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Design, Synthesis, and Antibacterial Activity of a Multivalent Polycationic Calix[4]arene–NO Photodonor Conjugate

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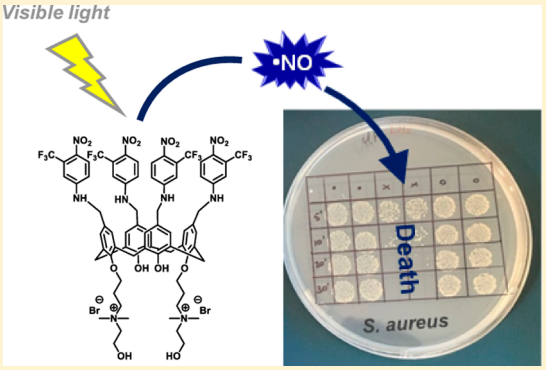
S *Supporting Information*

ABSTRACT: The role of nitric oxide (NO) as an antimicrobial and anticancer agent continues to stimulate the search of compounds generating NO in a controlled fashion. Photochemical generators of NO are particularly appealing due to the accurate spatiotemporal control that light-triggering offers. This contribution reports a novel molecular construct in which multiple units of 3-(trifluoromethyl)-4-nitrobenzenamine NO photodonor are clustered and spatially organized by covalent linkage to a calix[4]arene scaffold bearing two quaternary ammonium groups at the lower rim. This multivalent calix[4]arene–NO donor conjugate is soluble in hydro-alcoholic solvent where it forms nanoaggregates able to release NO under the exclusive control of visible light inputs. The light-stimulated antibacterial activity of the nanoconstruct is demonstrated by the effective bacterial load reduction of Gram-positive *Staphylococcus aureus* ATCC 6538 and Gram-negative *Escherichia coli* ATCC 10536.



KEYWORDS: *Calix[4]arene, nitric oxide, photoactive nanoaggregates, antibacterial*

Nitric oxide (NO) is an ephemeral inorganic free radical that has stimulated a tremendous interest in the last two decades because of its critical role in a number of physiological and pathophysiological processes.[1] Depending on its concentration, doses, and site of action, NO can elicit beneficial or deleterious effects, ranging from regulation of nervous,[2] cardiovascular,[3] immune,[4] and hormonal[5] systems to cytotoxicity.[6] The cytotoxic effects, associated with oxidation of lipids, proteins, and DNA, have stimulated curiosity on NO as a multitarget anticancer[7] and antimicrobial[8] agent and motivated the design and development of molecular and macromolecular scaffolds able to deliver NO with an ambitious goal to tackle important diseases.[9,10] Many devices releasing NO under the action of an external stimulus have been described in the literature.[11−13] Among them, the photoresponsive systems are particularly appealing[14−18] in view of the great advantages light offers in terms of precise spatiotemporal control and powerful trigger, which does not affect physiological parameters such as temperature, pH, and ionic strength.

Derivatives of the 3-(trifluoromethyl)-4-nitrobenzenamine, generating nontoxic photodegradation products, revealed very effective NO photodonors.[19,20] To significantly increase the NO reservoir, multiple units of this chromophore have been confined in a restricted area by both covalent and noncovalent conjugation to different materials and molecular scaffolds.[21]

Calix[*n*]arenes are a family of polyphenolic macrocycles, characterized by relevant synthetic versatility and the presence of a hydrophobic cavity with remarkable hosting properties.[22] The calixarene skeleton can be variously functionalized at both the upper and lower rim to give a variety of derivatives[23,24] with a large range of applications[25] in different areas including pharmacology.[26] Rudkevich and Neri investigated the use of calix[4]arene derivatives as cages for storage and release of gaseous NO.[27,28] Recently, we have reported the first example of a calixarene-based system delivering NO on demand.[29] It was achieved by the noncovalent entrapment of a highly hydrophobic NO photodonor within supramolecular nanoaggregates of calix[4]arene derivative.[29]

On this basis, the robust clustering of multiple units of a NO photodonor within a single molecular scaffold by covalent linkage represents a promising approach to enhance the reservoir of NO per molecule. Herein, we report the synthesis

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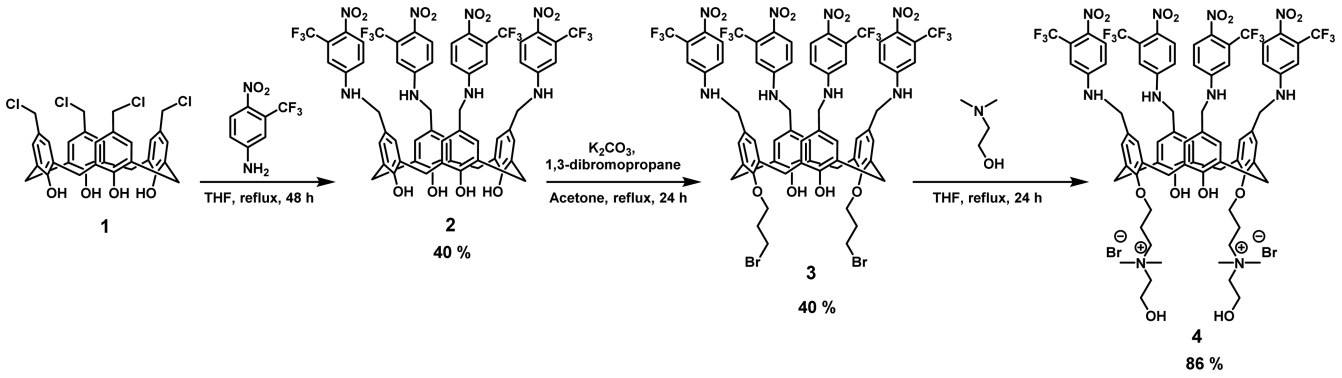
Scheme 1. Synthetic Route for the Preparation of Compound 4



of a novel multivalent photoresponsive NO donor, obtained by grafting multiple units of 3-(trifluoromethyl)-4-nitrobenzenamine onto the upper rim of a calix[4]arene backbone bearing two quaternary ammonium groups at the lower rim. The self-assembly of this amphiphilic conjugate in nanostructures was investigated by dynamic light scattering (DLS), and the light-stimulated NO release was measured directly by amperometric detection. The antibacterial activity of the calixarene−NO donor conjugate was proved on *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 10536 chosen as representative Gram-positive and Gram-negative bacteria strains.

The synthetic steps leading to the multivalent calix[4]arene−NO photodonor conjugate **4** are illustrated in Scheme 1. The *p*-chloromethyl-calix[4]arene (**1**), obtained by chloromethylation[30] of the commercial *p*-H-calix[4]arene, was treated with 3-(trifluoromethyl)-4-nitrobenzenamine in THF as a solvent to give compound **2** in 40% yield after chromatographic purification (Scheme 1). The signals in ^{1}H and ^{13}C NMR spectra of compound **2** and the presence of the pseudomolecular ion peak $[M - H]^-$ at 1295.2 in the ESI-MS spectrum evidenced that four 3-(trifluoromethyl)-4-nitrobenzenamine units were anchored at the calix[4]arene upper rim (see Supporting Information). To confer water-solubility to the calix[4]arene−NO donor conjugate, we devised to introduce two quaternary ammonium groups at the calix[4]arene lower rim. To this aim, compound **2** was reacted with 1,3-dibromopropane in the presence of potassium carbonate and acetone as a solvent to give intermediate compound **3** bearing two 3-bromopropyloxy pendants at the calix[4]arene lower rim in 1,3 distal position (Scheme 1). Finally, compound **3** was treated with *N*,*N*-dimethyl-ethanol amine in THF to achieve calix[4]arene derivative **4**. We chose *N*-dimethylethanol ammonium moieties as cationic polar head groups for the known ability of hydroxylamines to penetrate the bacterial membrane without harmful effect on eukaryotic cells[31] and the low cytotoxicity showed by other calix[4]arene derivatives bearing *N*-dimethylethanol ammonium moieties on eukaryotic cells.[26]

^{1}H NMR spectra of **4** (see Supporting Information) showed the presence of resonances at 3.34 ppm and at 3.62 and 4.07 ppm for choline CH_3 and CH_2CH_2OH protons, respectively. One AX system (3.49 and 4.25 ppm, J = 13.2 Hz) for the protons of the calix[4]arene methylene bridges evidenced that in compound **4** the calix[4]arene skeleton assumes a cone conformation in which the chromophore moieties are arranged on the same side with respect to the mean molecular plane (*all syn* orientation). The structure of calix[4]arene derivative **4** was further corroborated by an ESI−MS spectrum showing a peak at 778.2 corresponding to the doubly charged molecular ion (see Supporting Information).

The absorption spectrum of compound **4** in neat methanol is dominated by the typical band in the visible region with maximum at ca. 400 nm of the nitroaniline derivative chromophore[19] and an absorption in the UV region mainly due to the calixarene scaffold. The molar absorbance at 400 nm was 39,000 M^{-1} cm^{-1}, a value about 4-fold larger than that of the 3-(trifluoromethyl)-4-nitrobenzenamine in the same solvent. This is in excellent agreement with the presence of four NO photoreleasing units in the same scaffold and suggests that their circular arrangement onto the calix[4]arene backbone does not significantly affect the chromophore spectral properties. Compound **4** showed low solubility in water and in 10 mM PBS (pH 7.4), but dissolved well in a MeOH/PBS solvent mixture.

DLS analyses showed that in MeOH/PBS (1:4) compound **4** (0.1 mM) forms aggregates with an average hydrodynamic diameter of 271 nm (Z average) and polydispersity index of 0.2 (Figure 1). This feature offers the benefit to further enhance the concentration of NO donor in a restricted volume.

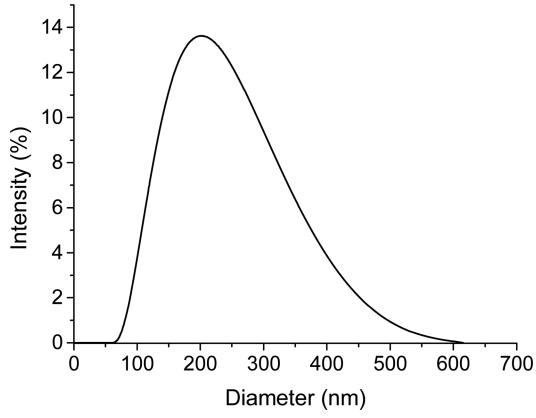


Figure 1. Intensity weighted hydrodynamic diameter distribution of the aggregates of **4** (0.1 mM) in MeOH/PBS (1:4) obtained by DLS.

The release of NO from the hydro-alcoholic solution of compound **4** was monitored by a direct detection using an ultrasensitive NO electrode. Figure 2A shows the NO release profile observed for the optically matched solution at the excitation wavelength of compound **4** and, for comparison, the model compound **5**. It can be noted that the NO release is strictly dependent on the light inputs as it starts in the presence of light, stops immediately in the dark, and starts again once the

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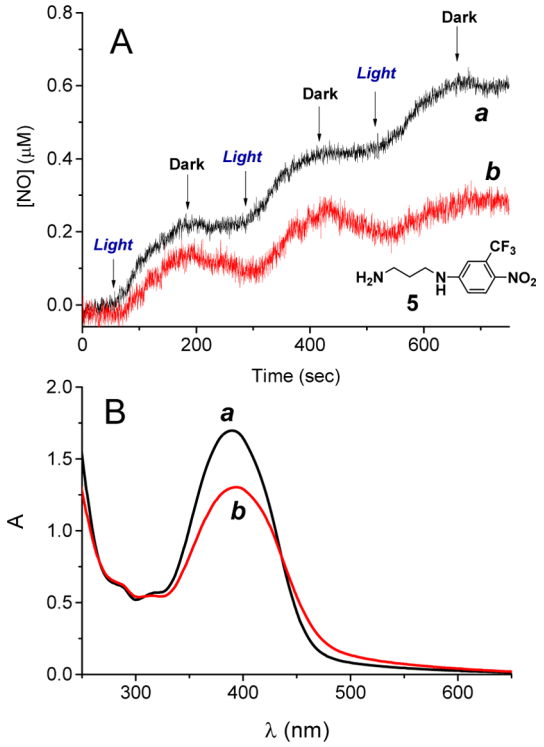

Figure 2. (A) Comparison of NO release profiles observed upon visible light irradiation (λ_{exc} = 405 nm) of optically matched MeOH/PBS (20/80) solutions of compound **4** (40 μM, a) and the model compound **5** (170 μM, b). (B) Absorption spectra of compound **4** (40 μM) before (a) and after the NO photorelease experiment (b).

light is switched on. Interestingly, the NO release efficiency of **4** is higher than that of the model compound **5**, despite the latter has a concentration ca. four times larger. Since the solutions are optically matched at the excitation wavelength, this result cannot be ascribed to a different fraction of absorbed photon by **4** with respect to **5**. Rather, an active role of the calixarene scaffold as suitable nanoreactor, which provides a low polarity environment and easily abstractable hydrogens close to the phenoxy-radical intermediate involved in the mechanism of the NO photorelease, appears the more likely. This is in excellent agreement with what we recently found in the case of the noncovalent nanoassembly between calixarene and the same type of NO photodonor.[29] Note that, in the present case, the concentration of calix is more than seven times smaller than that required by the noncovalent system, to obtain a similar amount of NO photoreleased. This is the result of the presence of the multiple NO photoreleasing units in a single scaffold that considerably increases the light harvesting properties of the whole construct.

Figure 2B shows the absorption spectra recorded before and after the NO photorelease experiment in the case of compound **4**. The spectral changes observed show a photobleaching of the visible band with a negligible shift in the absorption maximum. This spectral behavior is in very good agreement with the photochemical pathway leading to the NO release previously proposed in the case of the single NO photodonor unit and rules out the occurrence of significant side-reactions competitive with the NO release.

The biocide activity of compound **4** was investigated against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 10536, selected as specimens of Gram-positive and Gram-negative bacteria strains. Compound **4** was dissolved in a more biocompatible 10% EtOH/PBS hydro-alcoholic solvent (0.5 mM concentration). DLS analyses showed that also in this condition compound **4** formed nanoaggregates with diameter of 241 nm (Z average) and polydispersity index of 0.13. The images in Figure 3 show that the dark the hydro-alcoholic

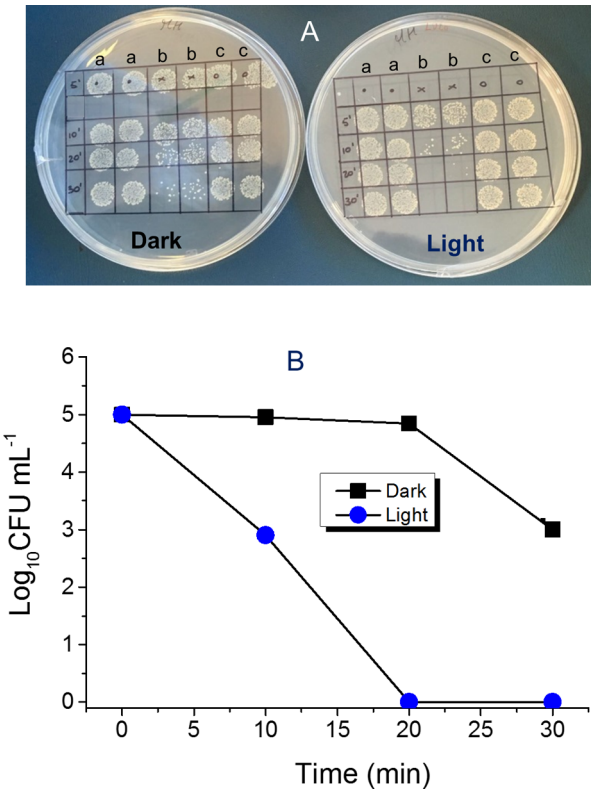

Figure 3. (A) Representative images for the antibacterial effect of a solution of compound **4** on *S. aureus* in the dark (left) and under illumination (right) at different times. Samples in duplicate: (a) control in PBS; (b) compound **4** (0.25 mM, 5% EtOH/PBS); (c) control in 5% EtOH/PBS. (B) Time-inhibition curves of *S. aureus* growth treated with compound **4** in the dark and under visible light irradiation (λ_{exc} > 400 nm).

solution of compound **4** significantly reduced the *S. aureus* bacterial count only after 30 min (reduction of 98.9% and 1.96-log in CFU/mL), whereas the biocide effect was more effective upon visible light irradiation, in a irradiation-time dependent fashion. In particular, we observed a significant biocide effect after 10 min (reduction of 99.2% and 2.1-log in CFU/mL) to reach a decrease in CFU/mL > 99.95% (reduction >3.31-log) after 20 min irradiation.

The antibacterial activity of calix[4]arene derivative **4** in the dark is ascribable to the perturbation of the bacterial cell wall. As reported for other quaternary ammonium compounds and polycationic calix[4]arene derivatives,[32−35] compound **4** may bind the negatively charged bacterial surface by ionic and hydrophobic interactions. Differently, no significant biocide effect of compound **4** was observed against *E. coli* ATCC 10536 in the dark, but an effective decrease of 93.5% in CFU/mL (1.2-log reduction CFU/mL) was observed after 30 min of irradiation (Figure 4). This behavior agrees with the greater resistance of Gram-negative over Gram-positive bacteria to quaternary ammonium salts[36] and supports very well the role of NO as a valid alternative to conventional antibiotics and

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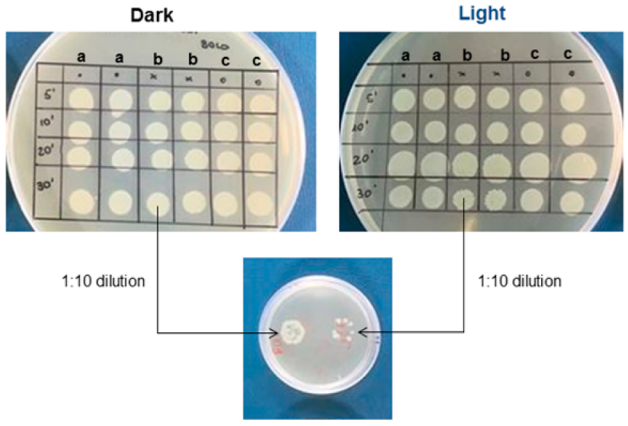

Figure 4. Representative images for the antibacterial effect of a solution of compound **4** on *E. coli* in the dark (left) and under illumination (right) at different times. Samples in duplicate: (a) control in PBS; (b) compound **4** (0.25 mM, 5% EtOH/PBS); (c) control in 5% EtOH/PBS.

disinfectants. Control experiments carried out with dermal human skin fibroblast cells as a control showed negligible (<15%) antiproliferative effect of compound **4**, under identical concentration reported in Figure 3, up to 30 min of irradiation.

The hydro-alcoholic colloidal solution of compound **4** might meet the requisites for a potentiated antiseptic and/or disinfectant. In principle, it combines the biocide properties of quaternary ammonium, phenol, alcohol, and NO.[37,38] The latter, due to its broad spectrum activity against bacteria, virus, fungi, and protozoa and inhibitory effects on the formation of bacteria biofilms,[39] is very appealing for the treatment of wounds but also for environmental biocontrol. NO also offers the advantages of a more effective biocide and multitarget mechanism of action useful to minimize the onset of resistance phenomena, other than the punctual light-controlled activation. In the prospect of pharmaceutical application, it is noteworthy that, analogously to antibacterial micellar nanoaggregates of benzalkonium or cetalkonium chlorides,[40] micellar nanoaggregates of calix[4]arene bearing multiple quaternary ammonium groups[26,29] showed low cytotoxicity against eukaryotic cells.

In conclusion, for the first time multiple units of a NO photodonor have been circularly clustered by a calix[4]arene scaffold bearing quaternary ammonium groups. This amphiphilic platform self-assembles in nanoaggregates in which the spatial arrangement of the NO donor allows an efficient light-controlled generation of NO. The released NO enhances efficacy and rate of the biocide effect of the calixarene−NO donor conjugate against *S. aureus* and triggers bactericidal activity against *E. coli*. As a result, the described construct combining the antibacterial properties of quaternary ammonium groups and NO into a single molecule represents an appealing potential alternative to conventional antibiotics for fighting bacterial resistance phenomena.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00228.

General experimental information; full synthetic procedures and characterization data for all new compounds; experimental procedure for the antibacterial and toxicity assay; NMR and ESI-MS spectra (PDF)

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Author Contributions

All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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