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Induction of Intracellular Reductive Stress with a Photoactivatable Phosphine Probe

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§SCS-DSM Award for best poster presentation in Chemical Biology

Abstract: Reductive stress is a condition present in cells that have an increased concentration of reducing species, and it has been associated with a number of pathologies, such as neurodegenerative diseases and cancer. The tools available to study reductive stress lack both in selectivity and specific targeting and some of these shortcomings can be addressed by using photoactivatable compounds. We developed a photoactivatable phosphonium probe, which upon irradiation releases a fluorescent molecule and a trialkyphosphine. The probes can permeate through the plasma membrane and the photoreleased phosphine can induce intracellular reductive stress as proven by the detection of protein aggregates.

Keywords: Photoactivation · Protein aggregation · Reductive stress · Tributylphosphine

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1. Introduction

1.1 *Reductive Stress*

Redox homeostasis is essential for regulating fundamental processes such as cellular signaling pathways, transcriptional and post-transcriptional activities, and protein folding.[1] Redox homeostasis can be defined as an intracellular equilibrium between oxidative and reducing species. A balanced redox environment is maintained by the reduced to oxidized ratios of redox molecular duos, such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H/NAD$^+$(P)) and glutathione (GSH/GSSG).[2,3] The reduced (GSH) and oxidized (GSSG) glutathione couple is the major redox buffer inside the cell and it is also responsible for maintaining individual redox environments needed in some organelles.[2]

For example, the cytosol needs, in general, a more reducing environment for the reversible oxidation and reduction of the cytosolic protein thiols and it has a range of GSH/GSSG of 30:1 to 100:1.[2] Mitochondria also need a more reductive potential and the reported range of GSH/GSSG is from 20:1 to 40:1. In contrast, the endoplasmic reticulum (ER) has a more oxidative environment necessary for the folding of secretory proteins through the formation of disulfide bridges.[2] Therefore, in the ER, the ratio of GSH/GSSG varies from 1:1 to 3:1 with millimolar concentrations of both components present.

When there is a shift in this balance, oxidative or reductive stress can occur, and these processes are linked to many pathologies, such as diabetes, inflammation, and neurodegenerative diseases.[4,5] Whereas oxidative stress has been studied intensively,[6] there is still a need for biological tools to investigate reductive stress. Pharmacological agents, such as *N*-acetyl-L-cysteine[2] or dithiothreitol (DTT)[7] can be used to effectively induce reductive stress, but these compounds lack selectivity and spatial resolution.

1.2 *Photoactivatable Compounds*

Photoactivatable molecules (often called photoremovable or 'photocaged') are an important class of compounds as they provide spatial and temporal control for the release of a variety of chemicals.[8,9] A photoactivatable probe is composed of a photocleavable protecting group (PPG) and an inactive molecule of interest, which is either directly covalently attached to the PPG or through a functional group.[10] By irradiating at a specific wavelength, the protecting group is removed and the molecule becomes active. In 1977, Engels and Schlaeger were presumably the first to employ this strategy in a biological system by photoreleasing cyclic adenosine monophosphate (cAMP).[11] Soon after, Hoffman and co-workers described the photolytic release of adenosine 5'-triphosphate (ATP) for use in studying the Na$^+$/K$^+$ pump.[12] These reports paved the way for a new research field and, over the years, there have been numerous reports of photoactivatable bioagents such as ions,[13] neurotransmitters,[14] lipids,[15] nucleic acids,[16] peptides,[17] and proteins.[18]

In general, the properties of the photoremovable protecting group (PPG) can be adjusted based on the desired application, but a good PPG should comply with the following criteria:[8,9] A) The photoreaction should be clean and occur with a high quantum yield of release, Φ_{rel}; B) the chro-

mophore should have strong absorption at wavelengths higher than 300 nm, where there is weaker absorption of light by the biological environment and less phototoxicity; C) the PPGs should be stable and soluble in the target media (most often aqueous, in case of biological applications); D) the photochemical byproducts should not be toxic and should not absorb at the wavelength of irradiation; and E) to facilitate monitoring the photoreaction, one of the photoproducts should be fluorescent.

Using different PPGs, a multitude of functional groups can be photoactivated, such as carboxylates, phosphates, sulfonates, alcohols, and thiols.[8,19]

Photoactivatable phosphines were first described by Weiss and co-workers in the 1970s. They reported the release of a free triphenylphosphine following a photolysis reaction, at 300 nm, of either a phosphorane[20] or a triphenylphosphonium[21] compound. Peranovich *et al.* then reported that irradiation of 9-anthracenenylmethyltriphenylphosphonium chloride, at 300 nm in degassed isopropanol, produced triphenylphosphine in 25% yield.[22]

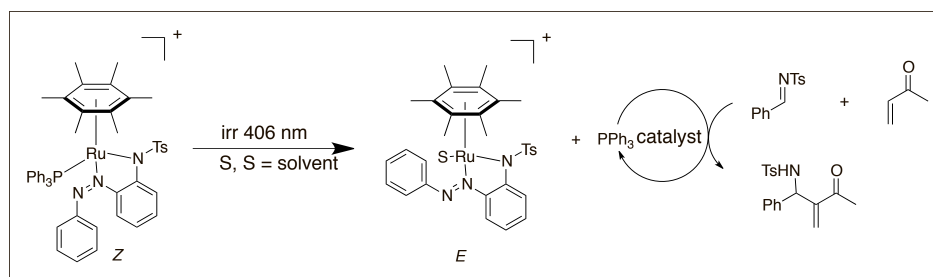
To the best of our knowledge, the first application of a photoactivatable triarylphosphine was reported in 2016 by Deo *et al.* when they released triphenylphosphine from a ruthenium-arene complex (Scheme 1).[23] The complex contained a photoswitchable *o*-tosylamide azobenzene ligand and, following irradiation with 406 nm light, changed its conformation from the *Z* to the *E* isomer. This isomerization released quantitative amounts of triphenlyphosphine, which was used as catalyst for the initiation of an aza-Morita–Baylis–Hillman reaction.

Photoactivatable triarylphosphines were described in the context of light-activated Staudinger–Bertozzi ligations.[10,24] As seen in Scheme 2, Lam and co-workers reported the cleavage of a triarylphosphine from 9-anthracenylmethyl diphenlyphosphonium chloride.[24] The released phosphinothioester was subsequently used as reagent in a Staudinger–Bertozzi ligation, which upon reaction with an azide formed an amide and triarylphosphine oxide as products.

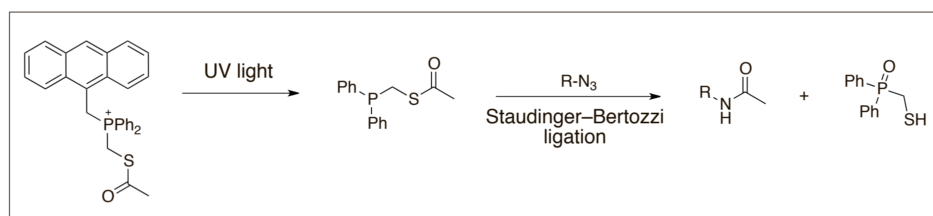
Shah *et al.* independently reported the photorelease of a triarylphosphine and its use as Staudinger–Bertozzi reagent (Scheme 3).[10] The liberated phosphine was used to label an azide-tagged glycoprotein present on the cell surface, either *in vitro*, in fixed mammalian cells, or *in vivo* employing zebrafish embryos.

All these reports focused on the photoactivation of triarylphosphines. The only literature precedence for the release of a trialkylphosphine, however, was published in 2012 by Zhuang and co-workers (Scheme 4).[25]

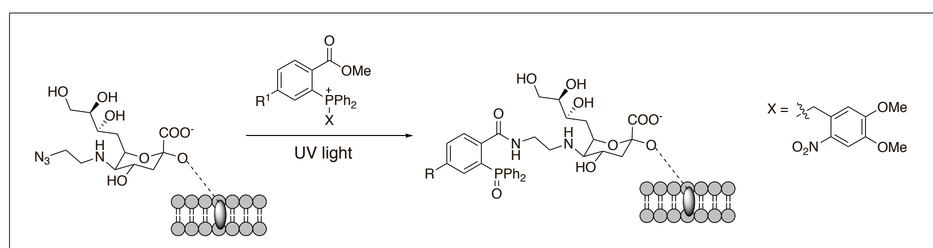
The authors described that tris(2-carboxyethyl)phosphine (TCEP), a water-soluble phosphine, quenched the fluorescence of the carbocyanine fluorophore Cy5, following the 1,4-addition of the phosphine to the polymethylene bridge of the dye. The fluorescence of the dye could be recovered after irradiation with UV light. Even though the Cy5–TCEP adduct is not thermally stable, the TCEP-induced fluorescence quenching was successfully applied in super-resolution microscopy and cell internalization assays.



Scheme 1. Photorelease of triphenylphosphine from a ruthenium complex and its subsequent use as a catalyst for an aza-Morita–Baylis–Hillman reaction.



Scheme 2. Photorelease of phosphinothioester and its use in a Staudinger–Bertozzi ligation.



Scheme 3. Photorelease of a triarylphosphine and its use as a Staudinger–Bertozzi reagent for labeling azido-tagged glycoproteins present on the cell surface.

2. Results and Discussion

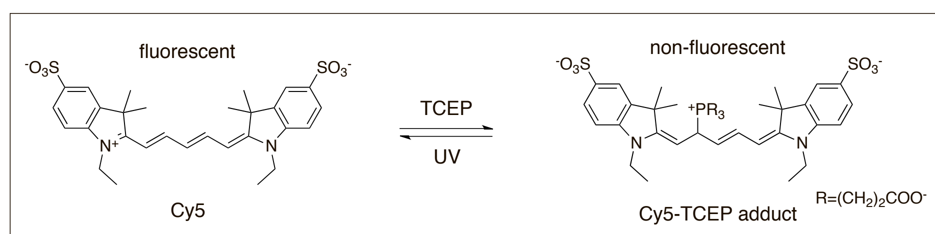
TCEP and other trialkylphosphines are known to reduce biological disulfide bonds.[26] In an intracellular environment, breaking disulfide bonds can lead to an accumulation of both reducing agents as well as misfolded proteins and the accumulation of unfolded proteins in the ER lumen has been known to induce ER reductive stress.[5,27]

The aim of our work was to develop a photoactivatable compound that upon photoactivation would release a trialkylphosphine, for inducing intracellular reductive stress, and a fluorescent reporter, for ease of monitoring, as the only photoproducts.[28]

The probe design was based on the thermally unstable Cy5–TCEP adduct and, using density functional theory (DFT) calculations, various substituents on the cyanine backbone were screened to increase the thermal barrier of the C–P bond cleavage. From this screening, we identified that a *N,N*-dimethylcoumarin–indolenine hybrid had the best predicted stability and was chosen as model compound for synthesis (Fig. 1A). We tuned the thermal stability of the probes further by varying the substituents on the indolenine core (Fig. 1B).

Probes **1a–d** were synthesized by Knoevenagel condensation of the *N,N*-diethylamino coumarin aldehyde **2** with Fischer's bases **3a–d** to result in the coumarin–carbocyanine dyes **4a–d** (Scheme 5). These dyes were subsequently treated with tributylphosphine to afford probes **1a–d**.

Absorbance and fluorescence spectra of dyes **4a–d** and probes **1a–d** were measured in phosphate buffered-saline (PBS). Dyes **4a–d** displayed an absorbance maxima (λ_{max}) at 570 nm, whereas probes **1a–d** exhibited λ_{max} at 415 nm because addition of the phosphine interrupts the conjugation of the molecules. Cuvette experiments were performed to determine the thermal stability of the probes, in the



Scheme 4. Conjugate addition of TCEP to Cy5 forms a non-fluorescent Cy5–TCEP adduct which can be reversed with UV light. TCEP = tris(2-carboxyethyl)phosphine.

dark, in PBS that was supplemented with GSSG. The role of GSSG was to trap the released phosphine, thus avoiding re-attack on the dye. Monitoring the increase in absorbance at 570 nm, we determined the half-lives of the probes in absence of light. The results of this experiment (Table 1, column 2) confirmed our hypothesis that an electron-donating group, like methoxyl, would decrease the thermal stability of the probes, whereas an electron-withdrawing group, like trifluoromethyl, would increase their stability.

Photoactivation experiments were carried out in a fluorimeter (Table 1, column 3). Solutions of probes **1a–d** in PBS with GSSG were irradiated with 415 nm light and the increase in fluorescence was monitored at 630 nm, where the dyes **4a–d** have their emission maxima. ^{1}H and ^{31}P NMR analyses confirmed the identity of the photoproducts as the corresponding dye and tributylphosphine. Whereas probes **1a–c** underwent photoactivation, no reaction was observed for **1d**. A combination of theoretical calculations and additional photophysical experiments indicated that the phosphine was released following a photoinduced electron transfer (PeT) from the indolenine core to the coumarin moiety. The trifluoromethyl group is too electron withdrawing, which lowers the energy of the HOMO level of the indolenine, disfavoring PeT, and thus probe **1d** was not photoactivatable.

With these probes in hand, we also proved that photoactivatable phosphines are capable of reducing GSSG to increase the concentration of GSH. Photoirradiation of **1c** in presence of GSSG was monitored employing high-performance liquid chromatography (HPLC, Fig. 2). As indicated by the chromatogram, the photoreaction produced GSH from the reduction of GSSG by the released trialkylphosphine.

Because compound **1c** had the best thermal stability and good photoactivation efficiency, it was chosen for live-cell experiments. Human cervical cancer cells (HeLa) were incubated with probe **1c** for 10 minutes in imaging medium, washed, and the fluorescence was recorded in the red channel (λ_{ex} = 561 nm; λ_{em} = 605/52 BP) before and after irradiation with a 405 nm laser (Fig. 3A–C). The increase in fluorescence upon irradiation proved that the compound is membrane permeable and the photoactivation is still functional in a cellular environment.

Next, we tested the ability of the photoreleased phosphine to increase the concentration of free thiols inside the cell. For this experiment, cells were pretreated with monobromobimane (mBB), a thiol sensitive fluorescent probe.[29] Following incubation of **1c** and irradiation, we detected a significant increase in the mBB signal, which indicated the generation of intracellular reducing species which could be either the generated thiols or the released trialkylphosphine (Fig. 3D–E).[30]

As mentioned before, common reductive stress indicators are the accumulation of unfolded proteins and aggregation following disulfide bond cleavage.[27] We employed Thioflavin T (ThT), a common fluorescent stain for amyloid protein aggregates,[31] to test whether protein aggregates are formed following irradiation of **1c**. HeLa cells that were irradiated with **1c** and then treated with ThT exhibited a significantly higher ThT signal than cells that were stained with ThT and irradiated, but had not been treated with **1c** (Fig. 3F–I).

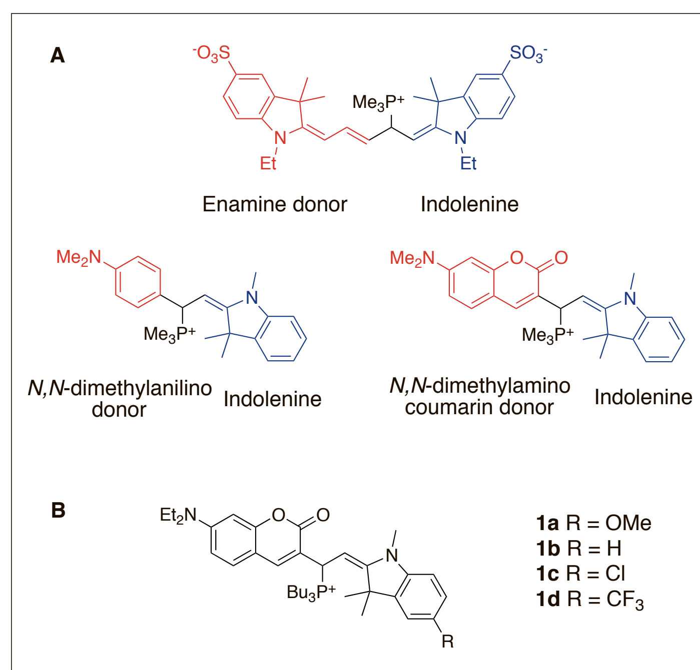
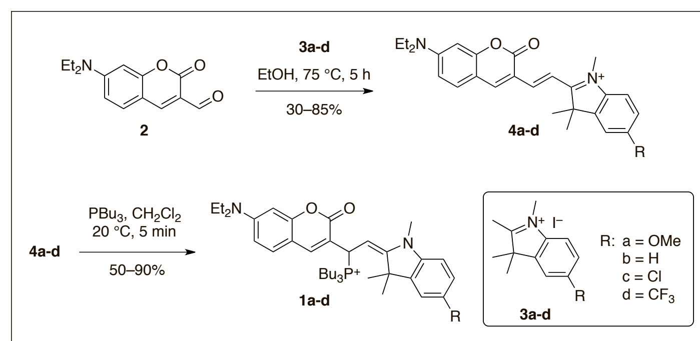


Fig. 1. New probe design based on computational modeling. A) Evolution of structures from the Cy5 core structure to the stable *N,N*-dimethylcoumarin–indolenine hybrid. B) Further tuning of the stability of the phosphonium adduct by variation of substituents on the indolenine core.

Table 1. Half-lives of phosphine release. (dark = thermal hydrolysis in absence of light; PA = hydrolysis during photoactivation with 405 nm light; n.d. = not determined).

probe	$t_{1/2\,(dark)}$ (min)	$t_{1/2\,(PA)}$ (min)
1a	9.2(2)	4(1)
1b	27.3(3)	11(2)
1c	85(2)	12(2)
1d	>120	n.d



Scheme 5. Synthesis of probes **1a–d**.

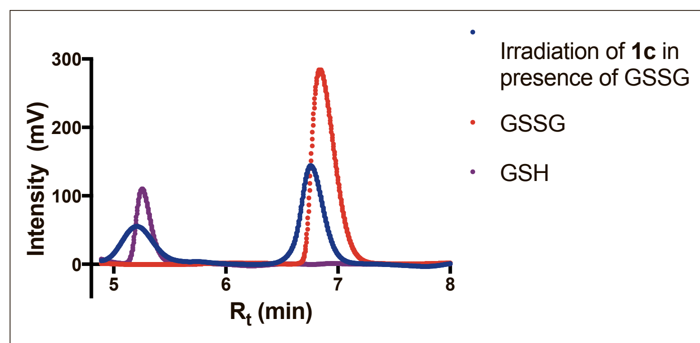


Fig. 2. *In vitro* reduction of GSSG upon photoactivation of **1c**.

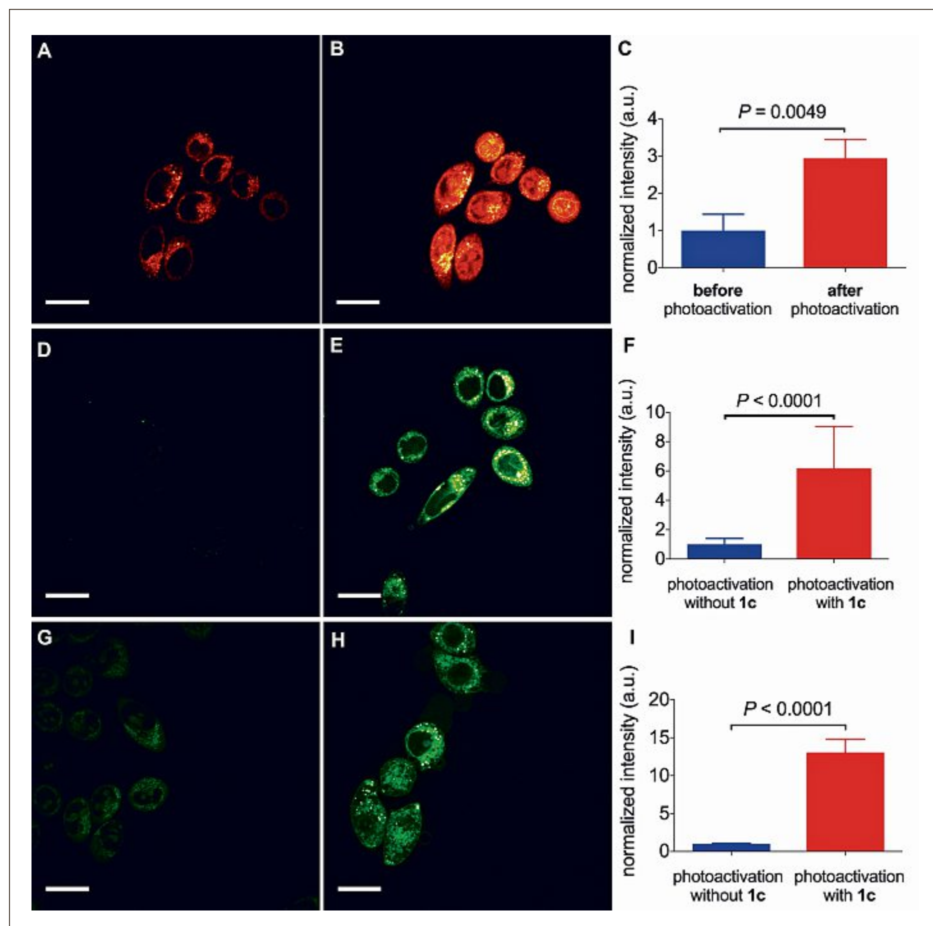


Fig. 3. Photoactivation of compound **1c** in live HeLa cells. A) Cells after 10 min incubation with **1c** (10 µM) before photoactivation. B) Same cells as in (A) after photoactivation (405 nm, 25 s). C) Quantification of the fluorescence intensity of **1c** in panels (A) and (B). D) Cells treated only with mBB (50 µM) and photoactivated. E) Generation of intracellular reducing agents in cells treated with mBB (50 µM) and **1c** (10 µM) after photoactivation. F) Quantification of the fluorescence intensity of mBB in panels (D) and (E). G) Cells treated with ThT (5 µM) and photoactivated. H) Generation of intracellular protein aggregates in cells treated with **1c** (10 µM) for 10 min, then photoactivated, washed, and stained with ThT (5 µM). I) Quantification of fluorescence intensity of ThT in panels (G) and (H). mBB=monobromobimane, ThT=Thioflavin; Scale bar = 10 µm.

In conclusion, a series of photoactivatable phosphonium probes were synthesized and their properties characterized. The probes are membrane permeable and can be activated with 405 nm light. Live cell photoactivation experiments resulted in an increase in intracellular reducing species and protein aggregates, which are telltale signs of reductive stress. Current work in our lab is focusing on targeting the probes to specific redox sensitive organelles, such as mitochondria and ER.

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