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RAPID COMMUNICATION

Construction of a reduction-responsive oligonucleotide via a post-modification approach utilizing 4-nitrophenyl diazomethane

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Abstract

Herein, we describe the construction of a reduction-responsive oligonucleotide by post-modification of an oligonucleotide with a diazo compound bearing a 4-nitrobenzyl group as a reduction-responsive cleavable moiety. High-performance liquid chromatography and mass spectrometry were used to reveal the introduction of a 4-nitrobenzyl group to the 5′-phosphate group of an oligonucleotide, and the subsequent reduction-triggered recovery of the original oligonucleotide. The protocol used for the preparation of this reduction-responsive oligonucleotide is simple and it will have various applications in the fields of chemical and synthetic biology.

Introduction

The installation of desirable chemical stimulus-responsive functionalities into bio(macro)molecules (e.g., nucleic acids and proteins) is important for the development of useful molecular tools in the fields of chemical and synthetic biology [1, 2]. The chemical approaches used to introduce stimulus-responsive groups into oligonucleotides (ONs) often include *pre*-modification of a stimulus-responsive moiety into a constituent monomer molecule (nucleotide) prior to the construction of a target ON. The *pre*-modification methods are generally based on chemical synthesis of the constituent monomer and the subsequent solid-phase synthesis of ONs, which enables site-selective introduction of stimuli-responsive groups into ONs. Alternatively, *post*-modification of a stimulus-responsive group after the construction of the ON has been less explored [3–10]. In fact, the majority of previously reported stimuli-responsive ONs have been constructed using *pre*-modification approaches [7–9]. Nevertheless, the chemical synthesis of long ONs is associated with technical limitations. Hence, the development of new chemistry-based *post*-modification methods for the preparation of stimuli-responsive ONs, as well as extension of the scope of existing approaches, is desirable [6].

Diazo compounds have been effectively applied in *post*-modification of bio(macro)molecules with modest to good selectivity [11, 12]. For instance, photo-caged nucleotides, such as caged adenosine triphosphate (ATP) [13], were constructed by reacting diazo compounds with phosphonate groups of the corresponding nucleotides. Subsequently, the same method was applied in a pioneering work involving the addition of diazo compounds into plasmid DNA to enable photo-controlled gene expression using photo-caged groups introduced randomly into plasmid DNA (e.g., 4,5-dimethoxy-2-nitrophenylethyl (DMNPE)-diazo or 6-bromo-4-diazomethyl-7-coumarin (Bhc)-diazo; Fig. 1a) [14–16]. More recently, Kala et al. demonstrated the selective introduction of a DMNPE group into terminal phosphate (monoester) groups instead of internal phosphodiester groups of double-stranded RNA (siRNA) using diazo compounds (**DMNPE-diazo**) [17], enabling photo-controlled RNA interference. Moreover, by employing the

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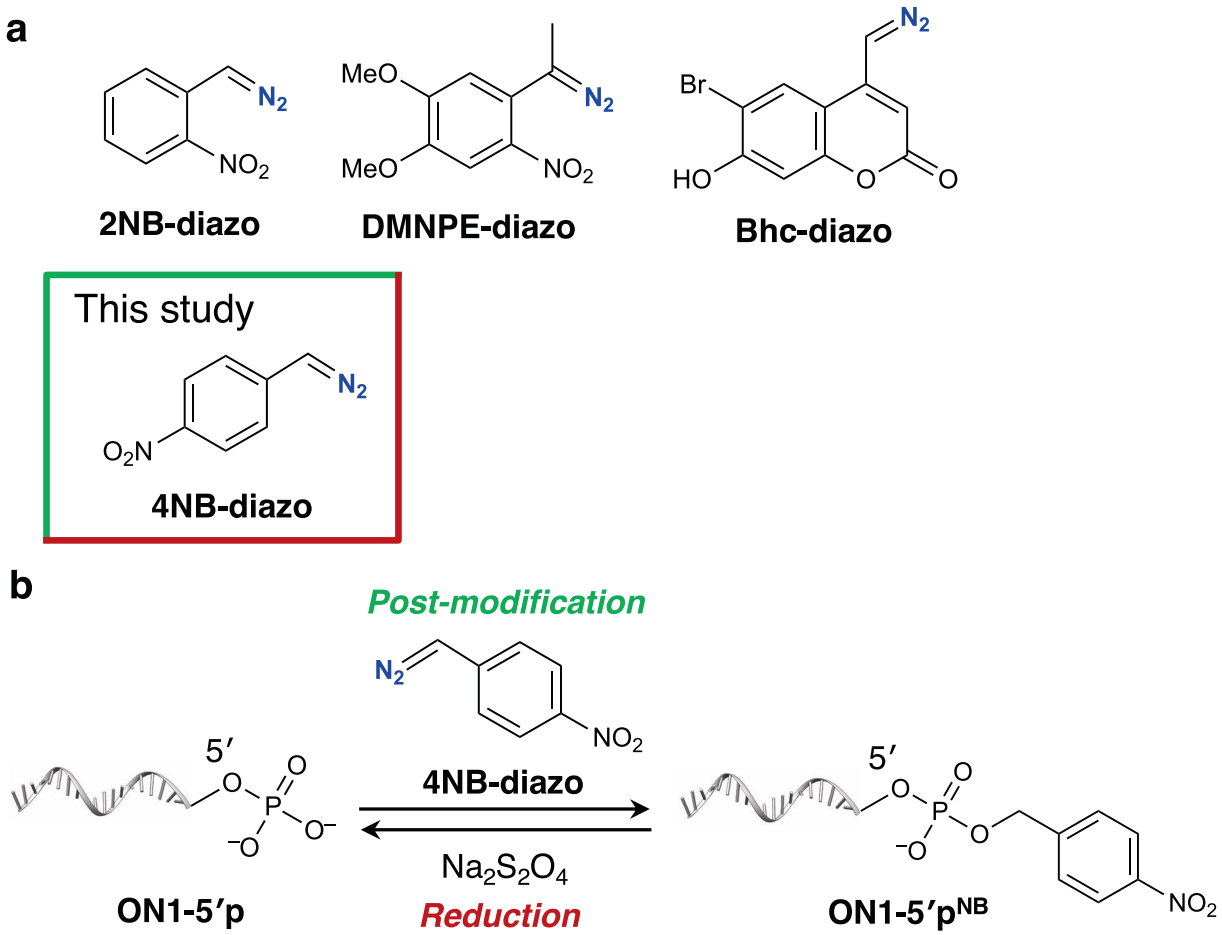

Fig. 1 **a** Chemical structures of aryl diazomethanes, including 4-nitrophenyl diazomethane (**4NB-diazo**), investigated in this study. **b** Reaction scheme showing *post*-modification of an oligonucleotide (ON) bearing a 5′-phosphate group (**ON1-5′p**) using **4NB-diazo** to introduce a reduction-responsive functionality

same method, the same group subsequently reported the installation of an additional azide handle, which could be modified via a click reaction [18]. Raines and Dennis investigated the reactivity of diazo compounds toward carboxylate and phosphate groups [19, 20] and determined that the reactivity depended on the pH as well as the presence and type of organic solvent. Therein, they putatively proposed that the nascent ion paired salt formed by the diazonium and oxo-anion species in a solvent cage by Coulombic forces plays an important role in their selective modification (*O*-alkylation) [20].

In this study, we describe the construction of a reduction-responsive ON by a *post*-modification method using a diazo compound bearing a 4-nitrobenzyl moiety (**4NB-diazo**) as the reduction-responsive cleavable group (Fig. 1b). We envisioned that this *post*-modification approach can enable the effective preparation of ON-based reduction-responsive molecular tools directly from ONs bearing phosphate groups, which can be useful in the fields of chemical and synthetic biology [1, 2, 21, 22]. To the best of our knowledge, the reactivity of this diazo compound toward biomolecules containing phosphate groups, including ONs, as well as the reduction responsiveness of the obtained ONs, has not been previously explored.

Results and discussion

To introduce the 4-nitrobenzyl (**4NB**) group as a reduction-responsive removable moiety in ONs employing a *post*-modification approach, we first prepared **4NB-diazo** (Fig. 1a) as the Bamford–Stevens reaction intermediate [23, 24] (Scheme S1). Briefly, according to a previously described method, we used a sterically hindered aromatic sulfonyl hydrazide (i.e., 2-mesitylenesulfonyl hydrazide) to synthesize the corresponding sulfonyl hydrazone precursor (Figs. S1, S2), which was converted to **4NB-diazo** using 1,1,3,3-tetramethylguanidine (TMG) as the base (**caution!** diazo compounds are presumed to be highly toxic and potentially explosive) [25]. In the present study, instead of utilizing less sterically hindered *p*-toluenesulfonyl hydrazide, **4NB-diazo** was efficiently prepared by employing

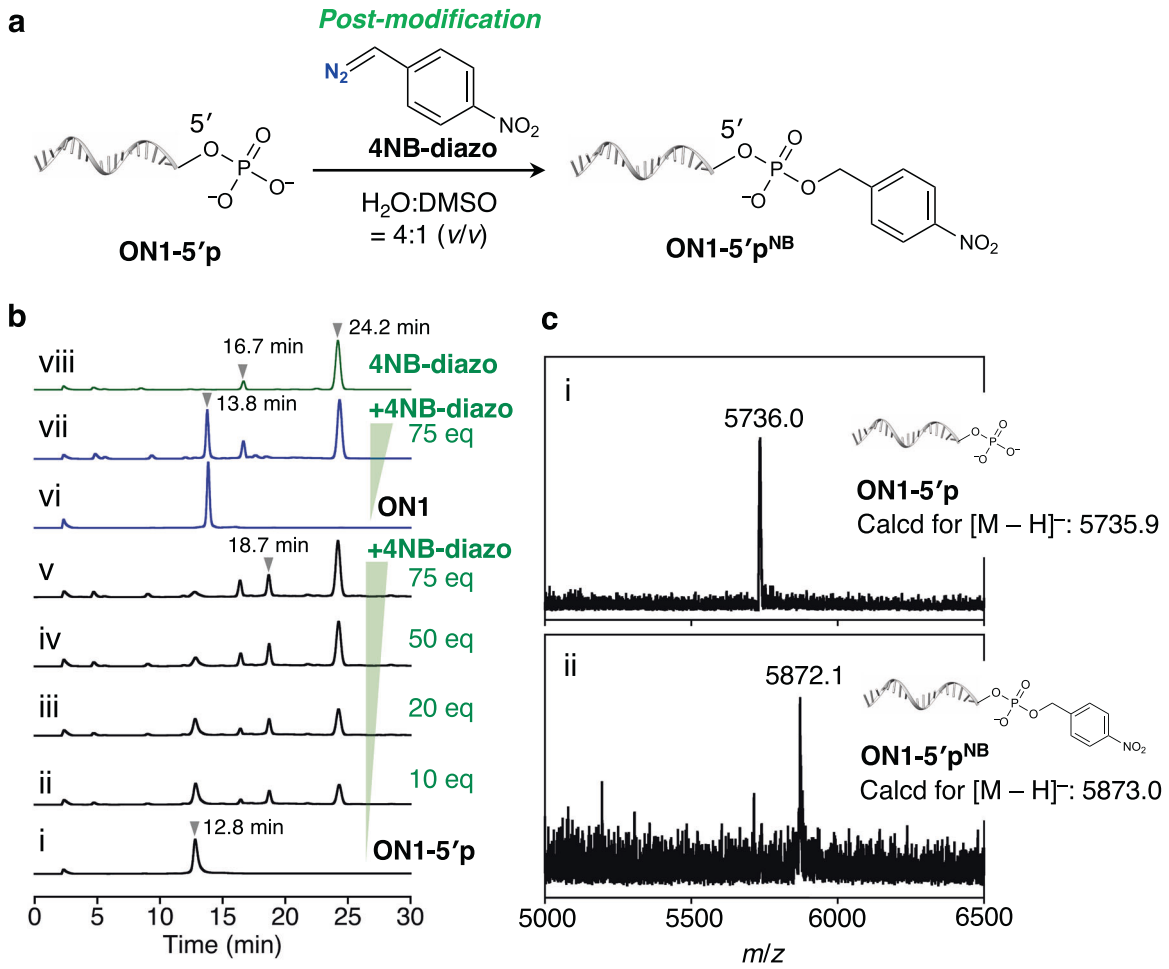

Fig. 2 **a** *Post*-modification of an ON bearing a 5′-phosphate group (**ON1-5′p**) with **4NB-diazo**. **b** Ion pair (IP) reversed-phase high-performance liquid chromatography (RP-HPLC) traces for **ON1-5′p** (i) before and (ii–v) 3 h after the addition of **4NB-diazo** (ii: 10 eq., iii: 20 eq., iv: 50 eq., and v: 75 eq.), **ON1** (vi) before and (vii) 3 h after the addition of **4NB-diazo** (75 eq.), and (viii) **4NB-diazo** without the addition of either **ON1-5′p** or **ON1** (detection wavelength = 260 nm; see Supplementary Information for the experimental details). **c** Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis of (i) **ON1-5′p** and (ii) **ON1-5′p^{NB}** (3-hydroxypicolinic acid [3-HPA] was used as the matrix in the negative detection mode). *Post*-modification reaction conditions: [**ON1-5′p**] = 63 μM, [**4NB-diazo**] = 3–19 mM (10–75 eq. against **ON1-5′p**), aqueous buffer (13 mM Tris-HCl containing 1.3 mM EDTA, pH 7.5):DMSO = 4:1 (*v*/*v*), ambient temperature

2-mesitylenesulfonyl hydrazide under milder conditions (data not shown). Owing to the instability and the potential explosive nature of the compound, the diazomethane derivative **4NB-diazo**, which was obtained as a pale brown powder, was used without further purification. Notably, ^{1}H nuclear magnetic resonance (NMR) analysis confirmed the identity and acceptable purity of **4NB-diazo** (Fig. S3).

We subsequently studied the stability of **4NB-diazo** under aqueous conditions. Because aryl diazomethane derivatives exhibit characteristic absorption bands at a wavelength of ~400 nm, monitoring the decrease in this absorbance using ultraviolet–visible spectroscopy enabled us to evaluate their stability. The half-lifetimes (τ) for **4NB-diazo** at pH 7.0 and 6.0 under aqueous conditions were evaluated to be 40 and 11 min, respectively (Fig. S4A). Rapid decomposition of **4NB-diazo** within ~10 s was noted at pH 5.0. A decrease in the half-lifetime of this compound under acidic pH is consistent with previous reports [19]. However, it is noteworthy that under the experimental conditions used in this study, the half-lifetimes for **4NB-diazo** were relatively longer than those for **2NB-diazo** (9.7 and 3.7 min at pH 7.0 and 6.0, respectively; Fig. S4B). In general, there is a tradeoff between stability and reactivity. Nevertheless, the half-lifetimes for **4NB-diazo** compared to those for **2NB-diazo** were deemed satisfactory for the subsequent investigation of the reactivity of **4NB-diazo** toward the phosphate groups in ONs.

To evaluate the *post*-modification of ONs by **4NB-diazo**, we employed an ON bearing a 5′-phosphate group (5′-pCCCTAGTTAGCCATCTCCC-3′ [19 nt]), hereafter referred to as **ON1-5′p**, where "p" denotes a phosphate group introduced at the 5′ position (Fig. 2a). The sequence

itself was not important in this study, but it was designed to contain all four bases. Various amounts of freshly prepared dimethyl sulfoxide (DMSO) stock solutions of **4NB-diazo** were added to an aqueous buffer solution of **ON1-5′p** (63 μM). Ion pair (triethylammonium acetate was used as the ion-pairing reagent, see Supplementary Information for the experimental details) reversed-phase high-performance liquid chromatography was performed to analyze the reaction mixture composed of an aqueous buffer (13 mM Tris-HCl containing 1.3 mM EDTA, pH 7.5) and DMSO in a 4:1 (*v*/*v*) ratio. As shown in Fig. 2b, the peak ($t_R = 12.8$ min) corresponding to **ON1-5′p** significantly decreased 3 h after the addition of **4NB-diazo** (50 and 75 eq.; Fig. 2b_vi, v). Concurrently, the appearance of a new peak ($t_R = 18.7$ min) was observed. On the basis of the area of the peak, the consumption of **ON1-5′p** was evaluated to be 81%. The peak at $t_R = 24.2$ min detected in the HPLC chart of the reaction mixture containing **4NB-diazo** (Fig. 2b_viii) in the absence of ONs was attributed to 4-nitrobenzyl alcohol, a hydrolyzed product of **4NB-diazo**, which was confirmed by comparison with an authentic sample of the compound (data not shown). To assign the new peak at $t_R = 18.7$ min, we conducted matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis. As shown in Fig. 2c_ii, the obtained spectrum exhibited a peak corresponding to **ON1-5′p^{NB}** (5′-p^{NB}CCCTAGTTAGC-CATCTCCC-3′). Accordingly, the HPLC peak shift from 12.8 to 18.7 min was attributed to the increased hydrophobicity of ON due to the introduction of the **4NB** group. The control experiments revealed that the ON without the 5′-phosphate moiety (**ON1**) only showed a negligible change in the HPLC chart under the same reaction conditions (Fig. 2b_vii). This result further supported our hypothesis that **4NB-diazo** mainly reacted with the

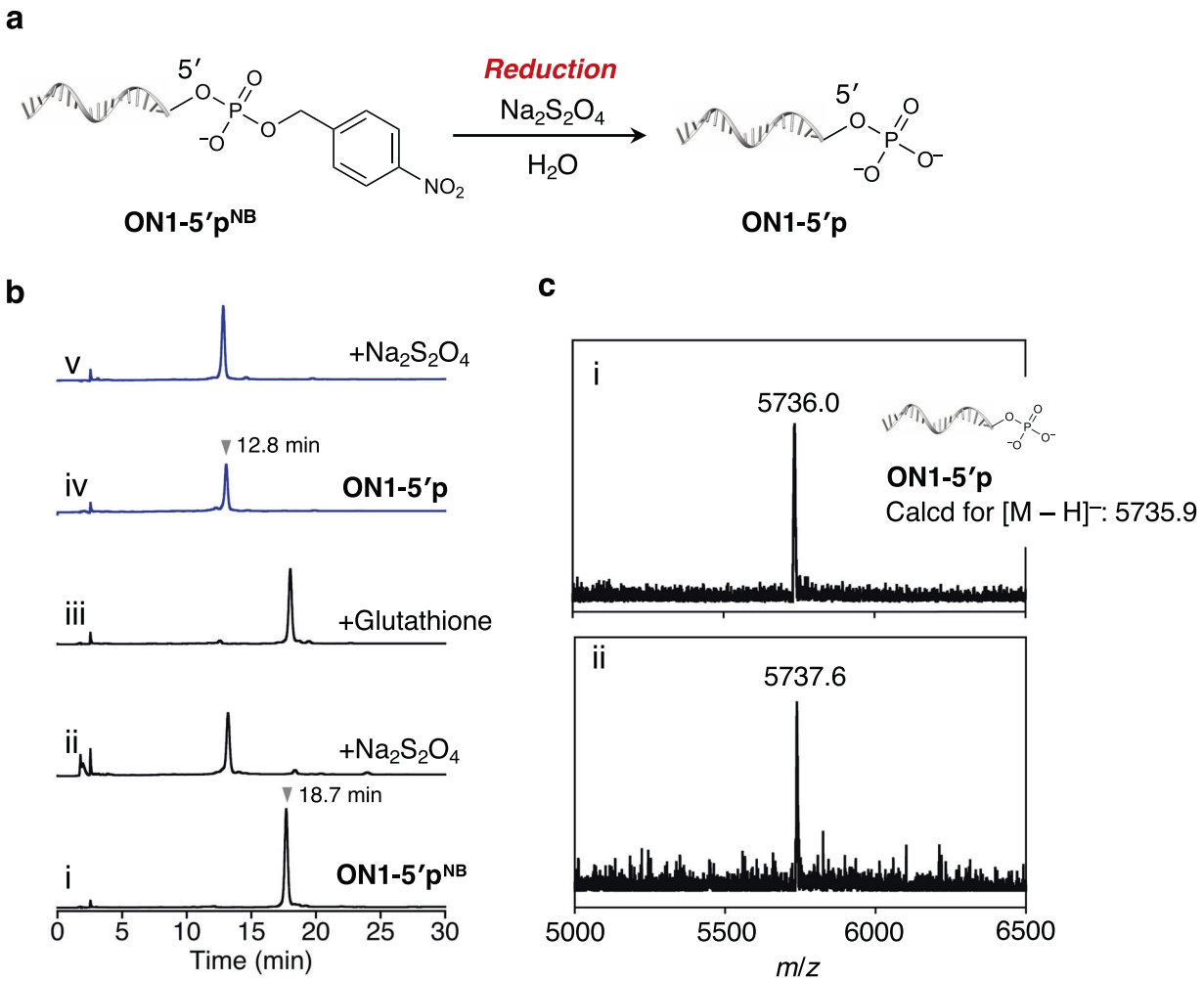

Fig. 3 **a** Reduction responsiveness of **ON1-5′p^{NB}** to regenerate **ON1-5′p**. **b** IP RP-HPLC traces for **ON1-5′p^{NB}** (i) before and 2 h after the addition of (ii) $Na_2S_2O_4$ and (iii) glutathione and **ON1-5′p** (iv) before and 2 h after the addition of (v) $Na_2S_2O_4$ (detection wavelength = 260 nm; see Supplementary Information for further details). **c** MALDI-TOF-MS spectra for (i) **ON1-5′p** and (ii) **ON1-5′p^{NB}** following the reduction reaction (3-HPA, negative mode). Reduction conditions: [**ON1-5′p^{NB}**] = 3.5 μM, [$Na_2S_2O_4$] = 7.0 mM or [glutathione] = 7.0 mM, 10 mM Tris-HCl (pH 7.5), ambient temperature

5′-phosphate group of **ON1-5′p** and not the internal phosphodiester [17], affording **ON1-5′p^{NB}**. These outcomes are comparable to the results obtained in previous studies of **2NB-diazo** [19].

We subsequently investigated the reduction-responsive propensities of **ON1-5′p^{NB}** obtained after HPLC purification (the isolated yield was evaluated to be ca. 20%). We employed $Na_2S_2O_4$ (7.0 mM as the final concentration in the medium) as the chemical reducing agent capable of reducing nitro groups [7, 8]. As shown in Fig. 3b_ii, the peak at $t_R = 18.7$ min attributed to **ON1-5′p^{NB}** almost completely disappeared within 2 h following the addition of $Na_2S_2O_4$. In addition, a new peak at $t_R = 12.8$ min, which corresponded to the original **ON1-5′p**, was detected. To assign this peak, we conducted MALDI-TOF-MS measurements, which clearly demonstrated a peak assigned to **ON1-5′p** (Fig. 3c_ii). No severe decomposition of the ONs was detected, which was evidenced by the lack of apparent new peaks in the analyzed retention time range. These outcomes were consistent with previously reported findings for $Na_2S_2O_4$-induced reduction of ONs without severe decomposition [7, 8]. Moreover, a control experiment involving the addition of the same amount of $Na_2S_2O_4$ to **ON1-5′p** (Fig. 3b_iv, v) showed no noticeable changes in the peak. As shown in Fig. 3b_iii, only marginal peak changes were observed in the HPLC chart for **ON1-5′p^{NB}** upon the addition of reduced glutathione (7.0 mM in the medium). This highlighted the selectivity of **ON1-5′p^{NB}** toward the reducing species [26]. Overall, the results obtained in the present study demonstrated that recovery of the original **ON1-5′p** can indeed be induced by the selective reduction-responsive function of **ON1-5′p^{NB}**.

Conclusion

In summary, we successfully constructed a reduction-responsive ON via a *post*-modification approach using a nitrophenyl diazomethane derivative. This *post*-modification approach is applicable to phosphate groups typically at the 5′- and/or 3′-end positions but not to internal phosphodiester groups of ONs; thus, the consequences of this approach might be selective but minimal. Nevertheless, the presence of a free 5′-phosphate group in ONs is crucial in several biological events, such as the resection of DNA ends by an exonuclease [26] and mRNA cleavage by Ago2 [27], suggesting that it might be possible to realize the reduction-responsive control of such biological events. Moreover, the developed simple protocol enabled the facile preparation of reduction-responsive ONs, which can be applied in the fields of chemical and synthetic biology. Further optimization of the process (e.g., improvement of the isolated yield) is necessary, but the described reduction-responsive functionality offers the possibility of constructing unique ON-based medicines because hypoxia-related conditions can trigger the reduction of nitroaromatics [28, 29]. Further research on this topic is currently ongoing in our laboratory.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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