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Hypocrellin B-based activatable photosensitizer for specific photodynamic effects against high H_2O_2-expressing cancer cells

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A novel tumor-related biomarker, H_2O_2-activatable photosensitizer 4 focused on the 1,3-dicarbonyl enol moieties of hypocrellin B (3), was designed and synthesized. 4 had a blue-shifted absorption band compared to 3, and showed negligible photosensitizing ability without H_2O_2. However, release of 3 from 4 by reaction with H_2O_2 regenerated the photosensitizing ability. Furthermore, 4 exhibited selective and effective photocytotoxicity against high H_2O_2-expressing cancer cells upon photoirradiation with 660 nm light, which is inside the phototherapeutic window.

Photodynamic therapy (PDT), which utilizes photosensitizers and light to induce selective destruction of target tissues, is a promising therapy for diseases such as cancer due to its advantages of minimal invasiveness and high spatiotemporal selectivity.[1] However, non-specific photodamage caused by sunlight exposure to healthy tissues remains a significant issue.[2] To address this problem, the development of activatable photosensitizers, which can turn on their photosensitizing abilities by reacting with a specific biomarker expressed in the target tissues, has attracted much attention.[3] For instance, H_2O_2, a reactive oxygen species (ROS), is a potential biomarker for several diseases such as cancer,[4] since excess production of ROS can induce oxidative stress, leading to various diseases.[5] In this context, we recently reported a H_2O_2-activatable photosensitizer **1** based on the 2-naphthol moiety **2** of the enediyne antitumor antibiotic, neocarzinostatin chromophore, which can be excited with long-wavelength UV light at 365 nm (Fig. 1a).[6] The hydroxyl group of the 1,3-dicarbonyl enol moiety of **2** is masked in **1** with a H_2O_2-reactive arylboronic ester[7] through a benzyl ether link. Consequently, the photosensitizing ability of **1** is suppressed compared to **2**, with decreasing absorbance in the long-wavelength UV region due to a blue-shift of the absorption bands. In this study, we focused on hypocrellin B (**3**) as a photosensitizer since it possesses 1,3-dicarbonyl enol moieties and can be excited with longer wavelengths of light, e.g., 660 nm (Fig. 1b). Hypocrellin B (**3**) is a natural perylenequinone pigment isolated from the fungus *Shiraia bambusicola* P. Henn.,[8] and has advantages of a wide visible absorption range extending up to the red region, which is inside the phototherapeutic window (600-900 nm),[9] a high singlet oxygen ($^1O_2$) quantum yield, a low dark toxicity, and fast clearance *in vivo*.[10] In addition, it is reported that methylation of the two hydroxyl groups of the 1,3-dicarbonyl enol moieties of **3** causes a blue-shift of the absorption bands.[11] Although **3** itself can be excited by highly tissue penetrating 660 nm light, by masking the two hydroxyl groups of the 1,3-dicarbonyl enol moieties of **3** with a H_2O_2-reactive arylboronic ester through a benzyl ether link, **3** could be

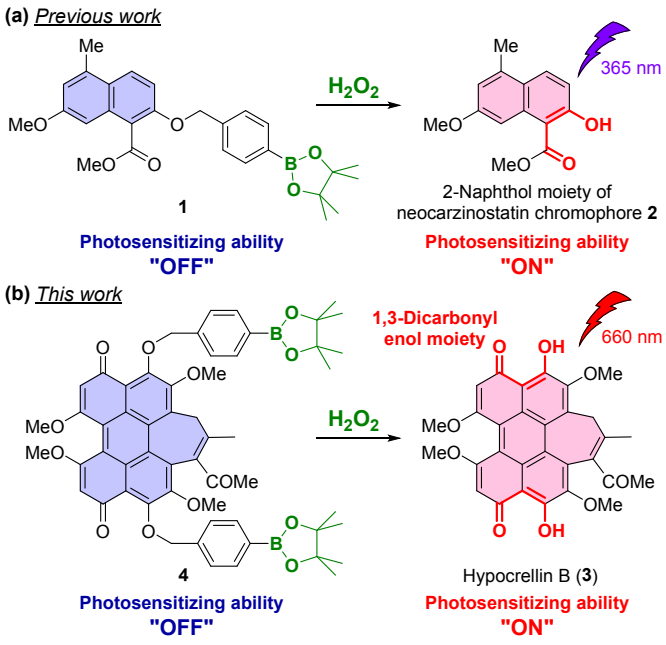

Fig. 1 Design concept of H_2O_2-activatable photosensitizer **4** and chemical structures of H_2O_2-activatable photosensitizers **1** and **4**, 2-naphthol derivative **2**, and hypocrellin B (**3**).

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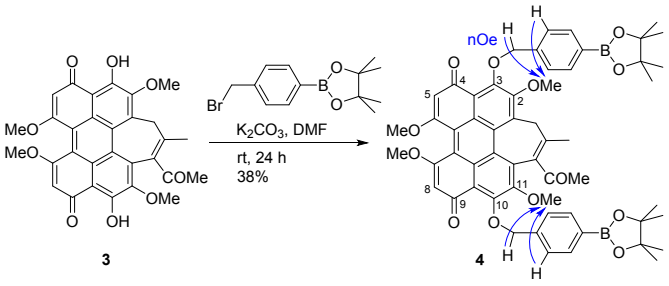

Scheme 1 Chemical synthesis of H_2O_2-activatable photosensitizer **4**. DMF = dimethylformamide.

converted to a H_2O_2-activatable photosensitizer **4**. We here present the chemical synthesis and biological evaluation of a H_2O_2-activatable photosensitizer **4** based on **3**.

The synthesis of the designed H_2O_2-activatable photosensitizer **4** was achieved by alkylation of the two phenolic hydroxyl groups of **3** with 4-(bromomethyl)phenylboronic acid pinacol ester (Scheme 1). The alkylated positions in **4** were confirmed by ^{1}H-NMR chemical shifts of H-5 and H-8, and nOe experiments. The reported ^{1}H-NMR chemical shifts of H-5 and H-8 of 3,10-dimethylated hypocrellin B are δ 6.13 and 6.12, whereas those of 4,9-dimethylated hypocrellin B are δ 6.83 and 6.82.[10b] Therefore, the ^{1}H-NMR chemical shifts of H-5 and H-8 of **4** (δ 6.16 and 6.16) were in good agreement with those of the 3,10-dimethylated derivative. In addition, nOe interactions were observed in two methoxy groups at the C-2 and C-11 positions upon irradiation of both the newly introduced benzylic and aromatic protons, indicating that the alkylated positions in **4** were the C-3 and C-10 positions, and not the C-4 and C-9 positions.

After chemical synthesis of **4**, UV/Vis spectra of **3** and **4** were measured. As shown in Fig. 2, **3** had an absorption band including the wavelength of 660 nm. On the other hand, **4** had a blue-shifted absorption band with a much lower absorbance around 660 nm compared to **3**, as expected.

To evaluate the photosensitizing abilities of **3** and **4**, photo-induced protein degradation assays[12] against bovine serum albumin (BSA) were performed under 660 nm LED irradiation (3 W, 33 mW/cm^2, and 2 h). The progress of the reaction was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3). Comparison of lanes 3 and

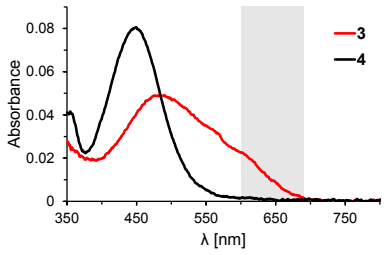

Fig. 2 UV/Vis spectra of **3** and **4** (5 μM) in 5% DMSO-PBS (pH 7.4, 10 mM). The highlighted wavelength range is the emission wavelength of the LED (660 nm) used in this study.

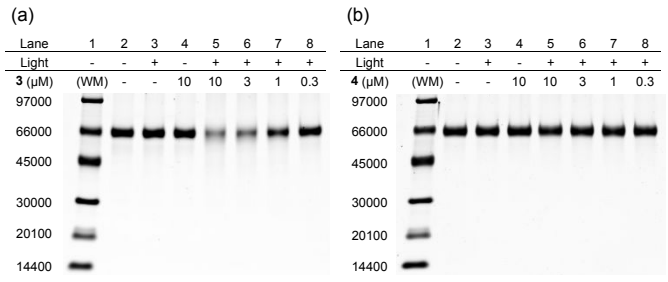

Fig. 3 Photodegradation of BSA using **3** and **4** under 660 nm light irradiation. BSA (1.5 μM) was incubated with (a) **3** and (b) **4** in 1% DMSO-PBS (pH 7.4, 10 mM) at 37 °C for 2 h under irradiation with a LED (660 nm, 3 W, and 33 mW/cm^2) placed 10 cm from the sample. The products were analyzed by tricine-SDS-PAGE. The gels were stained with Sypro Ruby protein gel stain. Lane 1: size marker; lane 2: BSA alone; lane 3: BSA upon photo-irradiation; lane 4: BSA + each compound (10 μM) without photo-irradiation; lanes 5-8: BSA + each compound (concentrations 10, 3, 1 and 0.3 μM, respectively) upon photo-irradiation.

4 with lane 2 in Figs. 3a and 3b showed that neither photo-irradiation of BSA in the absence of each compound nor treatment of BSA with each compound without photo-irradiation resulted in a change in the SDS-PAGE profile. In sharp contrast, remarkable fading of the bands corresponding to BSA was observed when BSA was treated with **3** under photo-irradiation conditions (lanes 5 and 6 in Fig. 3a), indicating that degradation of BSA took place. On the other hand, no significant degradation of BSA was observed when BSA was treated with **4** under photo-irradiation conditions (lanes 5-8 in Fig. 3b). These results showed that the photodegradation ability of **4** was significantly decreased compared to that of **3**, while **3** exhibited effective photodegradation ability against BSA upon photo-irradiation without any additives at neutral conditions.

To quantitatively evaluate the photosensitizing abilities of **3** and **4**, their $^1O_2$-generating abilities were measured using 1,3-diphenylisobenzofuran (DPBF) as a singlet oxygen scavenger.[13] A significant decrease in the absorption of DPBF at 410 nm was observed when DPBF was incubated with **3** upon 660 nm light irradiation (Fig. 4a). In contrast, no decrease was observed upon photo-irradiation when using **4** (Fig. 4b). Similar phenomena on the $^1O_2$-generating abilities were

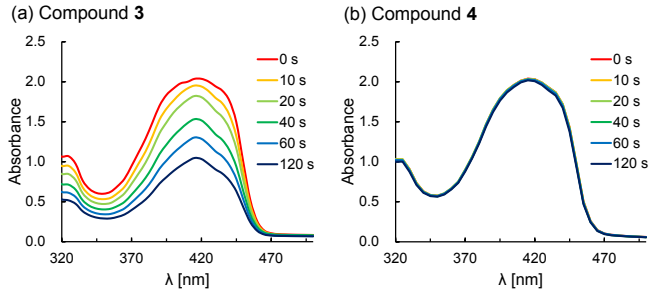

Fig. 4 Time-dependent changes in UV/Vis spectra of 1,3-diphenylisobenzofuran (DPBF) with **3** and **4** upon photo-irradiation. DPBF (500 μM) was incubated with (a) **3** and (b) **4** (5 μM) in 80% DMSO-PBS (pH 7.4, 10 mM) at rt for 0-120 s under irradiation with a LED (660 nm, 3 W, and 10 mW/cm^2) placed 20 cm from the sample.

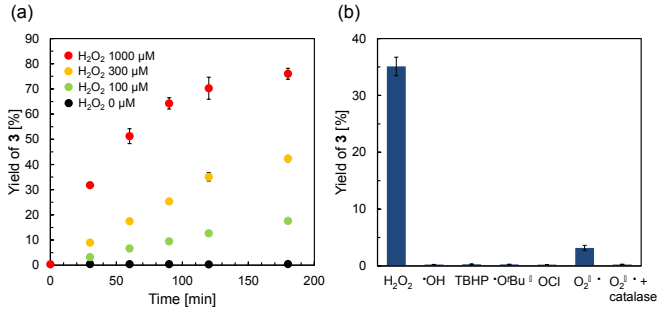

Fig. 5 (a) Responses of **4** to H_2O_2. **4** (20 μM) was incubated with H_2O_2 (0-1000 μM) in 5% DMSO-PBS (pH 7.4, 10 mM) at 37 °C for 0-180 min. The products were analyzed by HPLC/UV. (b) Responses of **4** to several reactive oxygen species (ROS). **4** (20 μM) was incubated with the indicated ROS (300 μM) in 5% DMSO-PBS (pH 7.4, 10 mM) at 37 °C for 120 min. The products were analyzed by HPLC/UV.

observed in electron paramagnetic resonance (EPR) experiments (see the ESI,† Fig. S1). These results indicated that the $^1O_2$-generating ability of **4** upon irradiation at 660 nm was effectively suppressed in comparison with that of **3**, which was consistent with the photo-induced BSA degradation results (Fig. 3). One of the plausible reasons for the differences in the photosensitizing abilities of **3** and **4** is the low absorbance of **4** at 660 nm, indicating that **4** cannot be readily excited to the S_1 state under 660 nm light irradiation.

Next, the reactivity of **4** against H_2O_2 was evaluated under neutral aqueous conditions. Progress of the reaction of **4** (20 μM) with H_2O_2 (0-1000 μM)[14] in 5% DMSO-PBS (pH 7.4, 10 mM) was monitored by HPLC/UV analysis. Compound **4** reacted with H_2O_2 and released **3** in H_2O_2 dose- and time-dependent manners, while **4** was stable in the absence of H_2O_2 (Fig. 5a). Additionally, the selectivity of **4** for H_2O_2 over other biologically relevant ROS species, such as hydroxyl radical (•OH), *tert*-butyl hydroperoxide (TBHP), *tert*-butoxy radical (•O^{t}Bu), hypochlorite ($^-$OCl), and superoxide anion ($O_2^{-\bullet}$), was evaluated. Fig. 5b shows that **4** responded to H_2O_2 with high selectivity. The apparent response to $O_2^{-\bullet}$ could be attributed to H_2O_2 produced from dismutation, because **4** showed a negligible response to $O_2^{-\bullet}$ in the presence of catalase, a H_2O_2 scavenger.[15] These results indicated that **4** selectively and effectively reacted with H_2O_2, and simultaneously released **3** under neutral aqueous conditions.

To confirm whether the $^1O_2$-generating ability of **3** was regenerated after reaction of **4** with H_2O_2, the DPBF assay was again conducted. A significant decrease in the absorption of DPBF was observed upon photo-irradiation when using **4** incubated with H_2O_2 (Fig. 6b), while no decrease was observed upon photo-irradiation when using the blank incubated with H_2O_2 (Fig. 6a). These results indicated that the $^1O_2$-generating ability of **3** was regenerated after reaction of **4** with H_2O_2.

Finally, to demonstrate selective photo-cytotoxicity against high H_2O_2-expressing cells, the photo-cytotoxic activity of **3** and **4** against B16F10 cells, a highly metastatic murine melanoma cell line that expresses high levels of ROS,[16] was evaluated. Normal human-lung fibroblast cells (WI-38) were used as a negative control in the assay. Cytotoxicity was tested with and without photo-irradiation using a LED (660 nm, 3 W, and 17 mW/cm^2) placed 15 cm from the sample using the MTT assay. The results are summarized in Fig. 7. Exposure of B16F10 cells and WI-38 cells to **3** or **4** without photo-irradiation resulted in no cytotoxicity against either type of cell (Figs. 7a-d). When B16F10 and WI-38 cells were treated with **3** with photo-irradiation, **3** exhibited non-selective and significant cytotoxicity against both types of cells in a dose-dependent manner (Figs. 7a and b). In sharp contrast, while **4** had negligible cytotoxicity against WI-38 cells even upon photo-irradiation, **4** showed selective and significant photo-

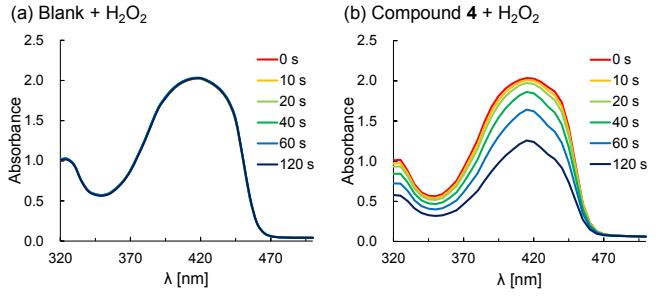

Fig. 6 Time-dependent changes in UV/Vis spectra of DPBF (a) without and (b) with **4** in the presence of H_2O_2 upon photo-irradiation. **4** (25 μM) was incubated with H_2O_2 (1 mM) in 5% DMSO-PBS (pH 7.4, 10 mM) at 37 °C for 2 h. The product was then incubated with DPBF (500 μM) in 80% DMSO-PBS (pH 7.4, 10 mM) at rt for 0-120 s under irradiation with a LED (660 nm, 3 W, and 10 mW/cm^2) placed 20 cm from the sample.

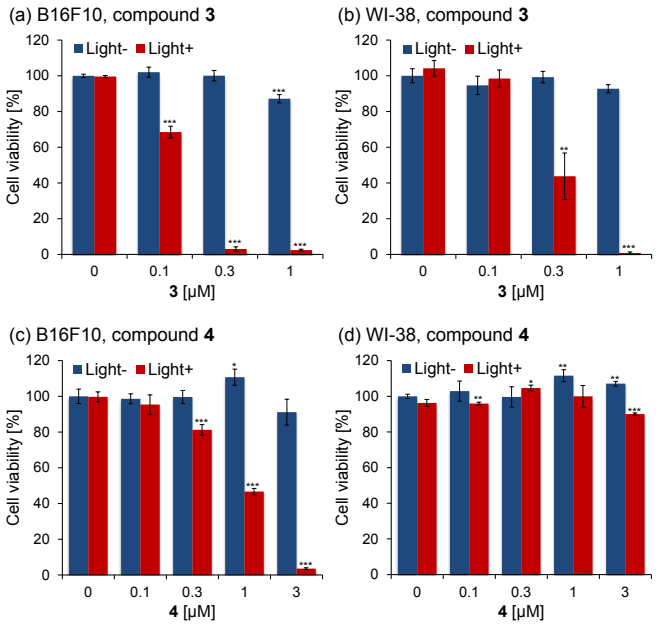

Fig. 7 Photo-cytotoxic activity of **3** and **4** against B16F10 and WI-38 cells. (a, c) B16F10 and (b, d) WI-38 cells were seeded into 96-well plates (4.0 × 10^3 cells). After 24 h, the cells were treated with the indicated concentrations of (a, b) **3** and (c, d) **4**, and incubated for 3 h at 37 °C, followed by incubation with or without photo-irradiation by a LED (660 nm, 3 W, and 17 mW/cm^2) placed 15 cm from the sample for 30 min. Samples were further incubated for 24 h at 37 °C, and then MTT reagent was added to each well and the cells were incubated for up to three additional hours. Absorbance at 540 nm was read using a plate reader. *p < 0.05, **p < 0.01, ***p < 0.001.

cytotoxicity against B16F10 cells (Figs. 7c and d). These results clearly indicated that **4** can selectively and effectively induce cytotoxicity against high H_2O_2-expressing B16F10 cells only under photo-irradiation conditions.

In conclusion, the novel H_2O_2-activatable photosensitizer **4** based on hypocrellin B (**3**) was designed and synthesized. The photosensitizing ability of **4** was significantly decreased compared to that of **3** under 660 nm light irradiation. However, release of **3** from **4** by reaction with H_2O_2 regenerated the photosensitizing ability. Cell assays showed that **4** exhibited selective and significant cytotoxicity against B16F10 cells, which overexpress H_2O_2, at low micromolar concentrations upon photo-irradiation. The results presented here will contribute to the molecular design of novel biomarker-activatable photosensitizers and help realize tumor-specific photodynamic therapy to minimize side effects.

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Author Contributions

DT and KT conceived and directed the project. TK and HN performed chemical synthesis and biological evaluation. The first draft of the manuscript was prepared by TK and DT, and the final draft was edited by all the authors.

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 (*a*) D. E. Dolmans, D. Fukumura and R. K. Jain, *Nat. Rev. Cancer*, 2003, **3**, 380; (*b*) A. P. Castano, P. Mroz and M. R. Hamblin, *Nat. Rev. Cancer*, 2006, **6**, 535; (*c*) S. Yano, S. Hirohara, M. Obata, Y. Hagiya, S. Ogura, A. Ikeda, H. Kataoka, M. Tanaka and T. Joh, *J. Photochem. Photobiol., C*, 2011, **12**, 46; (*d*) Q. Zhang, J. He, W. Yu, Y. Li, Z. Liu, B. Zhou and Y. Liu, *RSC Med. Chem.*, 2020, **11**, 427; (*e*) J. Zhang, N. Wang, Q. Li, Y. Zhou and Y. Luan, *Chem. Commun.*, 2021, **57**, 2305; (*f*) Y. Zhou, X. Ren, Z. Hou, N. Wang, Y. Jiang and Y. Luan, *Nanoscale Horiz.*, 2021, **6**, 120.

2 (*a*) A. E. O'Connor, W. M. Gallagher and A. T. Byrne, *Photochem. Photobiol.*, 2009, **85**, 1053; (*b*) G. Gunaydin, M. E. Gedik and S. Ayan, *Front. Chem.*, 2021, **9**, 691697.

3 (*a*) J. F. Lovell, T. W. Liu, J. Chen and G. Zheng, *Chem. Rev.*, 2010, **110**, 2839; (*b*) T. C. Pham, V.-N. Nguyen, Y. Choi, S. Lee and J. Yoon, *Chem. Rev.*, in press (DOI: 10.1021/acs.chemrev.1c00381).

4 (*a*) T. P. Szatrowski and C. F. Nathan, *Cancer Res.*, 1991, **51**, 794; (*b*) S. D. Lim, C. Sun, J. D. Lambeth, F. Marshall, M. Amin, L. Chung, J. A. Petros and R. S. Arnold, *Prostate*, 2005, **62**, 200; (*c*) H. Hagen, P. Marzenell, E. Jentzsch, F. Wenz, M. R. Veldwijk and A. Mokhir, *J. Med. Chem.*, 2012, **55**, 924; (*d*) M. Kita, J. Yamamoto, T. Morisaki, C. Komiya, T. Inokuma, L. Miyamoto, K. Tsuchiya, A. Shigenaga and A. Otaka, *Tetrahedron Lett.*, 2015, **56**, 4228; (*e*) J. Wang, Y. Zhang, E. Archibong, F. S. Ligler and Z. Gu, *Adv. Biosys.*, 2017, **1**, 1700084.

5 H. K. Seitz and F. Stickel, *Nat. Rev. Cancer*, 2007, **7**, 599.

6 T. Kitamura, S. Shiroshita, D. Takahashi and K. Toshima, *Chem. Eur. J.*, 2020, **26**, 14351.

7 For selected reviews, see: (*a*) D. Andina, J.-C. Leroux and P. Luciani, *Chem. Eur. J.*, 2017, **23**, 13549; (*b*) D.-J. Zheng, Y.-S. Yang and H.-L. Zhu, *Trends Anal. Chem.*, 2019, **118**, 625.

8 (*a*) H. Wu, X.-F. Lao, Q.-W. Wang, R.-R. Lu, C. Shen, F. Zhang, M. Liu and L. Jia, *J. Nat. Prod.*, 1989, **52**, 948; (*b*) T. Kishi, S. Tahara, N. Taniguchi, M. Tsuda, C. Tanaka and S. Takahashi, *Planta Med.*, 1991, **57**, 376.

9 (*a*) P. Agostinis, K. Berg, K. A. Cengel, T. H. Foster, A. W. Girotti, S. O. Gollnick, S. M. Hahn, M. R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette, B. C. Wilson and J. Golab, *CA Cancer J. Clin.*, 2011, **61**, 250; (*b*) M. Li, J. Xia, R. Tian, J. Wang, J. Fan, J. Du, S. Long, X. Song, J. W. Foley and X. Peng, *J. Am. Chem. Soc.*, 2018, **140**, 14851; (*c*) W. Lv, S. Chi, W. Feng, T. Liang, D. Song and Z. Liu, *Chem. Commun.*, 2019, **55**, 7037.

10 (*a*) Z. Diwu and J. W. Lown, *J. Photochem. Photobiol. A*, 1992, **64**, 273; (*b*) Z. Diwu and J. W. Lown, *J. Photochem. Photobiol. B*, 1993, **18**, 131; (*c*) Z.Diwu, *Photochem. Photobiol.*, 1995, **61**, 529; (*d*) E. P. Estey, K. Brown, Z. Diwu, J. Liu, J. W. Lown, G. G. Miller, R. B. Moore, J. Tulip and M. S. McPhee, *Cancer Chemother. Pharmacol.*, 1996, **37**, 343; (*e*) G. G. Miller, K. Brown, Å. M. Ballangrud, O. Barajas, Z. Xiao, J. Tulip, J. W. Lown, J. M. Leithoff, M. J. Allalunis-Turner, R. D. Mehta and R. B. Moore, *Photochem. Photobiol.*, 1997, **65**, 714; (*f*) W. Chin, W. Lau, C. Cheng and M. Olivo, *Int. J. Oncol.*, 2004, **25**, 623.

11 S. Xu, S. Chen, M. Zhang and T. Shen, *J. Org. Chem.*, 2003, **68**, 2048.

12 For selected reviews, see: (*a*) K. Toshima, *Synlett*, 2012, **23**, 2025; (*b*) K. Toshima, *Mol. BioSyst.*, 2013, **9**, 834.

13 (*a*) C. Hadjur, N. Lange, J. Rebstein, P. Monnier, H. van den Bergh and G. Wagniéres, *J. Photochem. Photobiol. B*, 1998, **45**, 170; (*b*) Y. Zhou, R. C. H. Wong, G. Dai and D. K. P. Ng, *Chem. Commun.*, 2020, **56**, 1078; (*c*) J. Sun, X. Cai, C. Wang, K. Du, W. Chen, F. Feng and S. Wang, *J. Am. Chem. Soc.*, 2021, **143**, 868.

14 It was reported that the intracellular concentrations of H_2O_2 in some cancer cells reach 10-100 μM.[4c,d] Therefore, we selected these concentrations of H_2O_2 including 100 μM in the reactivity experiments.

15 K. Koren, P. Ø. Jensen and M. Kühl, *Analyst*, 2016, **141**, 4332.

16 S. J. Park, Y. T. Kim and Y. J. Jeon, *Mol. Cells*, 2012, **33**, 363.