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Photocontrol of Antibacterial Activity: Shifting from UV to Red Light Activation

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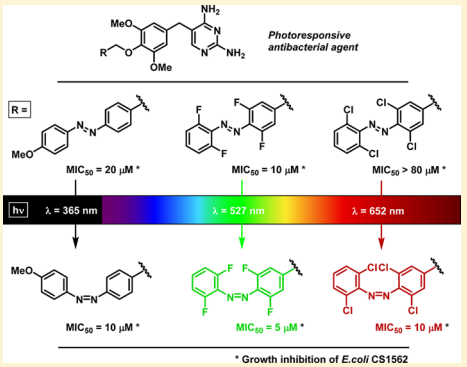
Supporting Information

ABSTRACT: The field of photopharmacology aims to introduce smart drugs that, through the incorporation of molecular photoswitches, allow for the remote spatial and temporal control of bioactivity by light. This concept could be particularly beneficial in the treatment of bacterial infections, by reducing the systemic and environmental side effects of antibiotics. A major concern in the realization of such light-responsive drugs is the wavelength of the light that is applied. Studies on the photocontrol of biologically active agents mostly rely on UV light, which is cytotoxic and poorly suited for tissue penetration. In our efforts to develop photoswitchable antibiotics, we introduce here antibacterial agents whose activity can be controlled by visible light, while getting into the therapeutic window. For that purpose, a UV-light-responsive core structure based on diaminopyrimidines with suitable antibacterial properties was identified. Subsequent modification of an azobenzene photoswitch moiety led to structures that allowed us to control their activity against *Escherichia coli* in both directions with light in the visible region. For the first time, full *in situ* photocontrol of antibacterial activity in the presence of bacteria was attained with green and violet light. Most remarkably, one of the diaminopyrimidines revealed an at least 8-fold difference in activity before and after irradiation with red light at 652 nm, showcasing the effective "activation" of a biological agent otherwise inactive within the investigated concentration range, and doing so with red light in the therapeutic window.



INTRODUCTION

The often life-saving treatment of infectious diseases is increasingly compromised by the emergence of antimicrobial resistance,[1] which can be traced back to a buildup of antibiotics in the environment due to their excessive application in both human health care and animal husbandry.[2] Another issue that plagues especially long-term antibiotic treatment is the common occurrence of severe adverse effects. A highly relevant case can be found in the fluoroquinolone antibiotics.[3] Quinolones are a class of synthetic, broad-spectrum antibiotics widely relied upon in the clinic since their discovery in the 1960s;[4] however, safety concerns regarding the treatment with fluoroquinolones are still being raised, with the FDA issuing the latest of numerous warnings as recently as 2016[5] (risks include selection of resistant pathogens,[6] tendon rupture,[7] and nerve damage[8]). Such a decline in the reliability of long-established classes of antibiotics is particularly troubling in the face of resistant pathogenic bacteria and the slow pace of innovation in antibacterial drug discovery over the past five decades.[9]

The emerging fields of photopharmacology[10] and photochemotherapy[11] offer a promising approach to deal with both systemic and environmental side effects resulting from chemotherapy (Figure 1). Rendering a drug photoswitchable allows for the spatial and temporal control over its biological activity with light. Light is, in principle, not harmful and easily delivered with high precision, which has already led to its application in antibacterial photodynamic therapy[12] for the formation of singlet oxygen. Examples of the successful utilization of photopharmacology, which is singlet oxygen independent, include photoswitchable antimicrobials[13] and antitumor therapy,[14] control of neuronal networks,[15] and vision restoration.[16] However, so far, the application of photopharmacology in a reversible manner (on/off switching of bioactivity with light) is often limited by the need of UV light to effect photoisomerization. UV light is toxic to healthy cells[17] and has a limited tissue penetration depth.[18] To ultimately

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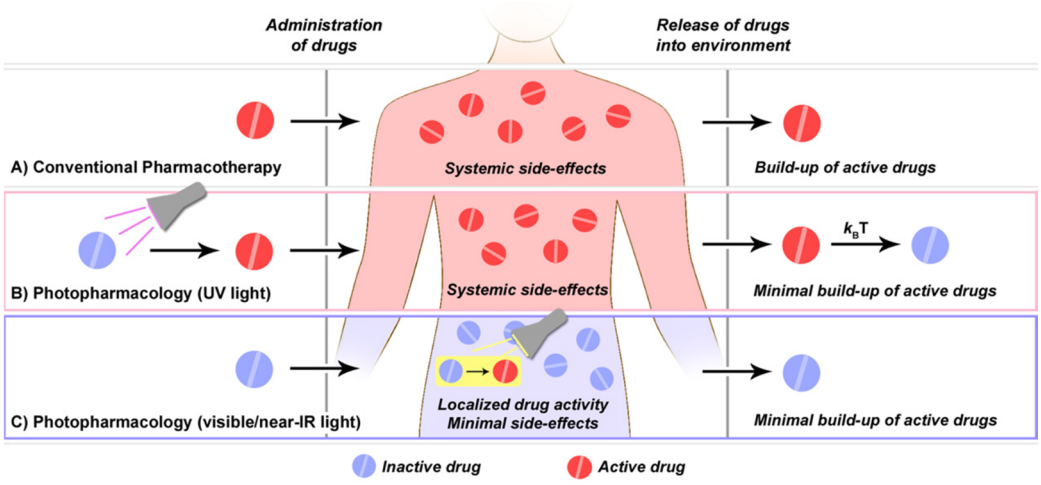
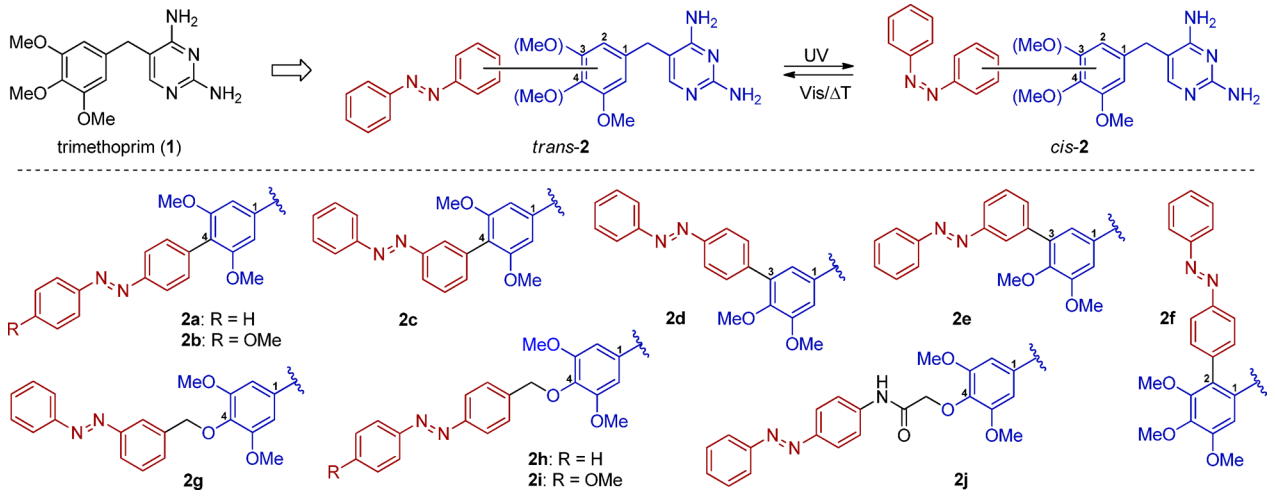


Figure 1. Potential scenarios for the application of photopharmacology compared to conventional pharmacotherapy. (A) Classic chemotherapeutic treatment without control over drug activity leads to systemic side effects and environmental buildup that is particularly damaging in the case of antibiotics, as this constitutes a major cause for the emergence of antimicrobial resistance. (B) Photopharmacological agents responsive to UV light may offer only a partial solution to these issues, as a drug with suitable properties may possibly be activated before administration by UV light irradiation, with the potentially unstable active state losing its activity over time, thus counteracting the environmental buildup. (C) Full control of photopharmacological agents outside as well as inside the human body to address both systemic and environmental side effects can only be realized if the photoswitchable drug is responsive to visible or near-IR light, both not harmful to cells and, particularly in the near-IR range, better suited to penetrate tissue.

Scheme 1. Structurally Diverse Set of UV-Light-Responsive Diaminopyrimidines



realize their full potential by achieving reversible control of activity *in vivo* (Figure 1C), photopharmacological agents should ideally be responsive to light in the therapeutic window between 650 and 900 nm.[18]

Here we report the control of antibiotic activity with red light for the first time and show *in situ* photocontrol of antibacterial activity in the presence of bacteria. In our effort to create photoswitchable antimicrobials while addressing the challenge to use visible or red light, we focused on antifolates, particularly on the antibiotic trimethoprim as a starting point for structural design. Antifolates are a class of drugs that interfere with the biosynthesis of folate, by inhibition of dihydrofolate reductase (DHFR) which catalyzes the reduction of dihydrofolate to the active cofactor tetrahydrofolate.[19] The latter plays a vital role in the biosynthesis of essential bacterial metabolites, such as the amino acids glycine and methionine, as well as purines and thymidine triphosphate. As a result, tetrahydrofolate depletion sets off a complex cascade that ultimately leads to the cessation of DNA, RNA, and protein synthesis.[20] Antifolates include antibacterial,[19,21] antiprotozoal,[22] and anticancer agents.[23] Among these, the antibiotic trimethoprim[24] shows a high selectivity toward bacterial DHFR compared to mammalian DHFR.[25] It is active against a broad spectrum of Gram-positive and Gram-negative bacteria (including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Hemophilus influenzae*) and widely used in the clinic (especially against urinary and respiratory tract infections).[26] However, treatment with trimethoprim is plagued by the emergence of bacterial resistance.[27] This makes it a particularly interesting candidate for photopharmacology, i.e., the development of photoresponsive analogues, as the spatial and temporal control over their activity could reduce excessive and unwanted exposure of bacteria to the active form of the drug, and thus drastically limit the number of bacteria with engaged resistance mechanisms.

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RESULTS AND DISCUSSION

The potential to modify the structure of trimethoprim[21,28] without losing affinity toward bacterial DHFR further encouraged us to use it as a basis for photoswitchable antibiotics. Initially we sought to employ a simple, modular approach to attach regular, UV light-responsive azobenzene photoswitches to the trimethoprim core structure in different ways, thus providing quick, synthetically facile access to a small collection of compounds that exhibit high structural diversity. In this way, we expected to identify a promising structural platform with the desired photopharmacological properties. Subsequent exchange of the azobenzene switch with a red-shifted analogue would enable photoisomerization with visible light, ideally with retention of the antibacterial properties.

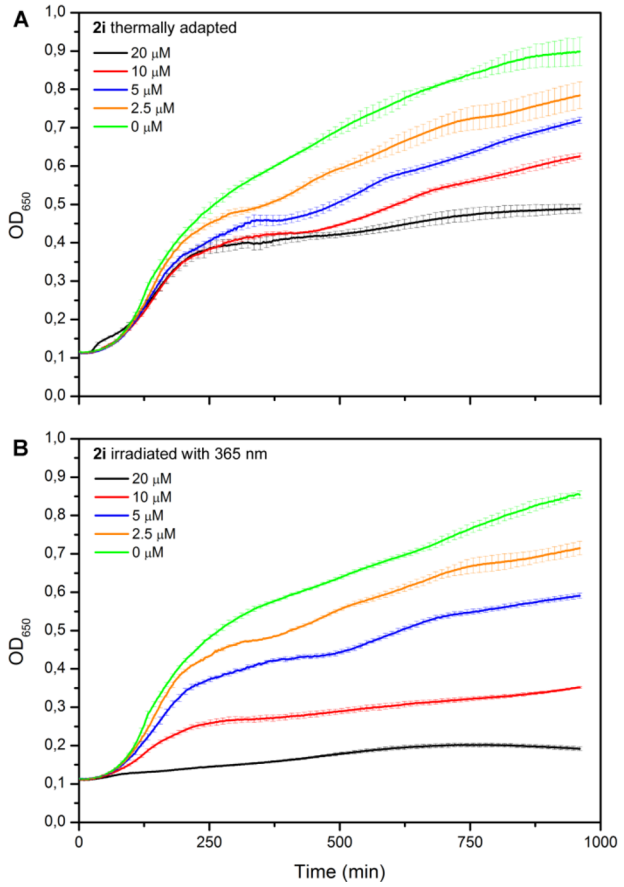


Figure 2. Bacterial growth curves of *E. coli* CS1562 at increasing concentrations of **2i**. (A) Samples after thermal adaptation. (B) Samples after irradiation with UV light at λ = 365 nm. Error bars show standard deviation calculated from measurements in triplicate.

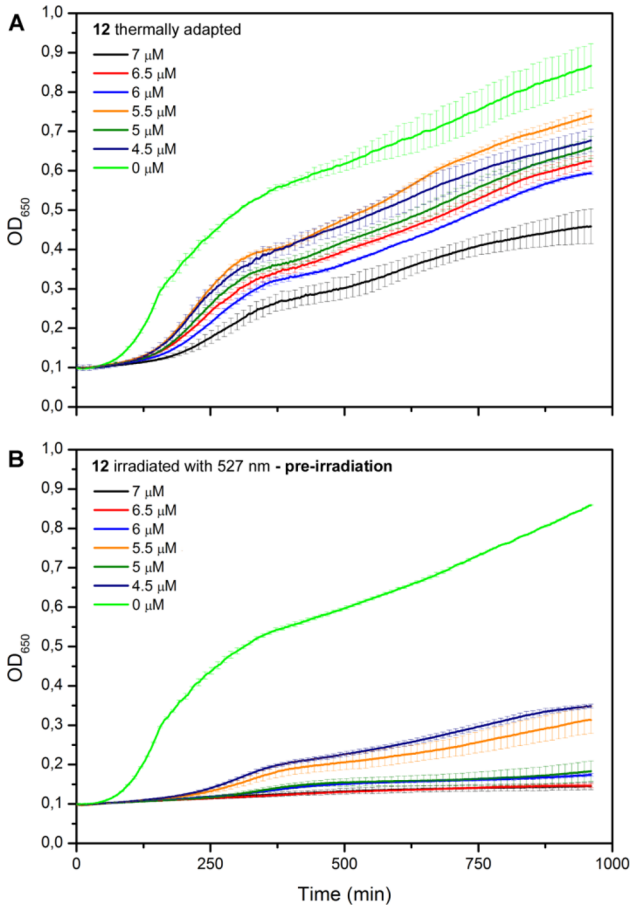
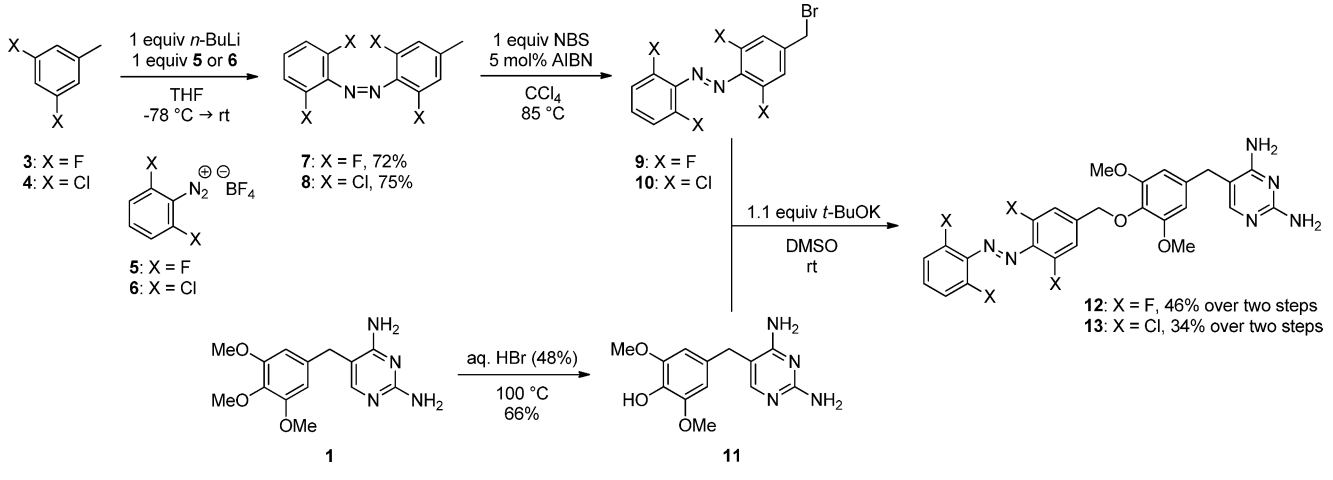


Figure 3. Bacterial growth curves of *E. coli* CS1562 at increasing concentrations of **12**. (A) Samples after thermal adaptation. (B) Samples after irradiation with green light at λ = 527 nm. Error bars show standard deviation calculated from measurements in triplicate.

Scheme 2. Synthesis of Red-Shifted Diaminopyrimidines 12 and 13



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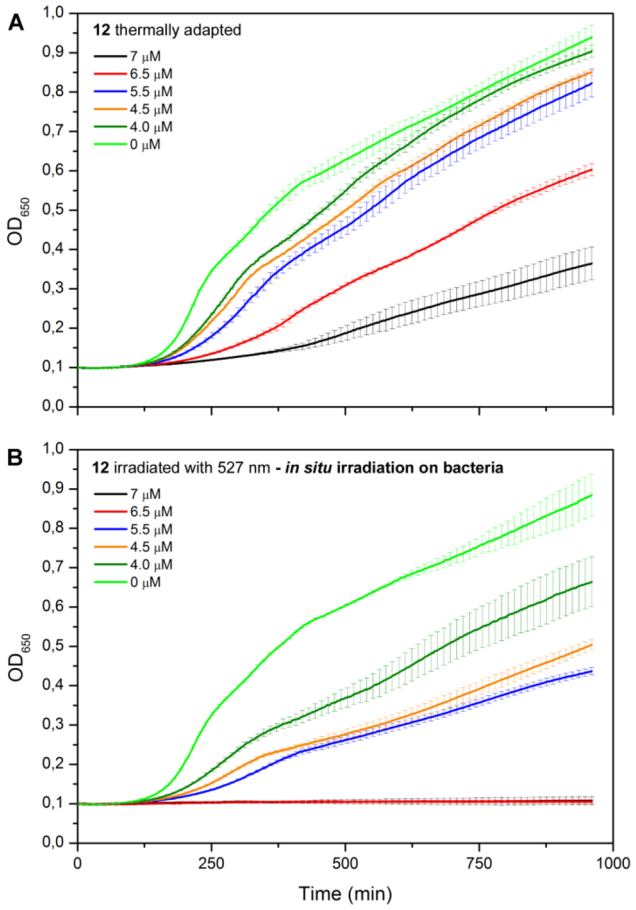

Figure 4. Bacterial growth curves of *E. coli* CS1562 at increasing concentrations of **12**. (A) Samples after thermal adaptation. (B) Samples after *in situ* irradiation with green light at λ = 527 nm in the presence of bacteria. Error bars show standard deviation calculated from measurements in triplicate.

The first stage of this approach is illustrated in Scheme 1: Photoresponsive diaminopyrimidines of the general structure **2** were generated by attachment of largely unsubstituted azobenzenes to the core structure of trimethoprim (**1**) (see also SI). Switches were either directly connected to the 2, 3, and 4 positions of the drug's methoxyphenyl unit by *Suzuki* cross-coupling (**2a–f**), or by alkylation of the free hydroxy group in the 4 position via different linkers (**2g–j**). Before starting with biological experiments, basic photochemistry of compounds **2a–j** was studied in DMSO to ensure satisfactory photoswitching behavior (see Figures S1–S10). In case of **2h**, NMR analysis revealed degradation of the compound upon irradiation with UV light, which was remedied by introduction of a methoxy group in the 4′-position, resulting in compound **2i**.

With this small library of structurally diverse compounds **2** in hand, their antibacterial activity was investigated against *E. coli* in 2-fold dilution series, both before and after irradiation with UV light at 365 nm (see Figure S43). With the exception of **2f** and **2j**, which proved to be mostly inactive up to a concentration of 20 μM, all compounds effectively inhibited bacterial growth with various degrees of potency (see Figures S26–S34). When comparing inhibition of growth by irradiated and non-irradiated samples, in most cases no significant difference was observed. However, **2i** stood out in that respect, as a considerable change in potency was evident upon *trans–cis* isomerization (see growth curves in Figure 2, which translate into a MIC_{50} of 20 μM before and 10 μM after irradiation). Fortunately, the *cis* isomer was found to be the more active one, setting the stage for targeted structural modifications of the photoswitch to specifically red-shift the wavelength for *trans–cis* isomerization (*vide infra*). In addition, the fact that the active *cis* isomer usually has a limited half-life and thermally isomerizes to the more stable—but less active—*trans* isomer, enables an intrinsic auto-deactivation pathway that would help to prevent the buildup of active drugs in the environment (cf. Figure 1B).[13a]

Prior to structural modification of **2i** to achieve the desired red-shift of its activating wavelength, we further investigated the actual nature of its observed effect on bacterial growth upon photoisomerization. From chemical actinometry and quantum yield determination (see Figures S43 and S45) we obtained $\phi_{(trans-cis)}$ = 0.18 and $\phi_{(cis-trans)}$ = 0.02 for **2i**, which showed that switching efficiencies are preserved when the azobenzene core was substituted with the trimethoprim motif. UV–vis analysis of **2i** in aqueous medium at 30 μM showed some form of aggregation of the compound in its *trans* state (Figures S11A and S13). Upon irradiation with 365 nm, the aggregates appear to dissolve during *trans–cis* isomerization, providing a possible explanation for the difference in potency. However, this effect was no longer observed at a concentration of 5 μM (Figure S11B), whereas a difference in antibacterial activity was still evident at that concentration. Furthermore, other diaminopyrimidines **2** exhibited similar aggregation behavior upon photoisomerization in aqueous medium (Figure S14), without any significant difference in activity between the respective photoisomers observed during antibacterial experiments. This encouraged us to proceed under the premise that the difference in antibacterial activity of **2i** effected by photoisomerization does indeed directly originate from structural changes on a molecular level, and is not caused by differences in solubility in the bacterial growth medium. We envisioned that further modification of the basic structure of **2i** would not only bring forth the desired red-shift of the activating wavelength, but also resolve any issue of possible aggregation in aqueous medium.

Among ongoing efforts to realize azobenzene photoswitches that can be addressed with visible light,[29] the groups of Hecht[30] and Woolley[31] made significant progress with the design and synthesis of azobenzenes bearing fluoro, chloro, or methoxy substituents in all positions *ortho* to the azo moiety. The influence of these substituents allows for the effective use of lower-energy n–π^* excitation to trigger *trans–cis* photoisomerization. Taking advantage of this approach in the creation of red-shifted analogues of structure **2i**, we were particularly interested in the tetrafluoro-substituted azobenzenes, since such a modification would provide us with a structure closest to **2i** in terms of size. In order to synthetically access the modified structure **12** (Scheme 2), we utilized the methodology, recently introduced by our group, for the facile preparation of *ortho*-substituted azobenzenes:[32] Lithiation of 3,5-difluorotoluene (**3**) and subsequent conversion with the corresponding diazonium salt **5** gave azobenzene **7** in good yield. After radical bromination, bromide **9** was used in an alkylation of phenol **11**, which in turn could be directly obtained from trimethoprim (**1**) by selective ether cleavage.[28b] Thus, photoresponsive diaminopyrimidine **12** was readily prepared by direct modification of the commercially available drug.

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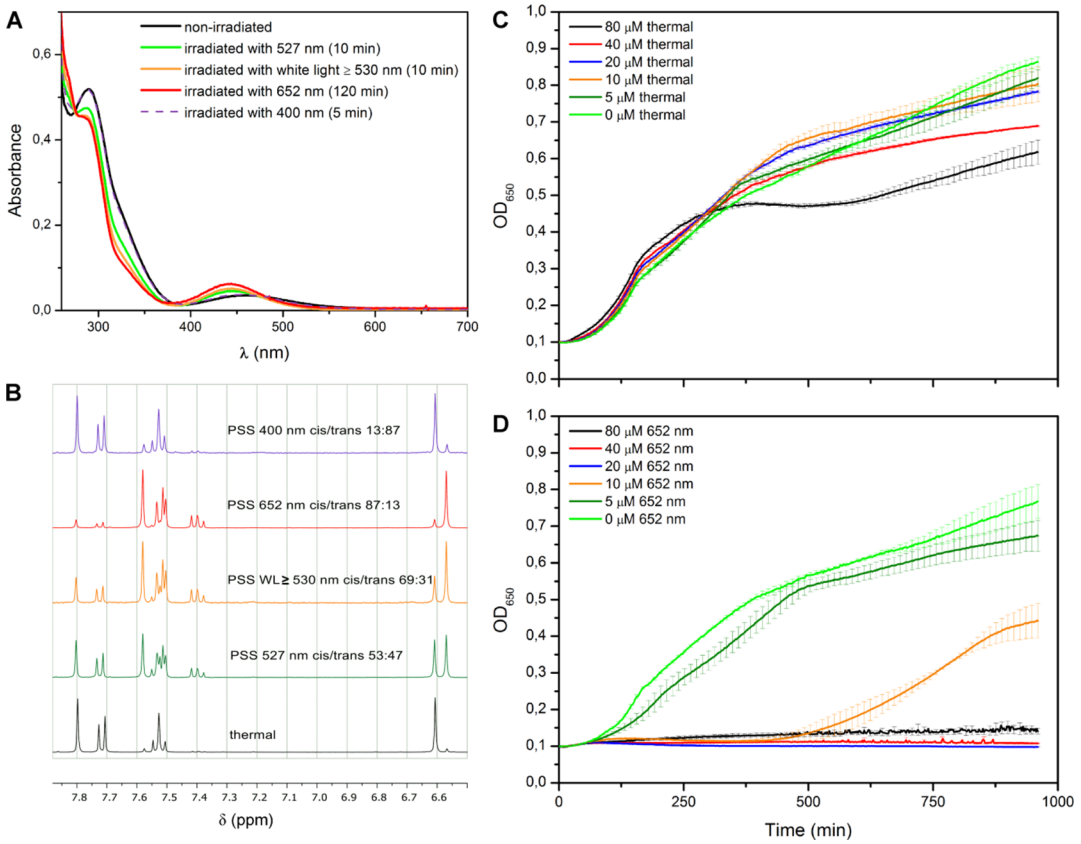

Figure 5. (A) UV−vis absorption spectra of **13** before and after irradiation with various light sources in DMSO. (B) Partial ^{1}H NMR spectra of **13** before and after irradiation with various light sources in DMSO-d_6. (C) Bacterial growth curves of *E. coli* CS1562 at increasing concentrations of **13** after thermal adaptation. (D) Bacterial growth curves of *E. coli* CS1562 at increasing concentrations of **13** after irradiation with red light. Irradiation was performed on a solution of **13** in DMSO for 2.5 h with red light at λ = 652 nm. Error bars show standard deviation calculated from measurements in triplicate.

Efficient *trans*−*cis* isomerization of **12** in DMSO was effected with green light at 527 nm (see Figure S44 and SI for specifications, for photostationary state (PSS) of *cis:trans* = 89:11 established by NMR analysis, see Figure S15D), while irradiation with violet light at 400 nm (see Figure S42 and SI for specifications) can be applied to switch back to the *trans* state. Photoisomerization could also be performed selectively in aqueous LB broth (used herein as medium for bacterial growth), with no sign of fatigue evident over numerous cycles at 37 °C (Figure S15B,C).

With a photoswitchable aminopyrimidine in hand that can be photoisomerized in either direction with visible light, we investigated its antimicrobial activity. Interestingly, **12** showed increased activity against *E. coli* when compared to **2i** in both isomeric forms.

Furthermore, we noted that a significant difference in antibacterial activity between the two forms remained at concentrations of 4−7 μM (Figure 3), with a genuine "activation" effect evident upon *trans*−*cis* isomerization with green light (translating into a change of MIC_{50} from 10 μM to 5 μM, see Figure S36). While these results were obtained for pre-irradiation of the samples in the absence of bacteria, analogous experiments with true *in situ* irradiation after inoculation of bacteria yielded a similar effect (Figure 4), as photoisomerization with nontoxic green light could be achieved without influencing the bacterial growth with the applied light. This marks an important next step toward the reversible photocontrol of an antibacterial agent *in vivo* with biocompatible wavelengths of light.

Having successfully realized control over bacterial growth with green light using photoresponsive aminopyrimidine **12**, we sought to take our approach a step further by red-shifting the wavelength of activation even more. For this purpose, diaminopyrimidine **13** (as a structural analogue of **2i** and **12**) was synthesized, featuring four chloro substituents in *ortho* positions of the azobenzene (see Scheme 2). Tetra-*ortho*-chloroazobenzenes have been reported to engage in *trans*−*cis* isomerization upon irradiation with red light between 630 and 660 nm.[31,33] Indeed, irradiation of **13** with red light at 652 nm (see SI for specifications) effected photoisomerization to a PSS of *cis:trans* = 87:13 (in DMSO, Figure 5A,B), albeit after prolonged irradiation times of 2−3 h. Faster isomerization was observed for irradiation with green light at 527 nm; however, this only resulted in a PSS *cis:trans* ratio of about 1:1. A compromise between irradiation time and PSS for the *trans*−*cis* isomerization of **13** could be reached by using a strong white light source in combination with a filter cutting off wavelengths below 530 nm: Using this light source, a PSS of *cis:trans* = 69:31 could be reached within 10 min. Photoisomerization of **13** also proceeded cleanly in aqueous medium and without fatigue over numerous cycles (Figure S18).

Due to our interest in the farthest possible shift of our photoswitchable agents' response toward the red, we immediately focused on the influence of red light irradiation on the antibacterial activity of **13**. In that regard, initial

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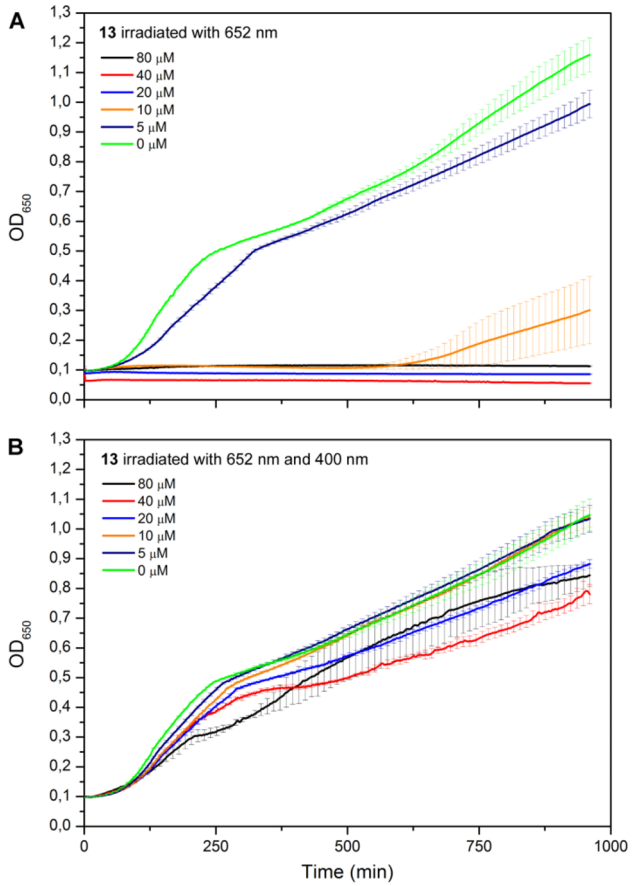

Figure 6. Two series of bacterial growth curves of *E. coli* CS1562 at increasing concentrations of **13** irradiated with red light at λ = 652 nm for 3 h in DMSO before treatment of bacteria. One series of bacteria (A) was protected from further irradiation. The other series (B) was irradiated with violet light at λ = 400 nm after inoculation. Error bars show standard deviation calculated from measurements in triplicate.

experiments performed analogously to the other compounds in this study (2-fold dilution series in 96-well plates) yielded disappointing results, with a noticeable but unsatisfactory difference in growth evident (Figure S39). However, we imagined that this lack of difference in activity before and after irradiation might well be due to ineffective photoswitching in the 96-well plates, as the light source was usually positioned at a distance of 11.5 cm from the plate to ensure maximum coverage. To verify this assumption, one-half of a divided stock solution of **13** in DMSO was irradiated for 2.5 h with red light at 652 nm at a distance <1 cm, before treating bacteria with the two separate samples in 2-fold dilution series. To our delight, this experiment revealed a dramatic photoactivation effect: Whereas non-irradiated **13** remained largely inactive with a MIC_{50} > 80 μM (Figure 5C), red light-irradiated **13** induced bacteriostasis down to 20 μM, with an observed MIC_{50} of 10 μM (Figure 5D). It is worth noting at this point that photoisomerization of **13** with red light at close proximity also works effectively in aqueous medium (Figure S20).

To exclude the possibility that factors such as changes in solubility upon photoisomerization have an undesired influence on the antibacterial activity of **13** in our experiments, we considered it vital to demonstrate that the photocontrol of its activity is reversible. Taking advantage of the fact that switching **13** back from its active *cis* form to the inactive *trans* form can be performed within the visible light range, experiments were conducted with violet light at 400 nm (cf. Figure 5A,B). This allowed us to treat bacteria with a red-light-irradiated, "activated" sample of **13** in two 2-fold dilution series, before irradiating half of the inoculated bacteria with 400 nm light, evidently without harming the bacteria in the process (Figure 6B). We were pleased to find that, indeed, samples irradiated with violet light were effectively "deactivated" again, with a MIC_{50} > 80 μM, just as previously observed for the *trans* isomer, properly restored (compared once again to the MIC_{50} = 10 μM of the active *cis* isomer protected from violet light irradiation, Figure 6A).

While the inefficient photoisomerization of **13** with red light prevented us from conducting red light irradiation experiments in the presence of bacteria in our model *in vitro* system, the results presented herein mark a significant development in our ongoing efforts to create photoresponsive antibiotics, as we were able, for the first time, to control the activity (8-fold difference) of an antibacterial agent with light beyond the 650 nm mark, which constitutes the lower margin of the therapeutic window,[18] bringing us an important step closer to future *in vivo* control of photoswitchable antibiotics.

CONCLUSIONS

We successfully developed photoresponsive, antibacterial diaminopyrimidines bearing azobenzene photoswitches, whose activity can be controlled by light of various wavelengths. Identification of a UV light-responsive core structure with suitable antibacterial properties and subsequent, targeted modification of the photoswitch moiety lead us to structures whose activity could be increased upon irradiation with green and red light. Remarkably, for the first time, these compounds allowed for the full *in situ* photocontrol of antibacterial activity with green and violet light, making it possible to trigger both the "activation" as well as "deactivation" of the antibacterial agent in the presence of bacteria. Most significantly, diaminopyrimidine **13** revealed an at least 8-fold difference in activity before and after irradiation with red light (previous efforts in our group show that similar differences are sufficient for spatiotemporal patterning of bacteria).[13a,b] More importantly, apart from showcasing the effective "activation" of a biological agent otherwise inactive within the investigated concentration range, we were able to do so while also shifting the wavelength of activation from the UV range toward red light in the therapeutic window.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b09281.

Synthetic schemes, experimental procedures, photochemical data, and bacterial growth curves, including Figures S1−S45, and NMR spectra of new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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