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Photo-induced oxidative damage to dissolved free amino acids by the photosensitizer polycyclic musk tonalide: Transformation kinetics and mechanisms

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ABSTRACT

Residue from the polycyclic musks (PCMs) in household and personal care products may harm human beings through skin exposure. To understand the health effects of PCMs when exposed to sunlight at molecular level, both experimental and computational methods were employed to investigate the photosensitized oxidation performance of 19 natural amino acids, the most basic unit of life. Results showed that a typical PCM, tonalide, acts as a photosensitizer to significantly increase photo-induced oxidative damage to amino acids. Both common and exceptional transformation pathways occurred during the photosensitization damage of amino acids. Experimental tests further identified the different mechanisms involved. The common transformation pathway occurred through the electron transfer from α amino-group of amino acids, accompanying with the formation of $O_2^{\bullet-}$. This pathway was controlled by the electronic density of N atom in α amino-group. The exceptional transformation pathway was identified only for five amino acids, mainly due to the reactions with reactive oxygen species, e.g. $^1O_2$ and excited triplet state molecules. Additionally, tonalide photo-induced transformation products could further accelerate the photosensitization of all amino acids with the common pathway. This study may support the protection of human health, and suggests the possible need to further restrict polycyclic musks use.

1. Introduction

Synthetic musks are ingredients used widely in household products, including perfumes, body lotion, shower gels, deodorants, hair conditioners, and sanitation products (Martinez-Giron et al., 2010; Reiner and Kannan, 2006; Struppe et al., 1997). Nitro-musks and polycyclic musks are two groups of the most widely used synthetic musks. Nitro-musks were used in the early 20th century, but have recently been shown to have active photosensitization properties (Karschuk et al., 2010; Lovell and Sanders, 1988; Parker et al., 1986; Vanhenegouwen, 1991); acute toxic and cancerigenicity (Carlsson et al., 2000; Kafferlein et al., 1998; Neamtu et al., 2000; Schnell et al., 2009; Schramm et al., 1996). As such, many countries and regions have prohibited their use. For instance, musk ambrette was withdrawn from the market due to its phototoxicity during use. In contrast, polycyclic musks are generally thought to be safe and has become alternative fragrances (Ford, 1998; Heberer et al., 1999; Regueiro et al., 2008; Santiago-Morales et al., 2012; Struppe et al., 1997). As a result, their production and use have increased rapidly with a worldwide production of approximately 6000 tons per year. Tonalide and galaxolide are two of the most dominant products, representing approximately 95% and 90% of the EU and U.S. polycyclic musk markets, respectively (Santiago-Morales et al., 2012).

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Unfortunately, polycyclic musks have been associated with ecotoxicological effects for specific organisms (Breitholtz et al., 2003; Chen et al., 2010; Skladanowski et al., 2005); endocrine disruption in humans has also been reported (Dodson et al., 2012). Ingredients containing polycyclic musks in household products, such as skin protectants, shampoo, and perfume, are easily left over skin surfaces, and may cause harm to humans (Wormuth et al., 2005). However, the potential risks risen by residual polycyclic musks on the skin of organisms and humans, especially the photosensitization properties of polycyclic musks are still currently lacking.

Solar light is a natural environmental factor. As such, it is important to validate the impact of the photo-induced transformation of polycyclic musks on human health as a result of using photosensitive ingredients in personal care products. The absorption of solar photons can induce the formation of photoexcited states in skin photosensitizers, and subsequently generate reactive oxygen species (ROSs) and other toxic photoproducts that mediate skin photooxidative stress (Wondrak et al., 2006). Several detrimental effects have been observed during the photosensitization process. For example, the photo-transformation of biomolecules can lead to apoptotic or necrotic signaling pathways and cell death (de Lucas et al., 2014). In addition, photosensitizers can accelerate the injury of cells and other organisms, and even may be associated with the development of human skin cancer (Robinson et al., 2013). All these negative effects could result from the sensitized photoalteration of critical biomolecules, as the macroscopic effects are believed to be consequences of changes at the molecular level. Some of these changes are likely due to photo-induced damages to structural proteins and amino acids.

Proteins are a significant target for oxidative cell damage. Amino acids are essential building blocks of protein, and are found in virtually every cell in human and other mammalian bodies. Since 20 amino acids are assembled in different combinations to create the tens of thousands of different proteins needed to sustain life, these natural amino acids are considered to be the basic units of life. Thus, investigating photo-induced damages to these basic units of life will help us fundamentally understand the photosensitization effects and the mechanisms of polycyclic musks on human health.

In this study, an important polycyclic musk, tonalide, was selected to study its damages to amino acids through photosensitization. Firstly, the study examined the photosensitized damage kinetics of 19 amino acids (all of the 20 natural amino acids except proline due to the inability to detect secondary amines) in the absence and presence of tonalide. Secondly, both experimental and computational methods were used to study the photosensitization mechanisms of amino acids at a molecular level. Finally, the solutions of tonalide irradiated for different period of time were collected to explore the photosensitization properties of tonalide's products. The goal of the study was to assess the risks and understand the potential pathopoiesia mechanisms of this potential organic pollutant under sunlight irradiation. In addition, the study was intended to provide a scientific basis for the early prevention and treatment of skin diseases.

2. Experimental section

2.1. Materials

Tonalide and *p*-Nitroanisole (PNA) were purchased from Adamas Reagent, Ltd. (Shanghai, China, purity > 95%). *O*-Phthalaldehyde (OPA, purity 97%) and nitro blue tetrazolium (NBT, purity 99%) were from Sengon Biotech Co., Ltd. (Shanghai, China). *N*,*N*-dimethyl-4-nitrosoaniline (RNO, analytic grade) was from AOPLLO Scientific Ltd. (Bredbury, England). N-acetylglycine (N-Gly), N(α)-acetyllysine (N-Lys), 2,4,6-trimethylphenol (TMP), imidazole and 19 amino acids were all analytical grade reagents used without further purification. The structural information and the abbreviation of 19 amino acids as well as acetyl amino acids are summarized in Table S1 in Supporting Information (SI).

Tonalide was primarily prepared in acetonitrile (HPLC grade) with a concentration of 20 mM, forming the storage solution, which was then diluted to required concentration just before the irradiation experiments. All other solutions were prepared using high purity deionized water (Millipore Corp., 18 MΩ cm); high-purity oxygen (O_2) or nitrogen (N_2) was used in some specific experiments to change the atmospheric environment of the reaction systems.

2.2. Photo-induced oxidation kinetics of amino acids

The photo-induced oxidative damage of each specific amino acid was assessed through experiments conducted in a 60 mL Pyrex glass tube reactor (diameter: 2.4 cm). The reactor was placed in a Pyrex glass cup with a double-walled cooling water jacket to keep the solution at a constant temperature throughout the experiments (Fig. S1). The reaction solution was prepared with acetonitrile and water at a 50:50 ratio, with a 30 mL volume. Photo-oxidative damage kinetics studies were performed in both acidic and alkaline solutions, with initial tonalide of 500 μM and amino acid concentrations of 50 μM, respectively. The acidic solution was maintained using a 2.5 mM phosphate ($C_{KH_2PO_4} : C_{K_2HPO_4} = 98:2$), and the alkaline solution was maintained using a 2.5 mM carbonate solution ($C_{Na_2CO_3} : C_{NaHCO_3} = 60:40$). Then, the actual pH values of the acetonitrile/water mixtures were calculated as 6.06 and 10.36, which were a little higher than the pH in pure water with the same buffer reagents (Gagliardi et al., 2007). The solution was stirred in the dark for 15 min to achieve equilibrium with respect to gas and temperature before being exposed to irradiation (dissolved oxygen was measured to be 15.6 mg L^{-1} with Winkler's method). Then, the light was turned on, and the 0.75 mL solution was sampled at different time intervals to determine amino acid concentrations. To assess photo-induced oxidative damage to amino acids under O_2 or N_2 saturated condition, the solution was sealed and bubbled with O_2 or N_2 for 20 min before the light was turned on. Samples were collected with a syringe under the positive pressure of specific gas. All experiments were repeated twice, and the average values were obtained for all studies.

A 300 W xenon lamp coupled with a sunlight simulated filter (Perfectlight, Inc., Beijing, China) was housed in one side of the reactor, and was used as the light source. The irradiance spectrum of actual and stimulated sunlight was measured with a spectrometer (USB 2000+, Ocean Optics Inc., USA). As Fig. S2 shows, the filtered light emission spectrum of xenon was similar to actual sunlight.

2.3. Quantitative and qualitative analysis methods

The apparatus and detailed methods associated with the use of high performance liquid chromatography (HPLC) and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) are included in SI.

The apparent quantum yield (ϕ_{aa}) of specific amino acid was calculated using the equation as follows (Kelly and Arnold, 2012):

$$\phi_{aa} = \frac{k_{aa}}{k_{PNA}} \sum_{\lambda} \frac{\varepsilon_{PNA}\lambda_{range}L_{\lambda}}{\varepsilon_{S}\lambda_{range}L_{\lambda}}\phi_{PNA}$$

where ϕ_{PNA} is the quantum yield of the chemical actinometer *p*-nitroanisole (PNA, $\phi_{PNA} = 0.00028$) (Dulin and Mill, 1982), k_{aa} and

k_{PNA} are the pseudo-first order rate constants for the elimination of specific amino acid and the actinometer, ε_s λ and ε_{PNA} λ are the molar absorptivities of the solution and actinometer at wavelength λ, respectively. λ_{range} is the difference between λ_n and λ_{n+1}. L_λ is the irradiance at wavelength λ, and L_λ was also determined by PNA as the actinometer.

Superoxide anions ($O_2^{\bullet-}$) were determined using 100 μM NBT (Onoue et al., 2008; Pathak and Joshi, 1984), which was reduced by $O_2^{\bullet-}$, resulting in an increased absorbance of the reaction solution at 560 nm ($Abs_{560\ nm}$). The variation of $Abs_{560\ nm}$ was analyzed using a Hengping 756PC ultra-violet spectrophotometer (Shanghai, China).

The presence of singlet oxygen ($^1O_2$) was characterized using electron spin resonance (ESR) with a Bruker A300 spectrometer at room temperature. The spin trapper was 1.36 g L^{-1} 2,2,6,6-tetramethyl-4-piperidone-N-oxyl radical (TEMPO) (Cui et al., 2012); the general instrument was set as follows: microwave power, 6.35 mW; modulation amplitude, 3 G; receiver gain, 1×10^3; time constant, 10.24 ms; sweep time, 42 s; center field, 3507 G; sweep width, 80 G. To increase detection sensitivity, a 200 W mercury lamp (Hamamatsu Corp., L9566-02), instead of a xenon lamp was used as the light source, which ensured the tonalide absorbed more photons and presented clear signals of $^1O_2$.

The photosensitization of tonalide solution evolved along with the photochemical reaction time was also evaluated, by collecting solutions irradiated for 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 min with initial concentration of 500 μM tonalide. Then, three chemical probes were respectively added to solutions collected at each time intervals as follows: 50 μM Gly was used to evaluate the oxidation towards α amino group; 100 μM TMP was used to examine the variation of excited triplet state molecules (ETMs) (Fenner et al., 2013); 50 μM RNO with 5 mM imidazole was used to study the level of $^1O_2$ by measuring the decrease of $Abs_{440\ nm}$ of the solution (Krishna et al., 1991; Pathak and Joshi, 1984). The solutions with different probes were further irradiated for 30 min, and the formation ($Abs_{440\ nm}$) or decrease (Gly and TMP) of specific probe was used to evaluate the photosensitization stress towards amino acids within every 30 min along with the photochemical reactions.

2.4. Computational methods

All quantum chemical calculations were performed using the Gaussian 09 computational software package (Frisch et al., 2009). The geometry optimization and frequency calculation of all amino acids were completed using the hybrid density functional B3LYP method with the 6–31 g(d,p) basis set; the B3LYP/6-311++g(d,p) level was used to calculate the electronic density of amino acids. The calculated results were further analyzed using Multiwfn software (Lu and Chen, 2012) to obtain the Hirshfeld electric charge of specific atom.

3. Results and discussion

3.1. Photo-induced damage of amino acids

Before photosensitization studies, the direct photolysis of amino acids were evaluated, and only tryptophan (Trp) and tyrosine (Tyr) were directly photo-degraded (Table S2), due to their absorption spectra overlapped with the spectrum of sunlight reaching the Earth's surface (λ > 290 nm) (Fig. S3s and S3t). The rate constants of direct photo-degradation of Trp in acidic, Trp and Tyr in alkaline solution were obtained as $2.55 \pm 0.03 \times 10^{-3}$, $7.33 \pm 1.11 \times 10^{-3}$ and $5.89 \pm 0.10 \times 10^{-3}$ min^{-1}, which contribute to 43.5%, 43.4% and 48.3% of the rate constant obtained in solution mixed with tonalide (Table S3). To explore the adverse effects of photo-induced transformation of tonalide on human health, 19 natural amino acids

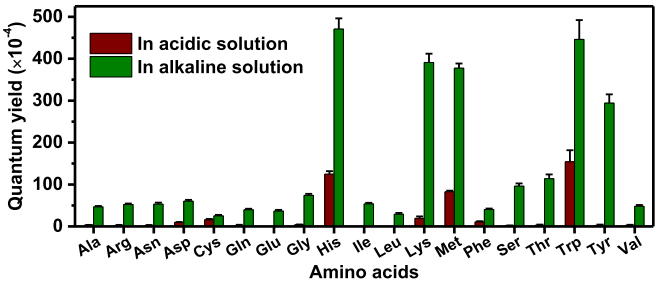

Fig. 1. The apparent quantum yields of 19 amino acids in acidic and alkaline solutions with 500 μM tonalide.

combined with tonalide were further irradiated with simulated sunlight. Herein, Fig. 1 and Table S3 summarize the quantum yields of 19 amino acids without considering the direct photolysis. For all other amino acids that cannot absorb sunlight directly because of absorption spectra below 290 nm in alkaline or acid solutions (Fig. S3c-u), it was found that photo-induced oxidative damage to these amino acids could also occur, with severe degrees in alkaline compared with acidic solution (Fig. 1 and Fig. S4). Thus, it can be concluded that natural amino acids could suffer photo-oxidation damage in the presence of tonalide, especially in alkaline solution, and regardless of whether those amino acids absorb sunlight.

This result suggests that the photo-induced oxidation of amino acids may be responsible for the photosensitization reaction of tonalide, which acts as a photosensitizer in the mixtures. As Fig. S3a shows, tonalide has an adsorption spectrum ranging from 200 to 320 nm, indicating that tonalide can absorb sunlight (λ > 290 nm) and may initiate the photochemical or/and photosensitization activity. This type of photosensitization effect could indirectly injure human skin, through skin photocarcinogenesis and photoaging (Wondrak et al., 2006). As such, exploring the photosensitization mechanism of amino acids may reveal potential negative impacts to skin.

The average photo-oxidation quantum yields of all amino acids were 22.58×10^{-3} and 3.45×10^{-3} in alkaline and acidic solutions (Table S3), respectively. This phenomenon suggests that tonalide is associated with amino acid damage through a common pH-dependent mechanism. Moreover, Fig. 1 shows that the quantum yields of several amino acids, such as His, Met and Trp, were significantly higher than others, regardless of acidity or alkalinity. This indicates that the specific reaction may also accompany the photosensitization oxidative damage of specific amino acids in the presence of the photosensitizer tonalide.

According to the previous report (Petroselli et al., 2008), the indirect reaction mediated by organic photosensitizer can occur through two different mechanisms: energy transfer reaction from the triplet state of the photosensitizer to the amino acids and the photosensitized oxidations, which can involve the generation of radicals, e.g., via electron and/or hydrogen transfer (type I mechanism), and/or the production of singlet oxygen ($^1O_2$), which is then involved in the subsequent oxidation process (type II mechanism). To understand the mechanisms behind the photosensitization effects of tonalide on amino acids, it is necessary to identify these two different transformation pathways.

3.2. Common photosensitization mechanisms

As described above, the photo-induced damage to natural amino acids when present with the photosensitizer tonalide was greater in alkaline conditions than in acidic ones. Considering the pH of human skin is influenced by skin secretions and by skin care

products, such as soap (Kottner and Surber, 2016; Takagi et al., 2015), it is very important to explore the reason why the photo-induced damage to amino acids was sensitive to ambient pH. Since the tonalide molecule lacks an ionized group, it is expected that the common photosensitization mechanisms were associated with the ionization state of amino acids, which would be changed with the pH of the solution.

Generally, amino acids have three kinds of ionization groups: α amino ($-NH_3^+$), carboxyl (−COOH), and ionization groups on the side chain (−R). Detailed reference information on the ionization properties of all tested amino acids are summarized in Table S4 (Nelson and Cox, 2008). Only 7 out of the 19 amino acid species have ionizable −R groups, indicating that the engagement of the −R group does not explain the common photosensitization mechanisms for all 19 amino acids. In addition, the pH value of the tested solution was 6.06 in the phosphorous buffer and 10.36 in the carbonate buffer, and the highest pKa value of −COOH among the 19 amino acids was only 2.38, suggests that the −COOH of each amino acid was almost fully ionized (as $-COO^-$ form) in both of the acidic and alkaline solutions. Therefore, the unchangeable $-COO^-$ part of the amino acid was also not involved in the common photosensitization mechanisms of amino acids.

The only remaining possible ionization group involved was the α amino ($-NH_3^+$) of amino acids. The pKa value of $-NH_3^+$ was between 8.80 and 10.28, indicating that all amino acids remained with their $-NH_3^+$ form in the acidic solution (pH = 6.06). However, when the solution pH increased to 10.36, $-NH_3^+$ was transformed to $-NH_2$, at ratios between 43.2% and 97.3% for 19 amino acids. The pH dependence of α amino group structure was in accordance with the increased photo-induced damage of amino acids in alkaline conditions. This asynchronous variation of $-NH_3^+$ form and oxidation quantum yields possibly imply that α amino group was the essential group involved in the common reaction mechanisms, further resulting in raising the photosensitization damage to all amino acids. In alkaline environment, the deprotonation of $-NH_3^+$ could increase the negative charge of α amino group. Accompany this variation, it was expected that the electron and hydrogen transfer reactivity of $-NH_2$ could be enhanced, and finally induced the increased oxidative damage of amino acids.

To test this assumption and further investigate the reaction mechanisms of the α amino group, glycine (Gly), as the simplest amino acid was employed as a model to depict the common sensitization mechanisms. Firstly, the electron transfer reaction occurred at the α amino group (type I mechanism) based on the measurement of $O_2^{\bullet-}$, which could be produced during the electron transfer reactions between sensitizers and some substrates, including amino acids (Gorner, 2007, 2008). With nitroblue tetrazolium (NBT) serving as the $O_2^{\bullet-}$ probe, the adsorption variation of $Abs_{560\ nm}$ which expressed the $O_2^{\bullet-}$ levels in different reaction systems was measured (Fig. 2). In the acidic solution, the adsorption variation pattern of $Abs_{560\ nm}$ in mixtures (tonalide + Gly) was similar to the solution with only tonalide. This indicates that $O_2^{\bullet-}$ was not produced by electron transfers from Gly in the acidic solution, and the type I mechanism probably did not occur. However, in the alkaline solution, the radical $O_2^{\bullet-}$ was formed in mixtures (tonalide + Gly) in comparison with solution only contain tonalide indicating that the electron transfer process occurred in the alkaline solution. Moreover, the photosensitization of tonalide could enhance the photo-induced oxidation damage of deprotonation Gly through electron transfer process, also aligned well with the finding that the amino acids were more easily photo-induced damaged in alkaline environment (Fig. 1). Therefore, it could be concluded that the type I mechanism was closely related to the common photosensitization pathway, and α amino group tend to be photosensitized in their $-NH_2$ form, with higher negative electron density.

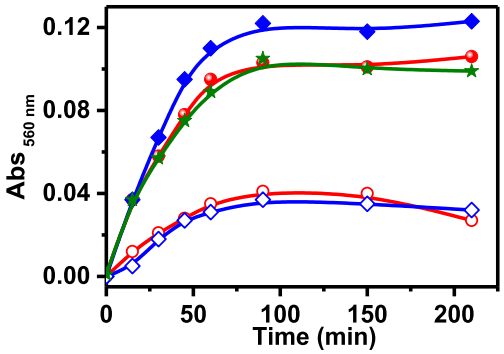

Fig. 2. The variation of $Abs_{560\ nm}$ in different solutions with 100 μM NBT in acidic solutions are expressed as a hollow shape, ○: tonalide, ◇: tonalide + Gly. Alkaline solutions are expressed as a solid shape, ●: tonalide, ◆: tonalide + Gly, ★:tonalide + N-Gly).

To confirm the relationship between N electron density and the photo-induced damage of amino acids in the presence of the photosensitizer tonalide, quantum chemistry was applied to calculate the N charge of α amino group in 19 amino acids (Table S5). For the $-NH_2$ form, the N atom was charged between −0.179 and −0.264, which were more negative than that for the $-NH_3^+$ form (−0.009 to 0.684). This result indicates the amino acids would be more sensitive to the oxidation damage raised by photosensitization in alkaline, rather than in acidic solution. Furthermore, the theoretical results suggested that the electronic density of N atom in α amino group could probably control the common photosensitization activities of amino acids. Hence, it is reasonable to conclude that amino acids could resist the photosensitization damage by tonalide when its N charge of α amino group ranged from −0.009 to 0.684.

To further confirm the common photosensitization pathways derived from α amino group, and the role of its electronic density was also considered. Gly was replaced by N-Gly, in which the electron-withdrawing acetyl group (CH_3CO-) substituting a hydrogen atom of $-NH_2$ in Gly. Thus, the N charge of α amino group in N-Gly could be significantly increased more positive than that of Gly (−0.264). The N atom in α amino group of N-Gly was calculated as −0.085, which was predicted theoretically that N-Gly is unlikely to be photochemically damaged by photosensitized tonalide through the common photosensitization pathway. To confirm this hypothesis, the photo-oxidation kinetics of N-Gly and the level of $O_2^{\bullet-}$ in N-Gly and tonalide mixtures were investigated (Fig. S5 and Fig. 2). The result shows that N-Gly was negligibly eliminated in the tonalide solution, and the variation of $Abs_{560\ nm}$ was comparable to the solution only contain tonalide. Combined with these two results, it could be concluded that an electron-withdrawing group such as CH_3CO- connected to $-NH_2$ could dramatically depress the reactivity of α amino group (Michaeli and Feitelson, 1994; Straight and Spikes, 1978). The antioxidation of N-Gly further revealed that the negative electronic density of α amino group was responsible for initiating the photosensitization oxidation of all amino acids.

In addition, the increased negative electronic density of the N atom in α amino group could induce another type I reaction mechanism: the hydrogen transfer reaction. To examine whether the hydrogen transfer occurred, the exchangeable H of α amino group was replaced by the D atom in the acetonitrile-D_2O solution (Abouelatta et al., 2009; Markle et al., 2011). Fig. S5 compares the elimination kinetics of Gly in acetonitrile-H_2O and acetonitrile-D_2O. The quantum yield of Gly in acetonitrile-D_2O was obtained as $2.70 \pm 0.24 \times 10^{-3}\ min^{-1}$, with a ratio of $\phi_{Gly,\ H_2O}/\phi_{Gly,\ D_2O} = 2.74$. The decrease of ϕ_{Gly} in D_2O indicates that the hydrogen transfers

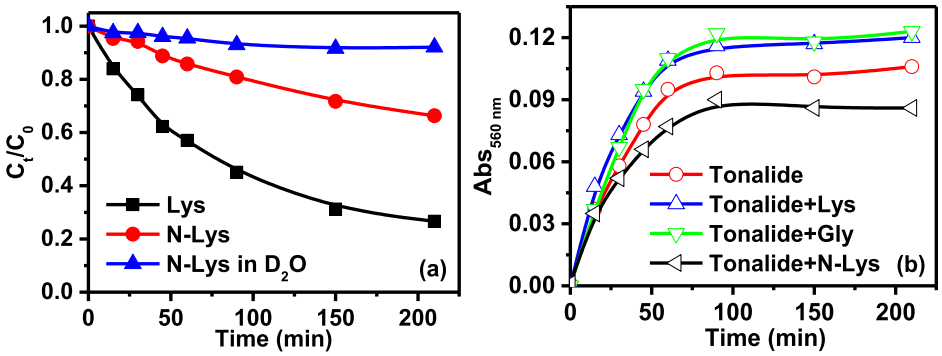

Fig. 3. (a) The elimination kinetics and (b) evolution of $Abs_{560\ nm}$ of 50 μM Lys and N-Lys in alkaline solutions with 500 μM tonalide.

also occurred during the photosensitized oxidation of α amino group. In addition, since the photo-induced damage of Gly in N_2 saturated solution was decreased as 54.1% of the air balanced condition, it indicates that the photosensitized oxidation of amino acids could not be fully attributed to the excited triplet state molecules (ETMs). Other oxidation intermediates or pathways such as the hydrogen transfer could also be involved in the common reaction mechanism, and the detailed discussion on this topic is provided in SI.

To summarize, under solar light irradiation, tonalide was first transformed into excited singlet state molecules (ESMs). Then these ESMs could react with O_2 through an electron transfer pathway, leading to the formation of radical cations and $O_2^{\bullet-}$ (Fasnacht and Blough, 2003). Furthermore, these radical cations and ESMs capture the electron from α amino group in amino acids, causing photo-induced oxidative damage to all tested amino acids. However, the photo-induced damage of Gly was not entirely inhibited in the oxygen-free condition, further confirming that the hydrogen transfer pathway occurred along with the electron transfer pathway. Both the hydrogen and electron transfer pathways were controlled by the electronic density of N atom in α amino group, and led to the common photosensitization mechanism across all tested amino acids.

3.3. Specific photosensitization mechanisms

Besides the common mechanisms, specific mechanisms were also observed during the photo-induced oxidative damage of several amino acids (Fig. 1). It shows clearly that 3 amino acids (histidine (His), methionine (Met) and Trp) in the acidic solution, and 5 amino acids (His, lysine (Lys), Met, Trp, and Tyr) in the alkaline solution, have significantly higher rate constants compared with others. This phenomenon motivated a closer look to clarify the special photosensitization mechanisms for these five amino acids. Based on a molecular structure analysis (Table S1), besides α amino group, Lys possesses another amino group in the R amino group, which of the N atom obtained negative charge was −0.247 in alkaline solution (Table S5). This value located in the electron charge ranged from −0.179 – −0.264, indicating that the R amino group may also be involved in the photo-induced damage of Lys together with α amino group. The hydrogen atom of Lys was thus further substituted by the deactivating group (CH_3CO-), namely N-Lys. The calculated charge of the N atom in −NH− of N-Lys was −0.091 (Table S5), which was close to the value of N-Gly (−0.085). This suggests that the photo-induced damage of N-Lys would completely occur in the R amino group, rather than in α amino group. As Fig. 3a shows, the photo-induced oxidation rate constant of N-Lys was significantly reduced, and $\phi_{N\text{-}Lys}$ ($9.88 \pm 0.36 \times 10^{-3}$) was only 25.3% of ϕ_{Lys} ($39.10 \pm 2.10 \times 10^{-3}$). The results indicate that the R amino group could enhance the oxidation damage of Lys, but the reactivity was still significantly lower than that of α amino group in the Lys system.

To further compare the initiating reaction mechanisms of N atom in R group with the N atom in α amino group, the electron transfer reaction mechanism was examined using the NBT probe (Fig. 3b). The comparable variation of $Abs_{560\ nm}$ of both amino acids indicates that the formation of $O_2^{\bullet-}$, a main contributor to the common pathways, occurred only from α amino group rather than R amino group of Lys. This hypothesis was further confirmed by replacing Lys with N-Lys. $O_2^{\bullet-}$ formation did not significantly decrease in the solution, indicating that it was impossible for the electron transfer to occur on the R amino group of N-Lys, so as Lys. Thus, the R amino group in Lys must react through a different mechanism, e.g. hydrogen transfer pathway. To confirm this, the photo-oxidative damage of N-Lys in acetonitrile-H_2O was evaluated and found to be faster than that in acetonitrile-D_2O (Fig. 3a), with a ratio of $\phi_{N-Lys,\ H_2O}/\phi_{N-Lys,\ D_2O} = 4.93$. The value was almost twice higher than the isotope effect obtained by Gly ($\phi_{Gly,\ H_2O}/\phi_{Gly,\ D_2O} = 2.74$), which only obtain α amino group. The difference of isotope effects confirmed that the $-NH_2$ in R and α amino group was photosensitized through different mechanisms, and the stronger isotope effects presented by Lys probably indicates the R amino group in Lys was resulted from the hydrogen transfer, rather than the electron transfer. Thus, the specific photo-induced damage mechanism of Lys is responsible for the R amino group, which reacts only through the hydrogen transfer pathway.

For other four amino acids (Met, His, Trp and Tyr), previous studies have suggested that specific photosensitization mechanisms are initiated by other ROSs in solution, e.g. $^1O_2$ (Boreen et al., 2008; Wilkinson et al., 1995). In this study, the formation of the $^1O_2$ in solution of tonalide was confirmed through ESR measurements, demonstrating the energy transfer from the ETMs of organic compounds, and the presence of $^1O_2$ in this work (Fig. S6). To further specify the roles of $^1O_2$ and ETMs, as well as other potential ROSs, the photo-induced oxidative damage kinetics of these special amino acids under specific conditions were estimated in Figs. S7a–g, and Table 1 summarizes their elimination rate constants.

A detailed discussion on the scavenging kinetic studies is provided in SI. In summary, during the photosensitization by tonalide, both $^1O_2$ and ETMs were found to contribute to the elimination of His, Met, Trp and Tyr. The exception was Trp, for which the degradation rate constant obtained with N_2 saturated condition was 11.99 and 2.19 times higher than the control group in acidic and alkaline solution, indicates the photosensitization of Trp might be dominated by ETMs. Generally, the contribution of $^1O_2$ could be estimated based on the comparisons between the control and the groups treated with NaN_3, and the results indicated that the

Table 1
The elimination rate constants of 4 amino acids photosensitized by tonalide.

Name	Buffer	Transformation rate constant ($\times 10^{-3}$ min^{-1})				
		Control	N_2 saturated	O_2 saturated	NaN_3	D_2O
His	phosphorous	2.67 ± 0.16	1.64 ± 0.09	3.06 ± 0.20	0.47 ± 0.03	13.42 ± 0.82
	carbonate	10.07 ± 0.55	7.86 ± 0.27	9.19 ± 0.41	3.55 ± 0.30	29.31 ± 1.90
Met	phosphorous	1.77 ± 0.06	1.22 ± 0.06	1.35 ± 0.06	1.01 ± 0.06	3.78 ± 0.17
	carbonate	8.10 ± 0.24	7.65 ± 0.13	5.39 ± 0.04	3.47 ± 0.08	12.33 ± 1.07
Trp[a]	phosphorous	3.31 ± 0.59	41.95 ± 2.11	0.28 ± 0.05	2.02 ± 0.20	6.29 ± 0.58
	carbonate	9.57 ± 0.99	20.91 ± 1.33	2.84 ± 0.41	4.36 ± 0.68	20.93 ± 1.50
Tyr[a,b]	phosphorous	–	–	–	–	–
	carbonate	6.31 ± 0.45	4.66 ± 0.30	5.36 ± 0.09	0.17 ± 0.04	23.54 ± 0.49

[a] The data has been corrected by deducting the degradation rate raised by direct photolysis.
[b] The elimination rate constant of Tyr was low in acidic solution (5.23×10^{-5} min^{-1}), which could be mainly contributed by the common reaction mechanism. Hence, the scavenging experiments were not performed due to the negligible contribution of ROSs.

contribution of $^1O_2$ was the greatest for Tyr, followed by His, Met and Trp. As to other potential reactive species (RSs) or transient intermediates involved in the reactions, previous reports (Bester, 2009; Lange et al., 2015) suggest that the $^1O_2$-mediated oxidation process of these amino acids could further generate a variety of RSs, including peroxide species, $^{\bullet}OH$, and photosensitizing intermediates. These RSs would also probably engage in the further oxidation damage of amino acids. In sum, the only hydrogen abstraction pathway on the R amino group of Lys, and the formation of $^1O_2$ and ETMs, contributed mostly to the specific photosensitization mechanisms of specific amino acids.

3.4. Photosensitization varied with photochemical reaction time

The complete photo-induced transformation of 500 μM tonalide was achieved within 210 min (Fig. S8). This indicated that tonalide, along with its photo-transformation products, could act as the photosensitizers during the oxidation damage of amino acids. As such, the photosensitization was also evaluated for both the parental tonalide and its photoproducts. Based on UPLC/MS/MS spectra, four photoproducts were identified with their structures summarized in Fig. S9. Due to the lack of authorized standards, it is hard to quantitatively evaluate the photosensitization of each photoproduct and the ROSs formed individually. As such, the variation of the average photosensitization ability presented by the whole tonalide solution was estimated by three specific probes: 50 μM Gly for photosensitiztion towards α amino group; 100 μM TMP for ETMs; 50 μM RNO with 5 mM imidazole for $^1O_2$. All experiments were conducted in an acidic solution, except Gly, which was tested in an alkaline solution. Then, the photosensitization of tonalide solution evolved along with the photochemical reaction time could be drawn as shown in Fig. 4.

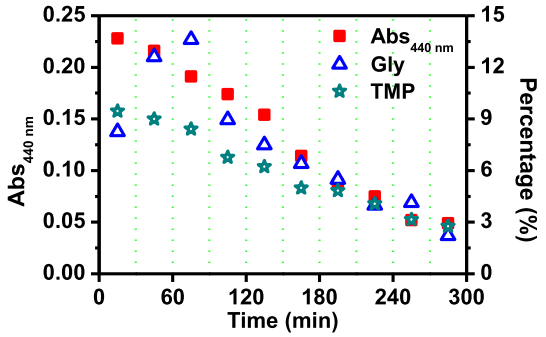

Fig. 4. The elimination of 50 μM RNO (□), 50 μM Gly (△) and 100 μM TMP (☆) within every 30 min in tonalide solutions which have been irradiated for 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 min (The initial concentration of tonalide was 500 μM. Left axis corresponds to the elimination extent of $Abs_{440\ nm}$, and right axis corresponds to the elimination efficiencies of Gly and TMP).

The elimination of $Abs_{440\ nm}$ and the constant reduction of TMP were found during the photochemical oxidative damage of tonalide, indicating that the levels of $^1O_2$ and ETMs in solution constantly decreased as the photosensitizer tonalide was eliminated. When tonalide was totally degraded after 210 min, the formation of $^1O_2$ and ETMs in solution had almost vanished. These results implied that tonalide rather than its photoproducts, yielded the reactive $^1O_2$ and ETMs, which are important RSs in the specific photosensitization mechanisms of His, Met, Trp and Tyr. As for the α amino group with the common mechanism, the electrophilic attacking ability towards α amino group first increased, and then decreased constantly after 90 min, indicating that the photoproducts formed in the middle photochemical transformation stage were highly electrophilic attackers. This result was consistent with the data in Fig. 2, where the $Abs_{560\ nm}$ predominantly increased after 30 min when the electrophilic photoproducts appeared. It was concluded that both tonalide and its photoproducts could photosensitize amino acids through common mechanisms, and some photoproducts contributed more significantly than the parental compound. However, the specific photosensitization reactions with $^1O_2$ and ETMs mainly occurred in the initial photochemical transformation stages involving tonalide rather than its transformation products.

3.5. Implications for PCM usage and skin protection

The studies above showed that both tonalide and its photoproducts could induce the photosensitization of free amino acids through both common and specific mechanisms. In addition, the generated ROSs could destroy human cells and potentially result in ROS-related diseases, such as cardiovascular and neurodegenerative diseases and even cancer (Brieger et al., 2012). The results suggest that the use of polycyclic musks as additives in personal care products may not be as safe as previously thought. Personal care products used on skin surfaces, such as perfumes, skin creams, and sunscreen cream, can protect the skin, but significant care may be needed if they include polycyclic musks. Polycyclic musk residuals on the skin surface may absorb sunlight and photosensitize amino acids, possibly damaging proteins, cellular systems, and causing human diseases.

Despite the reported results from this study, there are reasons to be optimistic. Firstly, natural sunlight strength is weaker than the xenon light adopted for the study, and the photosensitization reactions are likely to be inactive when musk is exposed to sunlight. Secondly, the pH of human plasma is approximately 7.4 (Petroselli et al., 2008), below the 10.36 as used in this study. Based on the pKa values listed in Table S4, the percentage of amino acids in the $-NH_2$

form at pH 7.4 ranged from 0.02 to 3.83%. The decrease in the number of these reactive molecules could also weaken the photosensitization effects caused by the polycyclic musk. Finally, most amino acids in cells function by becoming part of polypeptides or proteins, rather than acting as free molecules, where the amino acid is linked by a peptide bond. The carbonyl group of peptide bond could efficiently lower the negative charge of the N atom in α amino group, inhibiting the electron transfer and hydrogen transfer reactions described above. Given these factor, the common reaction mechanisms induced by polycyclic musk may in fact be negligible in daily lives.

However, despite these moderating factors, additional study is needed to more closely examine photosensitivity towards His, Met and Trp. This is important because these three amino acids can be dominantly photo-oxidized by $^1O_2$ and ETMs produced during the photochemical induction of polycyclic musks. These reactions could occur independent of the pH value. Future studies should also examine photosensitive damage to proteins when exposed to irradiated polycyclic musks, and to the cells of skin *in vivo*. Proteins containing amino acids that are sensitive to low levels of $^1O_2$ and ETMs may endure in sunlight, pointing to the need for a closer examination of the light factor.

4. Conclusions

This paper has explored the health effects of typical PCMs tonalide when exposed to sunlight, using both experimental and computational methods. The photosensitized oxidation performances of 19 natural amino acids were obtained, with the both common and exceptional transformation pathways. The major findings were:

1). PCMs tonalide residuals on the skin surface could act as a photosensitizer to significantly increase photo-induced oxidative damage to amino acids.
2). The most common transformation pathway occurred through the electron transfer from α amino-group of amino acids, accompanying with the formation of $O_2^{\bullet-}$. This pathway was controlled by the electronic density of N atom in α amino-group.
3). The exceptional transformation pathway was identified only for five amino acids, mainly due to the reactions with $^1O_2$ and excited triplet state molecules.
4). Transformation products of tonalide could further accelerate the photosensitization of all amino acids with the common pathway.
5). The use of PCMs as additives in personal care products may not be as safe as previously thought.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.03.006.

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