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Improved Photobactericidal Activity of Ultraviolet-A Light in Combination with Isomerizable *p*-Coumaric Acid Derivatives

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In order to improve the photobactericidal activity of ultraviolet-A (UV-A) mediated by reactive oxygen species (ROS), the present study focused on *trans*-coumaric acid (*trans*-CA), which is isomerized by UV-A. Generation of ROS was expected during the isomerization of *trans*-CA. *Trans*-CA derivatives, in which the carboxyl group was modified with a methyl, *n*-butyl or phenyl group, thereby changing the interaction with the cellular membrane by quenching the anionic properties of the carboxyl group and changing the UV adsorption properties, were used. The photobactericidal activities of *trans*-CA derivatives were evaluated by using UV-A light (wavelength 350 to 385 nm). The number of surviving *Escherichia coli* NBRC12713 was determined by colony-forming assay. Derivative 4c, which was esterified with a phenyl group, reduced survival by more than 5.0-log at a dose of 7.4 J/cm² and by 3.2-log at a dose of 4.9 J/cm². This synergistic activity may have been caused by the absorption of photon energy from UV-A, which is attributable to the UV spectrum of 4c. The photobactericidal activity was comparable to that of riboflavin, a known photo-activated agent. Isomerized molecules serve as a promising lead for improving the photobactericidal activity of UV-A by activating molecule-mediated ROS generation.

Key words : Photobactericidal activity / *p*-Coumaric acid derivatives / Ultraviolet-A / Isomerizable / Reactive oxygen species.

INTRODUCTION

Resveratrol is a natural phenolic compound, belonging to the stilbene family, that exists as both *trans*- and *cis*-isomers. The *cis*-isomer can be obtained from the *trans*-isomer after exposure to sunlight (Chen et al., 2007) or ultraviolet-A (UV-A) (at a wavelength of 366 nm) irradiation (Basly et al., 2000). Similarly, cinnamic acid, possessing a propenoic acid moiety on a phenyl ring, produces the *cis*-isomer by photo-isomeric conversion after irradiation with UV light (253 nm) (Chen et al., 2011). This photoinduced isomerization plays a role in cellular responses, such as that of *via* rhodopsin in vision (Birge and Hubbard, 1980) and bacteriorhodopsin in photosynthesis (Mathies et al.,

1991). In a photoactive yellow protein (PYP) from purple sulfur bacterium *Ectothiorhodospira halophila*, *trans*-coumaric acid (*trans*-CA) linked to a cysteine in the protein is the chromophore (Hoff et al., 1994). Excitation to the S₁ state of *trans*-CA, the π - π^* transition, triggers isomerization from the *trans*- to *cis*-form by absorption of photon energy during UV-A irradiation (320-400 nm) (Kort et al., 1996; Ryan et al., 2002), followed by the photo-reaction cycle of PYP. *All-trans*-retinal, the chromophore in rhodopsin and bacteriorhodopsin, in which an α , β -unsaturated carbonyl group, as well as *trans*-CA, is part of the structure, is frequently isomerized at the C₉ and C₁₃ positions by irradiation at 380 nm, and this is governed by both the π - π^* and *n*- π^* transitions (Deval and Singh, 1988).

Recently, a novel treatment approach based on photo-chemical reactions has been introduced for the control of microbial growth and viability (Chui et al.,

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2013). The photo-chemical reaction is responsible for the activation of photosensitizing agents by absorption of the appropriate wavelength, leading to the generation of reactive oxygen species (ROS) in the presence of oxygen, which disrupts the cell membrane (Girollo et al., 2009), membrane proteins (Segalla et al., 2002) or intracellular structures (Liang et al., 2013). The photosensitizing agent is initially transformed from its S_0 ground state into the S_1 excited state by both $\pi-\pi^*$ and $n-\pi^*$ transitions or either of these transitions, which may then relax to the more long-lived T_1 state. Eventually, interaction between the excited triplet state and molecular oxygen results in the production of ROS such as superoxide and hydroxyl radicals *via* electron or hydrogen transfer reactions, and singlet oxygen *via* energy transfer (Yin et al., 2013).

The present study focused on *trans*-CA, which is isomerized by UV-A light. The generation of ROS was expected during the isomerization of *trans*-CA, which is governed by the activated excitation state. Our aim was to improve the bactericidal activity of UV-A light mediated by ROS such as hydrogen peroxide and hydroxyl radicals (Hamamoto et al., 2007) *via* the utilization of photo-activated compounds, in a similar way to photobactericidal agents such as riboflavin (Liang et al., 2013), methylene blue (Girollo et al., 2009) and rose Bengal (Shrestha and Kishen, 2012). If excess ROS are produced by the combination of *trans*-CA and UV-A, viable cells can suffer extensive damage. To explore potential compounds, *trans*-CA and simple derivatives possessing photo-isomerizable moieties were used in this work. Derivatives based on *trans*-CA and its analogue *trans*-ferulic acid were synthesized in order to investigate the effects of molecular structure on antituberculosis (Yoya et al., 2009) and antimicrobial activities (Ergün et al., 2011), respectively. However, there have been (to our knowledge) few reports on the photobactericidal activity triggered by irradiation with various wavelengths. Candidates based on the *trans*-CA molecule were modified by a methyl, *n*-butyl or phenyl group at the carboxyl group in order to alter the interaction with cellular membranes by quenching the anionic properties of the carboxyl group, and to change the UV adsorption spectra. Bactericidal assays confirmed the improved bactericidal activity of UV-A light in the presence of *trans*-CA derivatives. The photo-induced bactericidal activity was compared with UV-A-activated riboflavin (Makdoui et al., 2010). The photoinduced activity of a *trans*-CA derivative and riboflavin was suppressed in the presence of catalase. In addition, the combination of the derivative and UV-A irradiation was found to enhance the generation of hydrogen peroxide when subject to fluorescence spectroscopic analysis. A light emitting diode (LED) emitting

UV-A light (350–385 nm) was used as the light source for the photo-reaction.

MATERIALS AND METHODS

Chemistry

All chemicals for the synthesis of compounds were commercially available, reagent-grade materials and were used without further purification. These reagents were purchased from commercial sources, including Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma-Aldrich Co., LLC. (St. Louis, MO), Kanto Chemical Co., Inc. (Tokyo, Japan), and Merck KGaA (Darmstadt, Germany). For syntheses requiring water-free conditions, solvents were dried using type-3A molecular sieves. Granules of sodium hydroxide were added to pyridines to ensure dryness. All reactions were monitored by thin-layer chromatography (TLC) using normal-phase (Merck silica gel 60 F_{254} , thickness 0.25 mm) and reversed-phase (Merck RP-18 F_{254S} , thickness 0.25 mm) thin-layer plates. NMR spectra were recorded with an NMR spectrometer (JEM-EX 400; JOEL, Tokyo, Japan) using tetramethylsilane as an internal standard. Purity of compounds was confirmed by TLC and by melting point analysis using a micro-melting apparatus (Thermo Fisher Scientific, Waltham, MA). Purity also was confirmed by HPLC with UV detection (Hitachi L-2000 HPLC system equipped with a COSMOSIL column (Cholest, 4.6×150 mm); Hitachi High-Technologies Corporation, Tokyo, Japan) before mass analysis. Mass spectra were recorded on an LC-MS system (Acquity UPLC-LCT Premier; Nihon Waters K.K., Tokyo, Japan). Crude compounds were purified on silica gel (silica gel 120, mesh 70–230 spherical; Nacalai Tesque Inc., Kyoto, Japan) using flash column chromatography. Except for synthesis reactions performed in aqueous solutions, all reactions were conducted under a stream of nitrogen.

Synthesis

As shown in Fig.1, two *trans*-CA derivatives (**4a-b**) were synthesized by direct modification of methyl or *n*-butyl groups. Another (**4c**) was derived by cleavage of a protective acetyl group from the respective precursors. These synthesis methods are as described in previous reports (Ergün et al., 2011; Narasimhan et al., 2004).

The identities of all synthesized compounds were confirmed by ^1H -NMR and mass spectrometry. The NMR spectra showed the *trans* (*E*) configuration, as demonstrated by the coupling constants (ranging from 15.6 to 16.9 Hz) of α -H and β -H on the double bond at the propenoic group. Purity of the synthesized

FIG. 1. Syntheses of the *trans*-CA derivatives (**4a-c**): (i) conc. H_2SO_4 , MeOH, reflux, 6 h, 79% (**4a**) or conc. H_2SO_4 , *n*-BuOH, reflux, 6 h, 76% (**4b**); (ii) acetic anhydride, pyridine, 4°C to rt, 3 h, 88% (**2**); (iii) oxalyl dichloride, phenol crystal, TEA, DMF, DCM, rt, 2 h, 86% (**3**); (iv) 35% hydrazine hydrate, AN, rt, 30 min, 80% (**4c**).

compounds, as demonstrated by TLC and HPLC, was 97% or higher. Therefore, our results showed that the synthesized *trans*-CA derivatives were the intended compounds. Physicochemical properties such as appearance, yield, m.p., R_f , and NMR and mass spectral analyses are summarized in the appendices section.

(*E*)-3-(4-acetyloxyphenyl)-2-propenoic acid (**2**)

O-Acetylation of *trans*-CA was performed with acetic anhydride in dried pyridine. Acetic anhydride (24.0 mmol, 3.0 equiv.) was added to a 4°C stirred solution of *trans*-CA **1** (8.00 mmol, 1.0 equiv.) in dried pyridine (10 mL). The solution was stirred for 3 h at room temperature and quenched with water, and the organic solvent in the reaction was evaporated. The purified target compound, **2**, was obtained by crystallization with a mixed solution of acetone and water.

(*E*)-phenyl 3-(4-acetyloxyphenyl)-2-propenoate (**3**)

Compound (**3**) was synthesized by reacting the acid chlorides of compound **2** with a phenol, as described previously (Ergün et al., 2011). DMF (1.50 mmol, 0.3 equiv.) and oxalyl dichloride (7.50 mmol, 1.5 equiv.) were added to a mixture of **2** (5.00 mmol, 1.0 equiv.) in 8 mL of dry dichloromethane (DCM). The reaction mixture was stirred for 15 min, and the solvent then was removed by thorough evaporation. The obtained acid chloride was again dissolved in 8 mL of dry DCM, and phenol crystals (10.0 mmol, 2.0 equiv.) and TEA (10.0 mmol, 2.0 equiv.) were added. The reaction was quenched with saturated ammonium chloride solution after a 2-h reaction. The DCM layer recovered from the reaction mixture was washed with brine, dried over sodium sulfate, and concentrated under reduced pres-

sure. Purification by flash chromatography on silica gel using a gradient elution with *n*-hexane/ethyl acetate (8:1) yielded the intended compound (**3**).

(*E*)-alkyl 3-(4-hydroxyphenyl)-2-propenoates (**4a-b**) and (*E*)-phenyl 3-(4-hydroxyphenyl)-2-propenoate (**4c**)

Concentrated sulfuric acid (0.20 mmol, 0.2 equiv.) was dissolved in a mixture of *trans*-CA **1** (1.00 mmol, 1.0 equiv.) and 5 mL of methanol or *n*-butanol using a previously described method (Narasimhan et al., 2004). The mixture was refluxed for 6 h and then quenched with ice cold water. After removal of the solvent, compound **4a** was purified by crystallization with a mixed solution of acetone and water. Compound **4b** was purified by flash chromatography on silica gel using a gradient elution with *n*-hexane/ethyl acetate (80:1).

As described in a previous report, hydrazine hydrate was used for cleavage of acetyl groups from compound **3** (Ergün et al., 2011). A solution of compound **3** (3.00 mmol, 1.0 equiv.) in 5 mL of AN was combined with 35% hydrazine hydrate solution (3.60 mmol, 1.2 equiv.) and stirred for 30 min. The reaction was quenched with acetic acid and water, followed by concentration by evaporation. The ethyl acetate layer was extracted from the residue and washed with brine, dried over sodium sulfate, and concentrated under reduced pressure. Purification by flash chromatography on silica gel using a gradient elution with *n*-hexane/ethyl acetate (20:1 - 15:1) yielded the intended compound (**4c**).

UV-A source and irradiation

The light source was an LED device, as described previously (Shirai et al., 2014); the spectrum and fluence rate were measured at a distance of 65 mm from the device (Fig.2). The fluence rate in the wavelength range (350 - 385 nm; peak wavelength: 365 nm) emitted by this device was 4.09 mW/cm². Total fluence dose was calculated based on the fluence rate and exposure time.

In vitro assay for photobactericidal activity

Bactericidal activity against *Escherichia coli* NBRC 12713 was determined by counting the number of colony-forming units (CFUs), as reported previously (Shirai et al., 2014); the bacterial suspension (2.0×10^7 cells/mL) was prepared as described in the same report. The suspension was distributed at 0.1 mL per well in 48-well culture plates (AGC Tecno Glass Co., Ltd., Tokyo, Japan) containing (per well) 0.01 mL of the test compound (10 mM in 80% DMSO) and 0.89 mL of sterile water. Plates were then incubated at 30°C with UV-A irradiation or with no irradiation (in a dark room).

FIG. 2. Emission spectrum of UV-A-LED used in this study. This UV-A-LED provides a spectral maximum at 365 nm. Fluence rate is 4.09 mW/cm² at a distance of 65-mm between the illumination source and UV meter.

Aliquots (0.15 mL from each well) were subjected to 10-fold dilution with SCDLP broth (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), followed by serial dilution with 0.8% (w/v) physiological saline containing 0.7% (w/w) Tween 80. Each serial dilution was plated on SCDLP agar (Nihon Pharmaceutical Co., Ltd.) plates, followed by incubation at 37°C for 48 h. Colony counts were determined and used to calculate bactericidal activity, expressed as the log survival ratio, such that the log survival ratio = $\log (N_t / N_0)$, where N_0 was the initial colony count before bactericidal treatment and N_t was the colony count after treatment for time t . *Trans*-CA and its derivatives (**4a-c**) were evaluated for their bactericidal activity, including comparison with that of riboflavin (Tokyo Chemical Industry Co., Ltd.).

In order to investigate the effects of ROS on bacterial inactivation using the combination of *trans*-CA derivatives and UV-A irradiation, a solution of catalase (from bovine liver; Wako Pure Chemical Industries Ltd.), dissolved in ion-exchange water, was added to the bacterial suspensions in a 48-well culture plate. The suspension was pre-incubated with bacteria at 37°C for 30 min before the addition of test compounds and UV-A irradiation. Catalase was added to the bacterial suspensions at final concentrations of either 100 or 200 U/well. Preliminary work (not shown) demonstrated that incubation (1 h, 37°C, in the dark) with catalase resulted in a loss of viability of less than 0.1-log. In another variation, instead of catalase, albumin (from Bovine serum, Cohn fraction V, pH 7.0; Wako Pure Chemical Industries, Ltd.) was added to the bacterial suspensions at final concentrations that were equal to the weight concentrations of catalase added.

Measurement of the UV spectrum

Solutions of *trans*-CA and derivative **4c** were prepared

with 80% (v/v) DMSO at 50 μM concentration. UV spectra were measured in a 1-cm path length cuvette with a spectrophotometer (U-3300; Hitachi Ltd., Tokyo, Japan).

Detection of hydrogen peroxide

Tested *trans*-CA and derivative **4c** were prepared at 1 mM in 80% DMSO, and then added to reaction mixtures at a final concentration of 5 μM. Hydrogen peroxide assay reaction (0.5 mL/well) was performed at room temperature in 48-well culture plates. Reaction buffer consisted of 50 mM Na⁺-K⁺ phosphate (pH 7.4) (P.B.). Hydrogen peroxide generation was investigated based on the fluorescence of a specific probe, BES-H₂O₂-Ac (measurement wavelength; λ_{ex} = 485 nm, λ_{em} = 515 nm; Wako Pure Chemical Industries, Ltd.), in response to peroxide (Shirai et al., 2014). The probe was formulated at 1.25 mM in DMSO, and was then diluted in P.B. to a reaction concentration of 5 μM in combination with the respective *trans*-CA. Following irradiation, 80-μl aliquots were transferred to 96-well black plates (Corning Inc., Corning, NY), and fluorescence was determined with a microplate reader (Infinite M200; Tecan Japan Co., Ltd, Kanagawa, Japan). For UV-A irradiation alone (no-additive) reactions, DMSO was added to a concentration of 0.4% in combination with the probe. The positive control consisted of hydrogen peroxide at 0.5, 1.0, 3.0 and 5.0 mM. As an antioxidant, L(+)-ascorbic acid (Kanto Chemical Co., Inc.) was added at a concentration of 40 mM to each reaction mixture in order to quench peroxide. As a positive control, antioxidant concentration was set at 5 mM.

Statistical analysis

All experiments were performed as three independent procedures, and the results are presented as means with error bars showing standard deviation. Inferential analysis was performed using a two-tailed, unpaired Student's *t*-test. *P* values of <0.05 were considered to be significant.

RESULTS AND DISCUSSION

Bactericidal activity

A combination of either parental *trans*-CA, its derivatives (**4a-c**) or riboflavin and UV-A irradiation was tested for bactericidal activity against *E. coli* using initial cell counts of 10⁶ CFU/mL. Test compounds were added to concentrations of 100 μM. Total illumination with UV-A (7.4 J/cm²) in the absence of exogenous compounds reduced viable cell counts by 3.6-log (Fig.3 (A)). Illumination in the presence of *trans*-CA yielded decreases in viable counts similar to those seen with illumination alone with increasing fluence doses

FIG. 3. Fluence dose-dependent changes in inactivation effects on *E. coli* after treatment with no-additive (A), *trans*-CA (B), **4a** (C), **4b** (D), **4c** (E), or riboflavin (F) in combination with (unshaded) or without (shaded) UV-A irradiation. Initial cells were suspended at densities of 10^6 CFU/mL. Data are presented as means $\pm$ SD ($n = 3$). Significant differences (* $P < 0.05$ and ** $P < 0.01$) were compared to the viability after treatment with each UV-A dose without an additive.

(Fig.3 (B)). Thus, the combination of the parent with UV-A did not enhance the bactericidal activity of UV-A. In contrast, the combined use with the ester derivatives of *trans*-CA (**4a-c**) showed significant decreases in viable cells at a 7.4 J/cm^2 UV-A, as compared with UV-A alone ($P < 0.01$); viable cell counts decreased by over 5.0-log (Fig.3 (C-E)). Combining derivative **4c** with a UV-A exposure at 4.9 J/cm^2 provided significant reductions in viable cell number (3.2-log), as compared to 1.9-log with UV-A alone ($P < 0.01$) (Fig.3 (E)). The reduction profile was comparable to that of riboflavin, which is photo-activated at 365 nm (Fig.3 (F)), thus demonstrating that combination with derivative **4c**,

FIG. 4. Typical UV absorption spectra of $50 \mu\text{M}$ *trans*-CA (shaded) and **4c** (unshaded) in 80% DMSO (A), and their absorbances, *trans*-CA (shaded) and **4c** (unshaded), respectively, in the range of 350 to 375 nm (B).

especially among combined treatments, improved the bactericidal effects of UV-A in a synergistic manner. None of the five tested compounds exhibited bactericidal activity in the absence of UV-A irradiation for 30 min (incubation time corresponds to the irradiation time to provide a 7.4 J/cm^2 UV-A dose), yielding reduction of 0.67-log for **4b** and less than 0.16-log for the others.

Inactivation of pathogens by riboflavin, using UV or visible light, is well documented and is induced by ROS. Significant non-specific oxidation formed 8-hydroxy-deoxyguanosine in calf thymus DNA under UV irradiation at 355 nm, which is similar to the peaks in the UV absorbance spectrum of riboflavin (Kumer et al., 2004). Thus, the UV spectra of the most potent photobactericidal derivative, **4c**, and parental *trans*-CA were measured. The maximum absorbances for parental *trans*-CA and **4c** were 1.195 and 1.189, respectively, which corresponded to wavelengths of 312 and 321 nm (Fig.4 (A)). The shift toward longer wavelengths resulted from modification of the phenyl group in the parental CA. The absorbances for both CAs in the range of 350 to 370 nm, the emission range of the present UV-A LED, were extracted from Fig.4 (A) (Fig.4 (B)). There were significant differences in absorbance; the parental CA scarcely absorbed light in this range, whereas derivative **4c** absorbed it strongly. This suggests that the derivative acts as a photo-activated agent with UV-A light, thus elevating ROS production during the photosensitization process.

Proanthocyanidin irradiated at 365 nm generates hydroxyl and superoxide radicals more effectively than with visible light (Nakumura et al., 2013). ROS generation is dependent on the wavelength at which the compound shows high absorbance, thus suggesting that the photo-reaction is caused by absorbed photon energy. Simple molecules, such as **4c**, with a photoisomerizable moiety such as *trans*-CA, may therefore be promising for the development of new photosensitizers to improve the photobactericidal effects of UV-A light. In

FIG. 5. Effects of ROS on the inactivation of *E. coli* by the combination of 100 μ M of either derivative (**4c**) or riboflavin with a fluence dose of 4.9 J/cm² UV-A. Cell suspensions (initial density, 10⁶ CFU/mL) were pretreated (30 min at 37°C) with catalase (100 or 200 U/well) or with equivalent concentrations of albumin. Data are presented as means $\pm$ SD (n = 3). Significant differences are shown as $P < 0.05$ (*) and $P < 0.01$ (**).

a future study, *trans*-ferulic acid, which is an analogue of *trans*-CA, will be examined as a new photo-agent, as the compound has almost the same UV absorption properties as derivative **4c**, showing maximum absorbance (ABS = 0.958) at 325 nm (data not shown).

Effects of ROS on the bactericidal mechanism

ROS was presumed to contribute to the synergistic anti-bacterial effects observed with the combination of derivative **4c** and UV-A treatment. Production of hydrogen peroxide was estimated in the presence of derivative **4c** upon UV-A photo-activation. The role of hydrogen peroxide was investigated in *E. coli* treated by the combination of **4c** (100 μ M) and 4.9 J/cm² of UV-A, as compared with riboflavin photo-activated under the same conditions. Figure 5 shows survival ratios after the combined treatment in the presence and absence of catalase. Addition of catalase significantly attenuated bacterial cell death for the derivative **4c** from 4.2-log to 1.3-log. Exposure to an equivalent weight concentration of non-enzymatic albumin indicated high bactericidal activity ($P < 0.01$), demonstrating that attenuation was related to the enzymatic decomposition of hydrogen peroxide. The effects of these substances on bactericidal activity was identical to those in the case of riboflavin (at 100 U/well catalase, although the data are not shown, the inhibition of bactericidal activity by riboflavin was observed to be less than that of 200 U/well and the difference in log survival ratios with or without catalase was one order of magnitude). Based on these results, we believe that the presence of ROS such as hydrogen peroxide contributed to the syner-

FIG. 6. Dose-dependent changes in fluorescence levels from a specific fluorescence probe reacting with hydrogen peroxide. (A) Fluorescence intensity was measured during a 30-min reaction with hydrogen peroxide in the dark, with (shaded) or without (unshaded) L(+)-ascorbic acid. Fluorescence levels for each concentration are expressed as the a.u. of fluorescence, as compared to the intensity obtained before addition of peroxide: (unshaded) a.u. = 1.25 + 1.98 $\times$ (hydrogen peroxide conc.) ($r = 0.999$); (shaded) a.u. = 0.497 + 0.918 $\times$ (hydrogen peroxide conc.) ($r = 0.999$). (B) Fluorescence levels are shown against the total fluence dose of UV-A. Shaded or unshaded circles indicate treatment with derivative **4c**, with or without L(+)-ascorbic acid, respectively. Levels with UV-A treatment alone are shown as unshaded squares. Levels for each treatment are expressed as the a.u. of fluorescence, as compared to the values obtained from each sample before irradiation. Data are presented as means $\pm$ SD (n = 3). * $P < 0.05$ and ** $P < 0.01$, as compared to each sample before irradiation.

gistic efficacy of the combined treatment procedures.

Generation of ROS by UV-A irradiation

Photo-isomerization of *trans*-CA modified with phenyl groups (**4c**) was investigated by ¹H-NMR analysis, as described previously (Kort et al., 1996). At a dose of 7.4 J/cm² from the present UV-A device, the derivative (tested at 1 mM in DMSO-d₆) isomerized to *cis*-isomer at a ratio of 35% (data not shown). Excitation of the derivative by UV-A light may be due to the transfer of an electron to molecular oxygen in order to generate superoxide radicals, followed by hydrogen peroxide, as ROS were detected as an excited unsaturated fatty acid ester following UV-A illumination (Xia et al., 2006). Production of hydrogen peroxide was estimated in the presence of derivative **4c**, which showed the highest bactericidal activity upon UV-A photo-activation.

Reactivity of the fluorescence probe used with hydrogen peroxide depended linearly on concentration. Addition of ascorbic acid as an antioxidant interrupted fluorescence (Fig. 6 (A)). Generation of hydrogen peroxide was investigated with or without derivative **4c**. As shown in Fig. 6 (B), the combination of **4c** and UV-A light resulted in a large and significant ($P < 0.01$) increase in fluorescence levels, which were dependent

on fluence dose, thus suggesting the production of peroxide. In the dark, **4c** maintained its initial fluorescence intensity during the measurement period (data not shown). UV-A alone scarcely induced fluorescence. On irradiation in the presence of **4c**, the inclusion of antioxidant clearly suppressed the increase in fluorescence. *Trans*-CA scarcely affected the bactericidal activity of UV-A (Fig.3 (A)) and the fluorescence profile was similar to that seen with the addition of antioxidant (data not shown). The combination of derivative **4c** and UV-A clearly increased hydrogen peroxide levels in solution.

Excess ROS generation, causing oxidative damage to bacterial cellular components, may be responsible for the observed bactericidal activity. We believe that ROS production results from electron or hydrogen transfer to molecular oxygen by mediating activation of photo-excited derivatives. Specifically, **4c** might show synergy with UV-A with regard to the generation of ROS. Hydroxyl radicals are typically a short-lived and strongly oxidative species; presumably, radicals could be produced by photolysis of hydrogen peroxide. In this work, the production of hydrogen peroxide was estimated based on relative changes in fluorescence intensity. However, other major ROS such as superoxide radicals and singlet oxygen were not specifically detected by spectroscopic methods. A more direct ROS assay using the electron spin resonance method would permit such qualitative and quantitative analyses (Nakamura et al., 2013).

CONCLUSION

We explored the application of *trans*-CA and its derivatives incorporating a photo-isomerizable molecule to photo-activated sterilization. The intent was to develop new photo-activated agents to improve the bactericidal activity by UV-A-triggered photo-reactions. The bactericidal activity of *trans*-CA derivatives, including the parent, was investigated in combination with UV-A illumination. A *trans*-CA derivative (**4c**) substituted with a phenyl group exhibited significantly higher bactericidal activity when combined with UV-A exposure. The synergistic activity could be caused by ROS production after absorption of UV light energy. The suppression of bactericidal activity with the addition of catalase and the production of hydrogen peroxide, as detected by fluorescence spectroscopy, suggest that photo-activation of the derivative results in excess ROS production, which may provide a mechanism for the bactericidal activity.

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APPENDICES

(E)-3-(4-Acetyloxyphenyl)-2-propenoic acid (2)

White crystal; yield, 88.0%; m.p., 217-220°C; R_f with SiO₂-TLC (*n*-hexane : ethyl acetate = 2:1, v/v), 0.18; ¹H-NMR (400 MHz, CD₃OD, δ in ppm): 2.28 (s, 3H, CH₃), 6.51 (d, 1H, J = 15.9 Hz, ethylene α -CH), 7.18 (d, 2H, J = 8.6 Hz, 4-acetyloxyphenyl group β -CH), 7.59 (d, 1H, J = 15.6 Hz, ethylene β -H), 7.74 (d, 2H, J = 8.6 Hz, 4-acetyloxyphenyl group α -CH); ESI-MS (m/z) for C₁₁H₁₀O₄, calculated: 206.06 [M]⁺, found: 207.067 [M+H]⁺.

(E)-Phenyl 3-(4-acetyloxyphenyl)-2-propenoate (3)

White crystal; yield, 86.2%; m.p., 113-114°C; R_f with SiO₂-TLC (*n*-hexane : ethyl acetate = 4:1 v/v), 0.34; ¹H-NMR (400 MHz, CDCl₃, δ in ppm): 2.33 (s, 3H, CH₃), 6.59 (d, 1H, J = 16.0 Hz, ethylene α -CH), 7.17 (m, 4H, 4-acetyloxyphenyl group β -CH and Ar group α -CH), 7.25 (t, 1H, J = 7.3 Hz, Ar group γ -CH), 7.41 (t, 2H, J = 7.8 Hz, Ar group β -CH), 7.61 (d, 2H, J = 8.2 Hz, 4-acetyloxyphenyl group α -CH), 7.85 (d, 1H, J = 15.6 Hz, ethylene β -H); ESI-MS (m/z) for C₁₇H₁₄O₄, calculated: 282.09 [M]⁺, found: 283.103 [M+H]⁺.

(E)-Methyl 3-(4-hydroxyphenyl)-2-propenoate (4a)

White crystal; yield, 79.2%; m.p., 144-145°C; R_f with SiO₂-TLC (*n*-hexane : ethyl acetate = 2:1 v/v), 0.50; ¹H-NMR (400 MHz, DMSO-d₆, δ in ppm): 3.81 (s, 3H, CH₃), 6.30 (d, 1H, J = 16.3 Hz, ethylene α -CH), 6.86 (d, 2H, J = 8.6 Hz, 4-hydroxyphenyl group β -CH), 7.42 (d, 2H, J = 8.6 Hz, 4-hydroxyphenyl group α -CH), 7.64 (d, 1H, J = 15.9 Hz, ethylene β -H); ESI-MS (m/z) for C₁₀H₁₀O₃, calculated: 178.06 [M]⁺, found: 179.071 [M+H]⁺.

(E)-Butyl 3-(4-hydroxyphenyl)-2-propenoate (4b)

Yellowish crystal; m.p., 82-85°C; yield, 76%; R_f with SiO₂-TLC (*n*-hexane : ethyl acetate = 2:1 v/v): 0.6; ¹H-NMR (400 MHz, CDCl₃, δ in ppm): 0.96 (t, 3H, J = 7.47 Hz, *n*-butyl group CH₃), 1.43 (m, 2H, *n*-butyl group CH₂), 1.69 (m, 2H, *n*-butyl group CH₂), 4.19 (s, 2H, *n*-butyl group CH₂), 5.17 (s, 1H, OH), 6.30 (d, 1H, J = 15.9 Hz, ethylene α -CH), 6.83 (d, 2H, J = 8.6 Hz, 4-hydroxyphenyl group β -CH), 7.43 (d, 2H, J =

9.0 Hz, 4-hydroxyphenyl group β -CH), 7.62 (d, 1H, J = 15.9 Hz, ethylene β -H); ESI-MS (m/z) for $C_{13}H_{16}O_3$, calculated: 220.11 $[M]^+$, found: 221.118 $[M+H]^+$.

(E)-Phenyl 3-(4-hydroxyphenyl)-2-propenoate (4c)

White crystal; yield, 80%; m.p., 144–146°C; R_f with SiO_2 -TLC (n -hexane : ethyl acetate = 4:1 v/v), 0.21; 1H -NMR (400 MHz, CD_3OD , δ in ppm): 6.53 (d, 1H, J = 16.3 Hz, ethylene α -CH), 6.84 (d, 2H, J = 8.6 Hz, 4-hydroxyphenyl group β -CH), 7.14 (d, 2H, J = 8.2 Hz, Ar group α -CH), 7.24 (m, 1H, J = 7.5 Hz, Ar group γ -CH), 7.41 (t, 2H, J = 7.9 Hz, Ar group β -CH), 7.54 (d, 2H, J = 8.6 Hz, 4-hydroxyphenyl group α -CH), 7.80 (d, 1H, J = 15.9 Hz, ethylene β -H); ESI-MS (m/z) for $C_{15}H_{12}O_3$, calculated: 240.08 $[M]^+$, found: 241.087 $[M+H]^+$.

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