecular Probes, Life Technologies, NY). Fluorescence intensity was then measured by flow cytometry using a FACS Calibur (BD Bioscience). DCFDA is a probe for H₂O₂ and other peroxides, while DHE is an intracellular probe that preferentially measures superoxide anion.
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Figure 1.
Structures of the positional isomers of NOSH-ASA.
o-NOSH-ASA analogs

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 2-((4-(nitrooxy)butanoyl)oxy)benzoate (1)

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 5-methoxy-2-((4-(nitrooxy)butanoyl)oxy)benzoate (4)

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 5-chloro-2-((4-(nitrooxy)butanoyl)oxy)benzoate (5)

m-NOSH-ASA analogs

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 3-((4-(nitrooxy)butanoyl)oxy)benzoate (2)

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 4-methoxy-3-((4-(nitrooxy)butanoyl)oxy)benzoate (6)

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 2-chloro-3-((4-(nitrooxy)butanoyl)oxy)benzoate (7)

p-NOSH-ASA analogs

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 4-((4-(nitrooxy)butanoyl)oxy)benzoate (3)

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 3-methoxy-4-((4-(nitrooxy)butanoyl)oxy)benzoate (8)

4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 3-chloro-4-((4-(nitrooxy)butanoyl)oxy)benzoate (9)

Figure 2.

Compounds **1**, **2**, and **3** inhibit proliferation by altering cell cycle progression and inducing apoptosis. HT-29 cells were treated with the indicated concentrations of compounds **1** (upper panel), **2** (middle panel), and **3** (lower panel) for 24 h and analyzed for A) proliferation by PCNA antigen expression; B) cell cycle phases by PI staining and flow cytometry; C) apoptosis by Annexin V staining and flow cytometry. In (A) and (C), results are mean $\pm$ SEM for 3 different experiments performed in duplicate, * $P < 0.05$ compared to control, comparison between treatment groups was performed by one-factor analysis of

variance (ANOVA) followed by Tukey's test for multiple comparisons. In (B), results are representative of two different experiments, which were within 10% of those presented here.

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Figure 3.

Compounds **1**, **2**, and **3** induce ROS levels. HT-29 cells were treated with **1** (50 nM), **2** (250 nM), and **3** (500 nM) representing their respective IC₅₀ for cell growth inhibition, for 1 h followed by staining with a general ROS probe DCFDA (A) or DHE which detects superoxide anions in cells (B). Values are the mean $\pm$ SEM of three independent experiments. *P < 0.05 compared to vehicle-treated controls, comparison between treatment groups was performed by one-factor analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons.

Scheme 1.

General synthetic scheme for NOSH-ASA positional isomers (**1-9**). a) DCC, DMAP, DCM, 4-bromobutyric acid, 0°C-rt, 12h, b) AgNO₃, CH₃CN, 70°C, 6h, c) KH₂PO₄, H₂O₂, CH₃CN, NaClO₂, d) ADT-OH, (5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione), DCC, DMAP, DCM, 0°C-rt, 12h.

Table 1

IC₅₀ values at 24 hr for cell growth inhibition.

Compound	Origin/Cell line, IC ₅₀ , nM	
	Colon HT-29	Colon HCT 15
Aspirin	>5,000,000	>5,000,000
<i>ortho analogs</i>		
1	48 ± 7 [*] , †	57 ± 5 [*] , †
4	200 ± 50 [*]	380 ± 150 [*]
5	400 ± 150 [*]	170 ± 30 [*]
<i>meta analogs</i>		
2	220 ± 80 [*]	110 ± 15 [*]
6	430 ± 30 [*]	330 ± 30 [*]
7	210 ± 110 [*]	140 ± 30 [*]
<i>para analogs</i>		
3	450 ± 125 [*]	380 ± 30 [*]
8	130 ± 30 [*]	220 ± 5 [*]
9	60 ± 10 [*] , §	330 ± 30 [*]

HT-29 and HCT 15 colon cancer cell lines were treated with various concentrations of aspirin and compounds **1–9**. Cell numbers were determined at 24 h from which IC₅₀ values were calculated. Results are mean ± S.E.M. of three to five different experiments done in triplicate.

^{*} P < 0.001 compared to aspirin,

[†] P < 0.05 compared to **2** and **3**,

[§] P < 0.05 compared to **3**. Comparison between treatment groups was performed by one-factor analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons.