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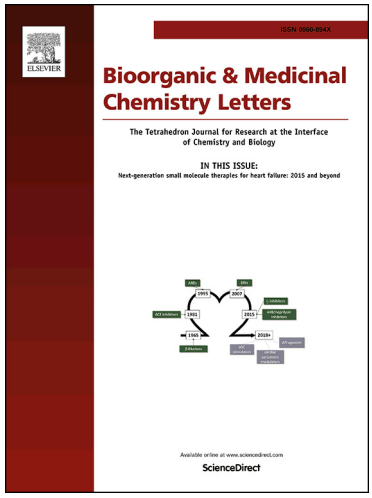

A fluorogenic H_2S-triggered prodrug based on thiolysis of the NBD amine†

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A fluorogenic H_2S-triggered prodrug based on thiolysis of the NBD amine†

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ABSTRACT

Based on thiolysis of the NBD amine, a H_2S-triggered prodrug has been designed and synthesized for localized production of ciprofloxacin under micromolar H_2S. Activation of the prodrug can be monitored through fluorescence in real-time. We envision that thiolysis of the NBD amine could be readily used for development of other H_2S-triggered prodrugs in the future.

Introduction

Recent studies deomonstrated that endogenously produced H_2S possess important physiological functions, and H_2S is named as the third gasotransmitter after NO and CO.[1,2] Accumulating evidence suggests that H_2S influences a wide range of physiological and pathological processes, including modulation of blood vessel and cardioprotection,[3] endogenous stimulator of angiogenesis,[4] mitochondrial bioenergetics,[5] and crosstalk with MicroRNA.[6] H_2S also plays important roles in tumour biology, and it is suggested that both inhibition of H_2S biosynthesis and elevation of H_2S concentration beyond a certain threshold could exert anticancer effects.[7] In plants, H_2S participates in seed germination, plant growth and development, as well as the acquisition of stress tolerance including cross-adaptation and cadmium tolerance.[8,9] In bacteria, H_2S is a key player in maintaining intracellular redox balance to counteract a lethal degree of oxidative stress induced by ampicillin.[10] Due to its widespread existence and functions, we spectulate that H_2S might be employed to develop H_2S-triggered prodrugs with improved properties.

One major challenge in developing H_2S-triggered prodrugs and fluorescence probes is the development of a chemical reaction to effectively differentiate the reactivity of high concentration of biothiols (millimolar) versus low concentration of H_2S (micromolar).[11] To address this issue, we and Pluth *et al.* have developed thiolysis of the NBD (7-nitro-1,2,3-benzoxadiazole) amine for design of H_2S-specific fluorescence probes since 2013.[12] The fluorescence of the fluorophore can be quenched by the NBD moiety in aqueous solution, whereas the fluorescence will be restored upon the thiolysis of the NBD moiety. To this end, a series of NBD-based H_2S probes with emissions from blue to near-infrared range have been developed by our groups.[13] In this work, we continue to investigate this field and develop a fluorogenic H_2S-triggered prodrug (Fig. 1) based on thiolysis of the NBD amine for the first time.

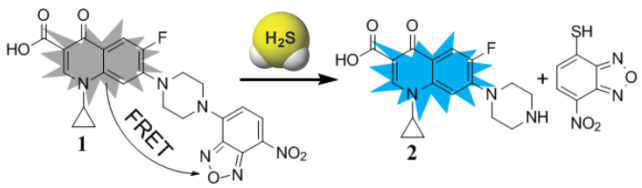

Fig. 1 Schematic illustration of H_2S-triggered release of the prodrug **1** to produce ciprofloxacin **2** and NBD-SH accompanying with fluorescence increase.

A fluorogenic prodrug can be used to *in situ* produce active drug accompanying with significant fluorescence increase.[14] A modification of prodrug is commonly designed by conjugating a parent drug with a promoiety, which could be eliminated by a specific reaction under a certain condition.[15] Therefore, selective delivery of the active drug can be achieved with a specific

NBD-capped ciprofloxacin **1** as a fluorogenic prodrug (Fig. 1), which can be activated by micromolar H_2S.

The synthesis of the prodrug **1** can be prepared from direct coupling of NBD-Cl and **2**.[16] The facile and economic synthesis is important for the wide use of the prodrug and probe. The chemical structure of **1** was confirmed by ^{1}H NMR, and high-resolution mass spectra (HRMS). It is noted that the signals for ^{13}C NMR were quite weak due to the poor solubility of **1** in common deuterated solvents.

With the prodrug in hand, we firstly tested the absorbance spectra of **1** in the presence of H_2S (using Na_2S as an equivalent) in PBS buffer (20 mM, pH 7.4). **1** showed relative low solubility in buffer (~ 17 μM, Fig. S1). As shown in Fig. 2a, **1** exhibited absorbance peaks at 350 nm and 490 nm, which should be assigned to the ciprofloxacin and NBD fluorophores, respectively. After reaction with H_2S, new absorbance peak appeared at 530 nm, which might be due to the production of NBD-SH.[12b] The intensities at 560 nm can be used to monitor the reaction kinetics to give the reaction rate of 12.9 $M^{-1}s^{-1}$ (Fig. S2). Such thiolysis reaction was further characterized by HPLC analysis (Fig. 2b). The reaction was nearly complete within 5 min, and the peak for **1** was completely disappeared at 40 min. These studies indicated the prodrug **1** could be activated by H_2S efficiently.

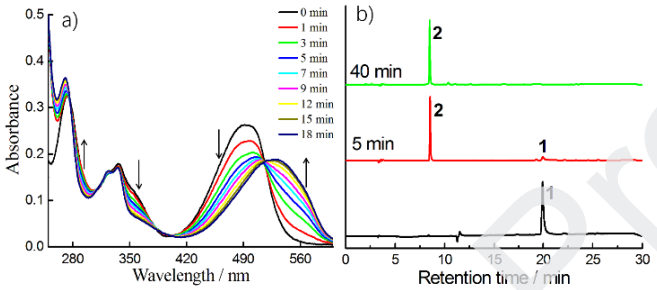

Fig. 2 (a) Time-dependent absorption spectra of **1** (10 μM) and Na_2S (200 μM) in PBS buffer (50 mM, pH 7.4). (b) Time-dependent HPLC traces of the reaction of **1** with Na_2S to give ciprofloxacin **2**.

The probe **1** showed very weak fluorescence upon excitation at 280 nm, indicating that the fluorescence from ciprofloxacin may be majorly quenched by the FRET effect (Fig. 1). After reaction with H_2S, large fluorescence turn-on was observed, with about 62-fold increase at 415 nm (Fig. 3a). The reaction kinetics of the prodrug during the activation is an important parameter to examine its biological applicability on account of the rapid catabolism of H_2S under physiological conditions. To this end, time-dependent fluorescence intensities at 415 nm for the probe in the presence of H_2S were acquired for data analysis (Fig. S3). The pseudo-first-order rate, k_{obs}, was found by fitting the data with a single exponential function. The reaction rate k_2 (12.0 $M^{-1}s^{-1}$) was obtained by linear fitting of the k_{obs} versus H_2S concentrations (Fig. 3b). Moreover, the fluorescence of the prodrug could be efficiently released by low-micromolar H_2S (Fig. S4). The prodrug cannot be activated by millimolar biothiols (10 mM GSH, 5 mM Cys, Fig. S5), which is consistent with the highly H_2S-specific reactivity of our thiolysis of the NBD amine.[13] In addition, we examined the reactivity of the prodrug with different concentrations of H_2O_2 (Fig. S6), the prodrug cannot be activated, even if the concentration of H_2O_2 is as high as 200 μM. These results imply that the prodrug can be

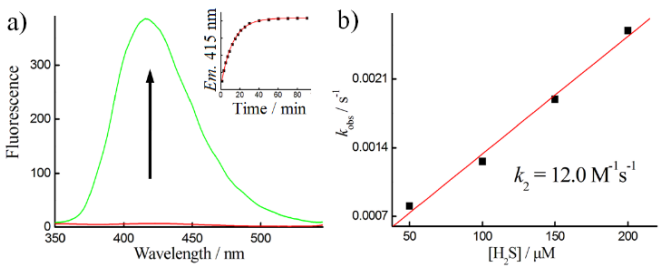

Fig. 3 (a) Time-dependent fluorescence spectra of **1** (5 μM) and Na_2S (100 μM) in PBS buffer (Ex. = 280 nm). Fluorescence intensity at 415 nm versus time is indicated inset. The red line represents the best fitting to give k_{obs} of 0.08 min^{-1}. (b) The linear relationship between concentrations of Na_2S and k_{obs}. The slope of the linear fitting gives k_2

Encouraging with the above results, we next employed **1** for antibacterial tests under different conditions. The concentration of H_2S in *E. coli* strains of urinary tract infections is between 300-600 μM approximately.[17] *E. coli* was chosen as a model bacterium in our study. After incubation of 30 nM **1** and *E. coli* in the LB medium for 12 h, the OD_{600} value was recorded for each sample to show the bacterial concentration (Fig. 4). The prodrug **1** or H_2S, L-Cys or **1** + GSH (Fig. S7) did not show obvious inhibition on the bacterial growth. However, the combined usage of 30 nM **1** and 50 μM H_2S resulted in significant inhibition activity against *E. coli*, and such inhibition effect could be directly visualized by naked eye (Fig. 4). L-Cys can induce *E. coli* to produce endogenous micromolar H_2S (Fig. S8), which can be also employed to activate the prodrug. However, a mixture of NBD-diethanolamine and H_2S (that can produce NBD-SH) has no inhibition on bacterial growth. The results implied that 1) the prodrug can be activated by exogenous or endogenous H_2S; 2) the release of ciprofloxacin produced a bactericidal effect, not the by-product NBD-SH.

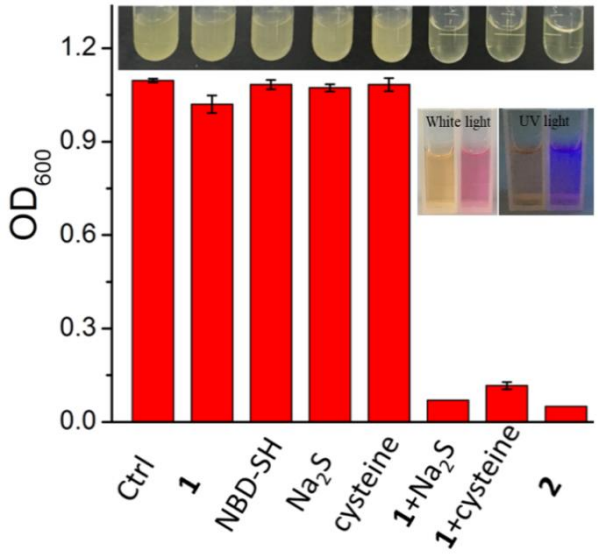

Fig. 4 OD_{600} values of *E. coli* in the different cultured conditions. Experimental conditions: 30 nM **1**, 30 nM NBD-SH, 50 μM Na_2S, 5 mM L-Cys, 50 μM Na_2S + 30 nM **1**, or 5 mM L-Cys + 30 nM **1**, 30 nM **2** was added into the LB medium as indicated below each bar. Inset: photographs of *E coli* in tubes under the conditions corresponding to each column. Images of **1** (15 μM) with or without H_2S (400 μM) in PBS buffer under white light or 254 nm UV light is indicated inset.

concentration dependent inhibition of bacterial growth. Various concentrations of **1** in the absence and presence of H_2S were added into the culture medium. After incubation at 37 ºC for another 12 h, the OD_{600} of the medium was measured for each sample (Fig. 5). As expected, the prodrug **1** at low concentration showed weak inhibition effect on the bacterial growth, while the addition of H_2S or L-Cys significantly increased the inhibition effect of **1**. However, the prodrug could also inhibit bacterial growth at high concentrations (> 0.2 μM), which may be explained by the inhibitory mechanism of ciprofloxacin. Firstly, the X-ray crystallography of ciprofloxacin in complex with DNA gyrase[18] showed that piperazinyl ring of ciprofloxacin protruding toward the protein surface had little interaction with its target protein. Secondly, the substituted piperazinyl ring of drug variants[19] (levofloxacin, gatifloxacin, moxifloxacin and C8-Me-moxifloxacin) were all effective antibiotics. Therefore, the inhibitory activity of prodrug **1** was reduced rather than completely lost. The MIC of the combination of prodrug **1** and H_2S (50 μM Na_2S or 5 mM L-Cys) was about 30 nM, and in this condition *E. coli* cell were almost killed (Fig. 5) within 12 hours. The NBD moiety even at micromolar concentrations did not have obvious effect on bacterial growth.

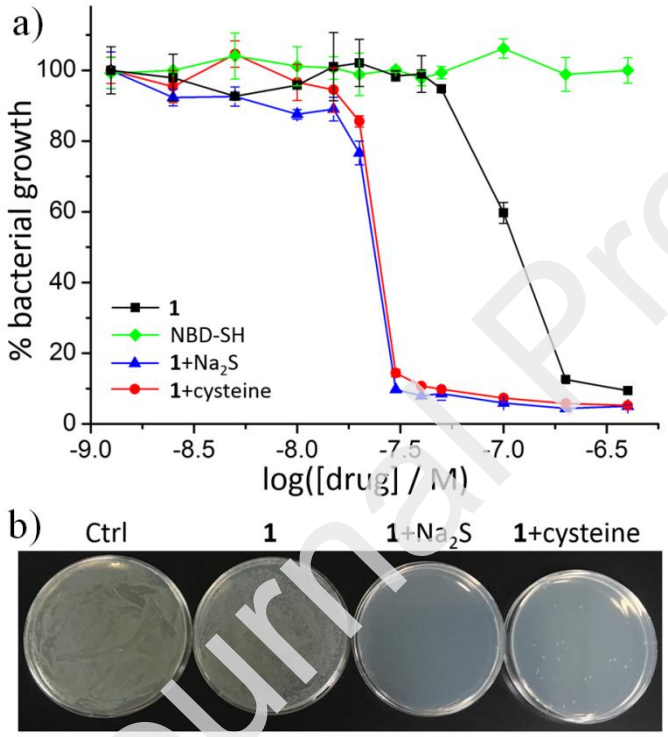

Fig. 5 (a) Concentration-dependent OD_{600} values of *E. coli* in the different cultured conditions as indicated inset. Experimental conditions: 30 nM prodrug **1**, 50 μM Na_2S, or 5 mM L-Cys were added into the LB medium. (b) Photos of bacterial growth inhibition for each culture plate.

In conclusion, we have designed and synthesized the first H_2S-triggered prodrug based on thiolysis of the NBD amine. The prodrug can be used for localized production of ciprofloxacin in the presence of micromolar H_2S. Activation of the prodrug can be indicated via significant turn-on (62-fold) fluorescence in real-time. We also envision that thiolysis of NBD amine could be generally used for development of other H_2S-triggered prodrugs, including that for sparfloxacin, grepafloxacin, pipemidic acid, and pethidine.

Acknowledgments

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Graphical Abstract

This work reports a fluorogenic H_2S-triggered prodrug for *in situ* production of ciprofloxacin under micromolar H_2S.

A fluorogenic H_2S-triggered prodrug based on thiolysis of the NBD amine†

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