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Preparation of a conjugation-ready thiol responsive molecular switch

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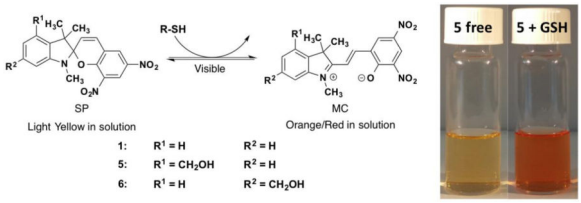
Abstract

In this work we synthesize molecular switches that are responsive to cysteine, homocysteine, and glutathione; three redox systems that make up the majority of the body's antioxidant defenses. Synthesized spiropyran isomers with conjugation-ready linkages showed good selectivity of response to these major antioxidant thiols over nucleophilic amino acids; however the position of the linking group can affect selectivity and reversibility of the switching response. An isomer with selectivity for cysteine against GSH and Hcy was identified.

Graphical abstract



Keywords

Spiropyran; Thiol; Colorimetric Sensor

1. Introduction and background of thiols

Oxidative stress has been shown to precede and contribute to the progression of diseases including cancer, cardiovascular disease, ulcerative colitis, and neurodegenerative disorders.[1, 2] Under normal physiological conditions, the production of reactive oxygen species (ROS) is important for a host of cellular processes including cell growth and

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differentiation. Sustained high levels of ROS, however, can lead to deleterious cellular events such as lipid peroxidation, protein and DNA modification, and eventually impaired cellular function or apoptosis. In a healthy individual the production of ROS is highly regulated by a number of antioxidant systems. Imbalances in these important antioxidant systems can leave the body vulnerable to oxidative stress which can lead to development of disease.[1]

Intracellular thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are important antioxidants that scavenge ROS. Antioxidants are important interceptors of ROS and alterations in antioxidant levels that accompany oxidative stress could serve as a biomarker for disease, or as an endpoint for monitoring therapeutic efficacy.[3] Several studies have illustrated that local administration of antioxidants can provide neuronal protection in patients with traumatic brain injury, prevent bone loss related to osteoporosis, and improve the immune response of aged mice.[4–6] Intracellular thiol concentrations are dominated by glutathione, with concentrations ranging from 1 – 10mM. Glutathione is also the major thiol antioxidant extracellularly with concentrations ranging from 0.5–1mM. At 0.1 mM cysteine concentrations are lower than glutathione but still ten to twenty fold greater than homocysteine concentrations. Glutathione, cysteine, and homocysteine are all important antioxidants for cellular defense and the depletion of these antioxidant thiol levels has been linked to a number of diseases.[7–14] For example, patients with Parkinson's disease had 30% lower GSH concentrations in the rostral and caudal regions of the brain than neurologically healthy control patients.[15] The ability to monitor thiol levels in tissues could provide a useful marker for understanding disease pathogenesis. Most methods for thiol quantification require destructive analysis of tissues.[16] Alternatively, noninvasive imaging modalities capable of identifying regions of oxidative stress and depleted antioxidant defenses would be highly desirable.

Current methods for in vivo quantification of thiols and other antioxidants include magnetic resonance spectroscopy (MRS) and electron paramagnetic resonance (EPR). However, both suffer from poor signal to noise ratios, require analyte concentrations in the high millimolar range, and have low resolution (cm^3), limiting these techniques to more advanced diseases with larger volumes.[17] A clear need exists for better non-invasive sensors of this important thiol redox system and its role in disease progression.

Magnetic Resonance Imaging (MRI) is an excellent method for visualizing antioxidant levels in vivo because of its ability to non-invasively obtain high resolution (0.5 mm^3) anatomical images.[18] However, MRI requires a biosensor to translate antioxidant levels into an MR signal. We have previously shown that spiropyran switches can be converted to redox active imaging agents through conjugation to a gadolinium chelate MRI contrast agent. In the presence of NADH in cells these probes produced contrast enhancement greater than 20%, opening up an attractive new class of imaging agents capable of characterizing the concentrations of redox active species *in vivo*.[19, 20] In this work we address the issue of developing spiropyran molecular switches that demonstrate thiol response with a functional handle for future use with MRI contrast agents.

Li et. al. have demonstrated that glutathione responsiveness requires two nitro groups on the chromene ring of a spiropyran switch,[21] and in our previous work the linker to the gadolinium chelate occupies one of these positions. In this study we investigated two other positions for functional modification of spiropyrans and characterize their effect on switching by determining the response, selectivity, and reversibility towards thiol sensing of these spiropyrans. We found that adding functionality to the indoline portion of a dinitro spiropyran had little effect on the thiol response of these spiropyrans. This work demonstrates thiol responsive spiropyran isomers ready for conjugation and overcomes the difficult task of adding functionality without significantly altering thiol response.[21, 22]

Results/Discussion

Spiropyrans are a well known class of molecular switching compounds that can be converted between the closed spiropyran form (SP) or the open merocyanine form (MC) in response to external stimuli (Scheme 2).[23] In this work we synthesized switches for antioxidant sensing, the presence of thiols induces ring opening; this can be reverted back to the closed form using visible light. This conversion can be easily observed by UV/Vis spectroscopy with the MC form absorbing strongly in the visible region while the SP form absorbs strongly in the UV region. Measurement of the MC absorbance peak was performed to monitor switching from SP to MC form induced by response to various amino acids.

Synthesis of spiropyrans **5** and **6** proceeded from the commercially available 3-aminobenzyl alcohol **2**, which was oxidized to the hydrazine under Sandmeyer conditions. Utilizing continuous extraction over 24 hours provided 50% mass recovery, and the crude aryl hydrazine underwent Fischer indole synthesis to generate indoles **3** and **4** as a mixture. The overall yield of both indole isomers was 32% over two steps, a significant improvement on previously reported yields over the same two steps[24]. Indoles **3** and **4** were methylated with iodomethane and the crude enamines were subjected to condensation with 2-hydroxy-3,5-dinitrobenzaldehyde in refluxing ethanol (24 h, 80 °C) to yield spiropyrans **5** and **6** (Scheme 1) in four steps with 2.5% and 1.5% overall yield. Spiropryan **1** (Figure 1) was prepared as reported by Buback et. al.[25] (supporting information)

Spiropyran **1**, featuring two nitro groups in the 6- and 8-positions of the chromene moiety, was reported in the literature to respond to primary thiols such as Cys, Hcy, and GSH, however, no switching to the MC form was reported in the presence GSSG.[21] We used this spiropyran as reference to test for positional effects of the introduction of linkers. Hydroxyl groups, which can readily be converted to the alkyne for conjugation to azide species, were placed in different positions on the indole ring of spiropyran **1** to form spiropyrans **5** and **6**. Spiropyrans **5** and **6** were successfully synthesized and structure identified by accurate mass spectroscopy, proton and carbon NMR spectroscopy (supporting information).

Following the synthesis, switching ability of spiropyrans **1, 5**, and **6** in response to Cys, Hcy, GSH and GSSG was investigated by UV/Vis spectroscopy. Representative data from single samples of spiropyrans **1, 5**, and **6** are shown in figure 2 to illustrate the relative responses for these spiropyrans in the presence of equimolar amounts of Cys, Hcy, GSH, and GSSG. All three spiropyrans exhibit a response to the sulfhydryl-containing amino acids as

illustrated by the strong absorbance increase centered around 500 nm. The disulfide GSSG was used as a control and the lack of response to GSSG supported the necessity of the sulfhydryl moiety for ring opening. Similar to previous literature reports, spiropyran **1** displays a similar response for all three thiols with cysteine and GSH perfectly overlapping, indicating the same extent of switching response for these two primary thiols (0.551 A.U., 0.548 A.U., at λ_{MC} respectively). The response to homocysteine was slightly lower (0.513 A.U. at λ_{MC}) and minimal response to GSSG (0.060 A.U. at λ_{MC}) was observed; these results recapitulate literature observations (Figure 2A).[21] Spiropyrans **5** and **6** show much greater variability between the three primary thiols. For all three isomers Cys produced the greatest changes in absorbance, resulting in final absorbances of 0.51 AU, 0.71 AU and 0.75 AU at the peak absorbance wavelength of the MC form (λ_{MC} for spiropyrans **1, 5**, and **6** respectively). Repeating these assays demonstrated that minor fluctuations in absorbance profile in response to analytes could be expected, resulting in no significant selectivity for thiols with spiropyran **6**. The aggregate data is discussed further in the next section. There is also a markedly higher baseline absorbance for spiropyran **5** after 5 minutes of visible light irradiation compared to the other two spiropyrans, **1** and **6**, suggesting that spiropyran **5** has a greater population of the MC form at equilibrium compared to spiropyrans **1** and **6** (Figure 2B). Longer visible light irradiation times were tested in an attempt to drive conversion to the closed form, but after 20 minutes of visible light irradiation this high baseline still persisted. (SI, Figure S1).

Selectivity

Building upon this initial characterization of thiol responsiveness, spiropyrans **1, 5**, and **6** were evaluated for their thiol selectivity by incubating with GSH, Cys, Hcy, and other nucleophilic amino acids. As expected, all three spiropyran solutions shifted to an orange color in the presence of Cys, GSH, and Hcy indicating a ring opening to the MC form, which is supported by the appearance of a strong absorbance centered around 500 nm (λ_{MC}) (Figure 3A). Figure 3 represents the ratio of $(A\text{-}A_0)/A_0$ for the λ_{MC} for each isomer respectively. Based on these ratios all three isomers showed selectivity for thiols over GSSG and other nucleophilic amino acids without primary thiols (p <0.01, Figure 3). Spiropyrans **1** and **6** demonstrated poor selectivity among the three primary thiols GSH, Hcy, and Cys (Figure 3). In contrast, spiropyran **5** exhibits the greatest response to cysteine at the λ_{MC} with a 5.29 fold increase from the baseline prior to incubation with thiol and reduced responsiveness to GSH (4.08 fold, $p < 0.05$), further reduced response to Hcy (3.82 fold, $p < 0.01$) indicating some level of selectivity over primary thiol (Figure 3B). Like spiropyran **1**, spiropyran **5** had no response to amino acids lacking a primary thiol (Figure 3B). The addition of the hydroxyl in this position seems to afford some degree of cysteine selectivity over the other sulfhydryl containing amino acids (GSH $p < 0.05$, Hcy $p < 0.01$).

While the values of $A\text{-}A_0/A_0$ appear to be smaller for Spiropyran **5**, it is important to note that this ratio may not be reflective of the response of the switch. While final absorbance values between spiropyrans **5** and **6** were similar in magnitude after incubation with Cys (Student t test, $p = 0.1727$) the higher baseline of spiropyran **5** reduced the $A\text{-}A_0/A_0$ values. The position of the linker placed on spiropyran **6** had little effect on selectivity, and spiropyran **6** behaved similarly to spiropyran **1** (Figure 3C).

Kinetics of interaction assays

The conversion from SP to MC forms was monitored over time at the λ_{MC} for each derivative. All three spiropyrans show similar saturation kinetics in the presence of thiols with a high initial rate of ring opening that gradually plateaus at longer incubation times (Figure 4). Spiropyran **5** exhibited an increased rate of ring opening in the presence of Cys relative to Hcy and GSH. Spiropyran **5** with Cys achieved 95% of the total absorbance change in the first 10 minutes and 100% by 15 minutes. Conversely, solutions of spiropyran **5** with GSH or Hcy demonstrate similar kinetics to those of spiropyrans **1** and **6**, with Hcy or GSH inducing nearly 60% conversion in the first 5 minutes and roughly 85% conversion after 10 minutes. Unlike spiropyran **5**, Spiropyrans **1** and **6** demonstrate similar kinetics between all three primary thiols with a 60% response in the first 5 minutes, 85% at 10 minutes, and saturation of response in the final 5 minutes. None of the spiropyrans showed isomerization to the mero form when incubated in the dark or with 1 equivalent of GSSG, further supporting that the ring opening reaction requires the presence of primary thiols only (purple line in all plots).

Reversibility assays

The nucleophilicity of the sulfhydryl moiety has been exploited for visualizing thiols by a number of different chromophores including squaraines,[26, 27] merocyanines,[28] naphthalimides,[29] maleimides,[30] chromenes,[31] boron dipyrromethanes (BODIPY),[32, 33] and quinones.[34] However, unlike these other chromophores the reaction of spiropyrans with thiols is reversible. This trait would be attractive in a thiol responsive MR probe for dynamic contrast enhancement that reflect the changing levels of antioxidant thiols in the region of interest.

To demonstrate that the response to a primary thiol is reversible, solutions of the spiropyrans containing cysteine were subjected to repeated rounds of visible irradiation to induce ring-closing to the SP form in the presence of a ring-opening primary thiol, illustrating the non covalent interaction and reversibility of this sulfhydryl catalyzed ring opening. Figure 5 illustrates the change in absorbance intensity of the MC peak of photoswitch solutions. After 20 minute incubation with cysteine in the dark, a solution of spiropyran **1** had an absorbance peak of 0.65 AU at 495nm. This peak drops to 0.068 AU after 5 minutes of visible light irradiation indicating complete conversion back to the SP form. After cessation of visible light irradiation the solution of **1** still containing 1.0 equivalent of cysteine was allowed to incubate in the dark for 20 minutes, and the nearly complete recovery of the MC absorbance was observed (Figure 5A). However, the cysteine response for spiropyran **1** did not regain the same absorbance intensity as the first cycle but showed minor fatigue with a final absorbance of 0.53AU after 4 cycles of visible light irradiation. While reversibility has been hypothesized for these dinitro spiropyran switches,[21] to our knowledge this is the first demonstration of reversibility.

While spiropyran **5** has a greater initial response to cysteine (0.74 AU) the switching fatigue is significantly greater compared to spiropyran **1** (Figure 5B). After two cycles of visible light irradiation the cysteine response decreases dramatically to 0.38 AU and nearly disappears completely (0.19 AU) after three rounds of visible light irradiation. It is unknown

why functionalization at this position leads to severe degradation of switching; this will be investigated in future studies. The limited recovery may limit the utility of spiropyran **5** as an activatable MR probe for dynamic applications, but may increase contrast for static applications as the agent would accumulate over time with activation.

In contrast spiropyran **6** exhibited complete and sustained reversibility after 4 cycles of irradiation (Figure 5C). These differing reversibility results suggest that these probes can be applied for applications suited to their strengths, for example spiropyran **6**s hows promise as a reversible probe for thiols but lacks selectivity, so it would be better suited for applications where dynamic information about thiols is valued over specificity; while spiropryan **5** demonstrated better selectivity for cysteine over other biologically relevant thiols but had poor reversibility, so it is best suited for applications requiring specific information about cysteine where amplification of signal by accumulation over time would be valued

Conclusions

In conclusion, we have synthesized two thiol responsive spiropryan isomers and investigated two positions of attachment of a functional group to allow future conjugation to contrast agents for MRI. The benzyl alcohol can be readily converted to the alkyne in high yields (~90%) through a Horner-Wadsworth-Emmons type olefination with dalkyldiazomethylphosphonates and manganese oxide.[35] This provides an alkyne terminated spiropyran for click reactions, providing access to spiropyran conjugation to any azide-bearing molecule/nanoparticle/surface from the benzyl alcohol.[36] These probes demonstrated that functionalization of the indoline ring of spiropyrans has little effect on thiol response but can impart probe selectivity. While spiropyran 5 demonstrated some thiol selectivity, these probes will most likely be glutathione reporters based on the overwhelming levels of GSH intracellulary (1–10 mM).[37] The same is true extracellularly and in plasma where GSH concentrations are 5–10 fold greater than cysteine, and up to 100 fold greater than homocysteine. All three probes do however, exhibit good seletivity over amino acids, indicating potential use as general thiol probes. All three probes exhibited noticeable color changes from yellow to orange, indicative of switching from SP to MC, upon addition of equimolar biothiols. To the best of our knowledge, this is the first demonstration that the sulfhydryl mediated ring opening of spiropyrans can be reversed and cycled several times, highlighting the reversibility of these switches in thiol sensing. This work provides access to spiropyran isomers ready for conjugation to any nanoparticle surfaces or molecules, bearing reactive azides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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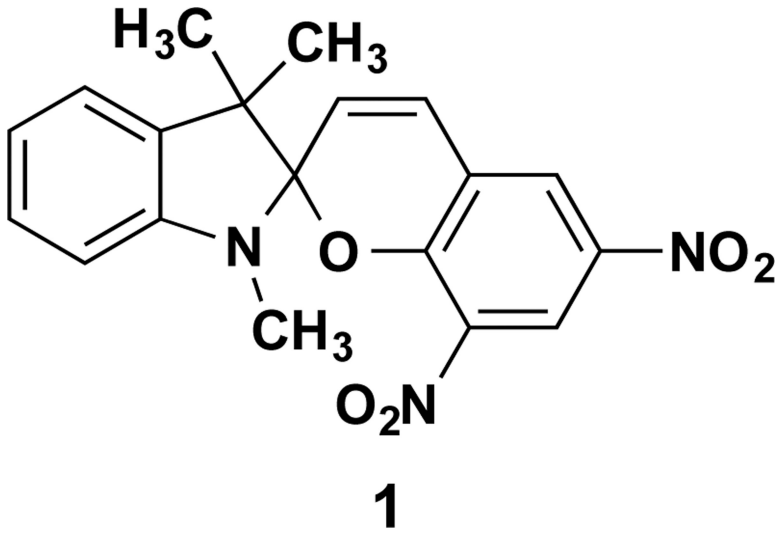

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Figure 1.
Unfunctionalized dinitro spiropyran.

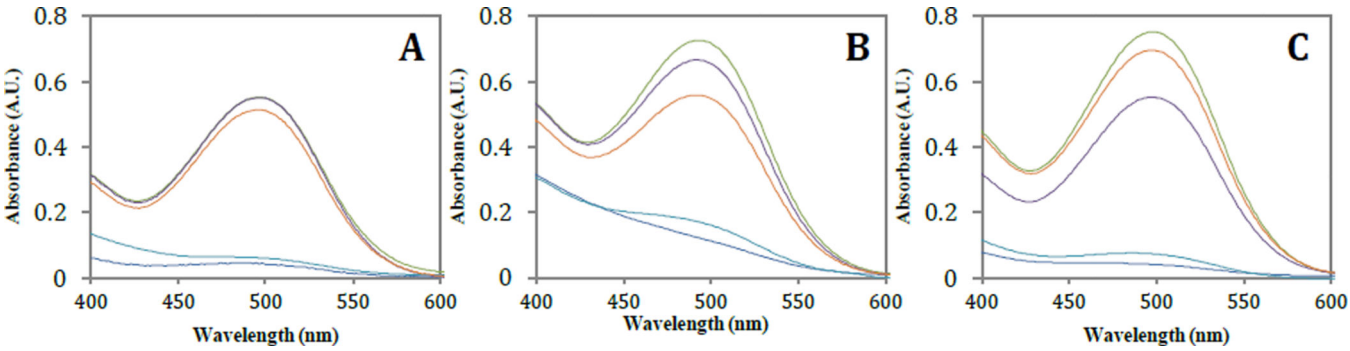


Figure 2.

Absorption spectra changes of **1** (Panel A), **5** (Panel B), **6** (Panel C) under different conditions: After 5 minutes of visible light irradiation (Blue) and upon addition of GSSG (light blue), Homocysteine (Orange), Glutathione (Purple), and Cysteine (Green). Final concentrations of all analytes is 5×10^{-5} M.

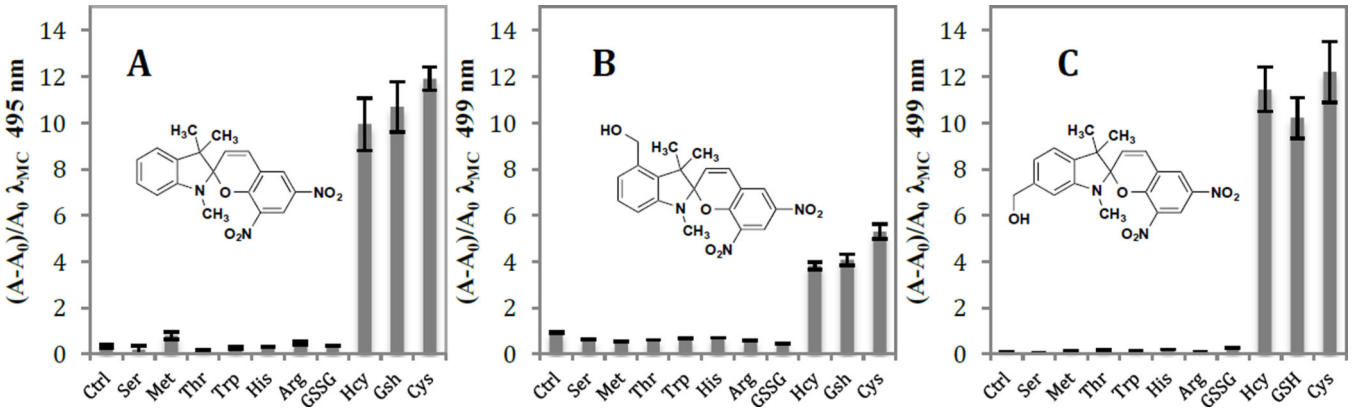


Figure 3.

Selectivity of Spiropyrans **1** (Panel A), **5** (Panel B), **6** (Panel C) towards various nucleophilic amino acids. Grey bars represent the ratio $(A\text{-}A_0)/A_0$ of spiropyrans **1, 5**, and **6** in the presence of various amino acids. Data shown is the average of three independent trials with standard deviation. All thiols significantly influenced swithing in contrast to the control and other amino acids, which were unreactive (p <0.01). (No statistical significance between thiols for spiropyrans **1** and **6**. For spiropyran **5** Cys response was greater than GSH (p < 0.05) and Hcy (p < 0.01), analysis by one way ANOVA).

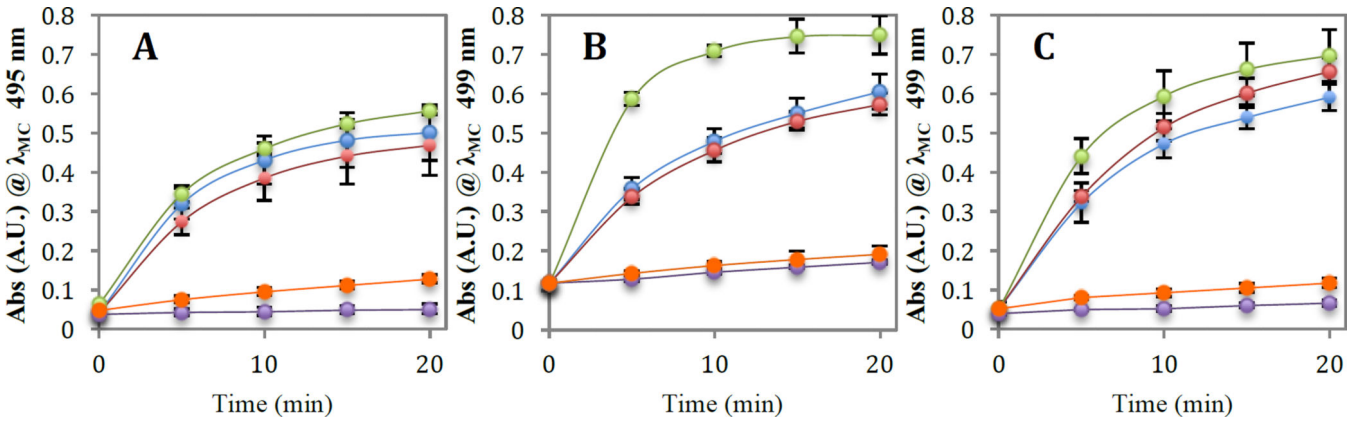


Figure 4.

Time course of ring opening reaction for spiropyrans **1** (Panel A), **5** (Panel B), **6** (Panel C) in the absence of any analyte (light blue), and upon addition of GSSG (purple), Hcy (red), GSH (orange), and cysteine (green). Final concentrations of all analytes is 5×10^{-5} M. Error bars represent the standard deviation of three trials.

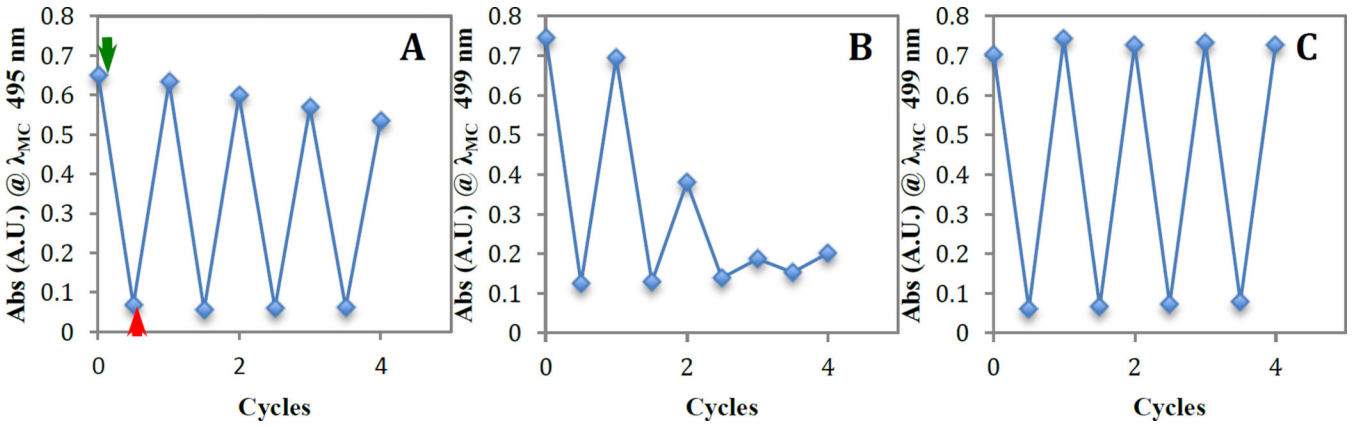
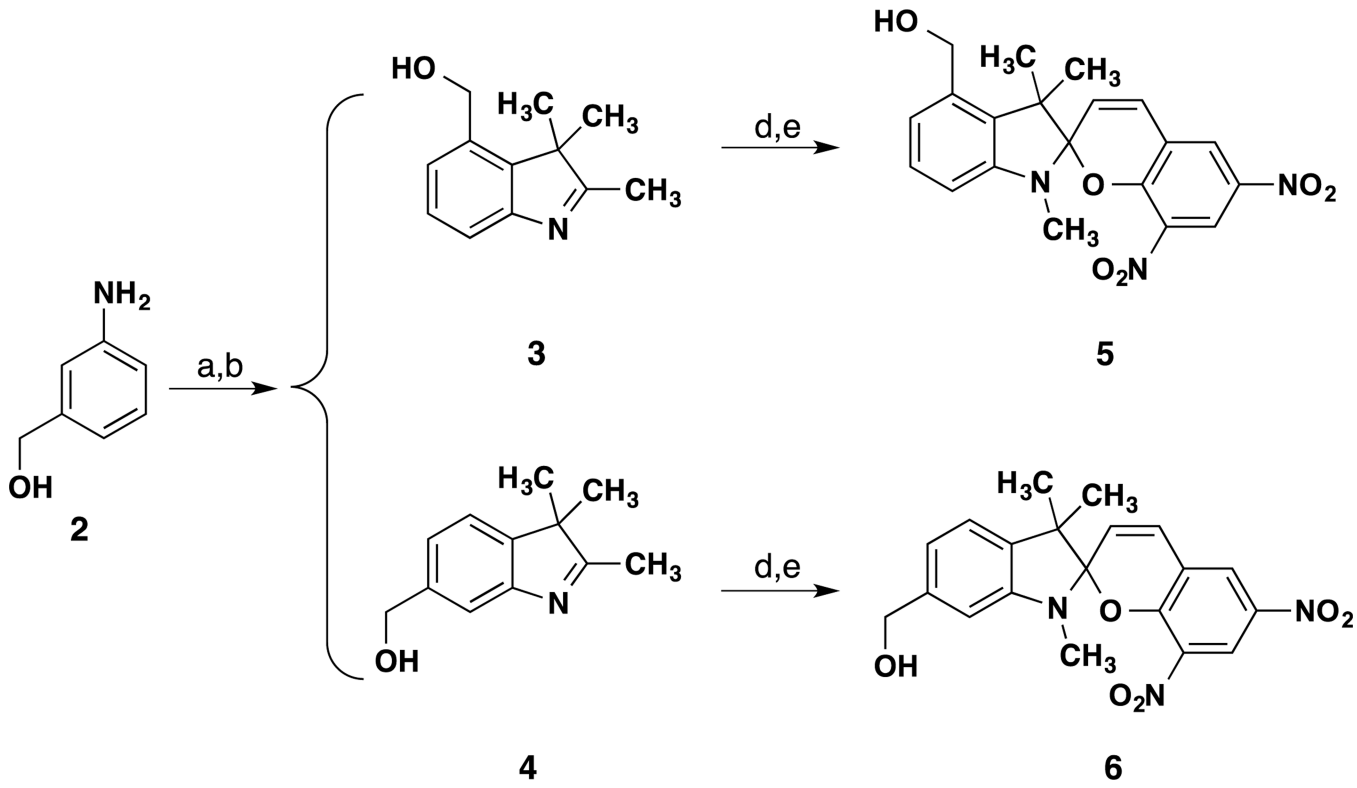


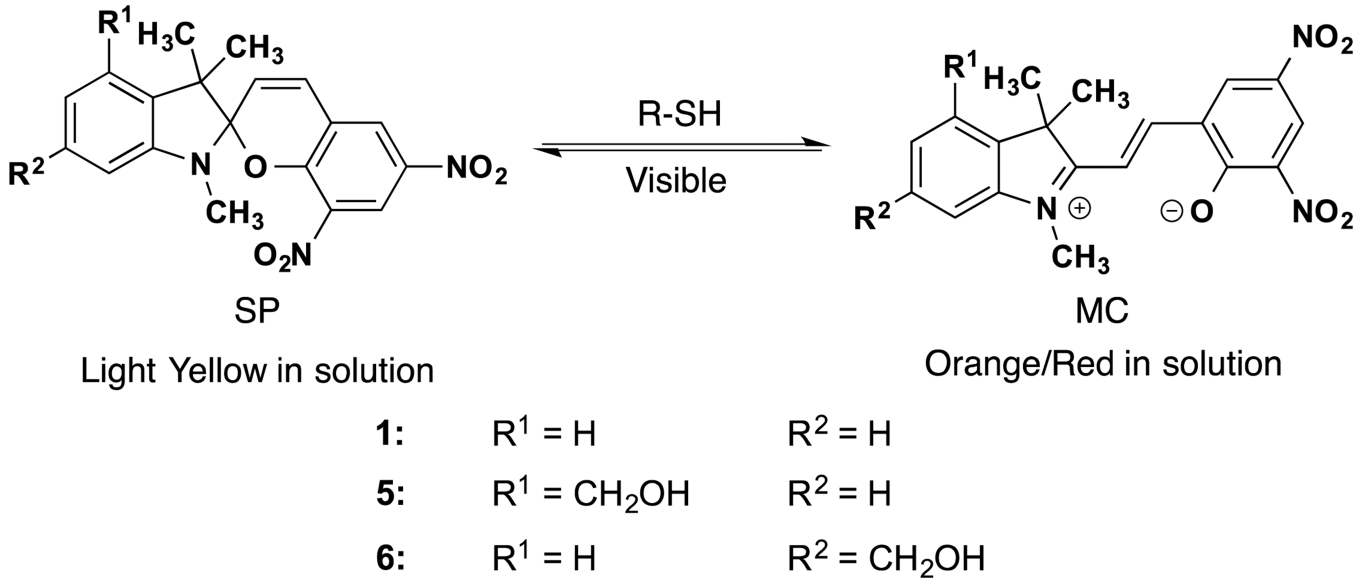
Figure 5.

Absorbance changes at the λ_{MC} for spiropyrans **1** (Panel A), **5** (Panel B), and **6** (Panel C) after 20 minute incubation with equimolar cysteine (Green Arrowhead) followed by 5 minute visible light irradiation (Red Arrowhead). This process was repeated four times on the same solution to determine the reversibility of thiol promoted ring opening.



Scheme 1. Synthesis of spiropyrans 5 and 6

Reagents and Conditions: (a) $Sn_2Cl_2 \cdot H_2O$, $NaNO_2$, HCl (12N), 0 °C; (b) 3-methyl-2-butanone, acetic acid, reflux; (d) CH_3I, $CHCl_3$, reflux; (e) 3,5-dinitrosalicylaldehyde, ethanol, reflux.



1: $R^1 = H$ $R^2 = H$

5: $R^1 = CH_2OH$ $R^2 = H$

6: $R^1 = H$ $R^2 = CH_2OH$

Scheme 2.
Sulfhydryl catalyzed ring opening of dinitro spiropyrans.