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Laser-induced in vitro isomerization of urocanic acid in UVA region and the origin of excited triplet state

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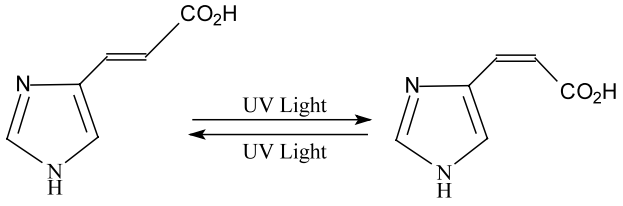
Abstract—We demonstrate here unambiguously that (*E*)-urocanic acid, an epidermal and photobiologically important chromophore, undergoes isomerization to the (*Z*) isomer upon excitation with UVA (320–400 nm) light using monochromatic 355 and 340 nm laser radiation. Additionally, chemical evidence is presented that supports the isomerization coming from the excited singlet state, thus proving unequivocally that the previously observed triplet state is populated from the excited singlet manifold via intersystem crossing rather a weak $S_0 \rightarrow T_1$ transition.

Urocanic acid (2-propenoic acid, 3-[1*H*-imidazol-4(5)-yl], UA) continues to attract interest of biologists, environmentalists, photochemists, photobiologists, medicinal chemists, and (photo)immunologists, primarily due to its presence in the upper layer of the mammalian skin and its excellent light absorbing properties.[1,2] It is synthesized in vivo from the essential amino acid histidine by the action of the enzyme histidase, a histidine-ammonia-lyase (EC 4.3.1.3), and accumulates in the epidermis in high concentration (ca. 30 mg/cm^2). Upon exposure to UV light, the naturally occurring isomer, *trans*, i.e. (*E*)-urocanic acid [(*E*)-UA], undergoes isomerization to produce *cis*, i.e. (*Z*)-urocanic acid [(*Z*)-UA]. The latter is of current immense (photo)biological interest because of its immunosuppressive properties in several animal models.[2]

Until recently, the photochemistry and photobiology of UA has been restricted to the UVB (280–320 nm) and UVC (200–280 nm) region of the electromagnetic spectrum due to its significant absorption in these regions. The most efficient photochemical reaction is (*E*)/(*Z*) isomerization (Eq. (1)), which shows a wavelength dependency.[3] Both spectroscopic[4] and theoretical[5] data support the presence of multiple electronic transitions within the broad structureless absorption band centered ca. 275 nm, and these account for the wavelength-dependent chemistry. However, there are recent reports describing UA photoreactivity in the UVA (320–400 nm) region, where it shows minimum absorption. This spectral region has become increasingly more important since more UVA radiation penetrates from the sun to the earth due to constant depletion of the stratospheric ozone layer.[6] Direct[7,8] as well as sensitized[2,9] photoisomerization has been observed upon excitation of UA with the UVA-I (340–400 nm) and UVA-II (320–340 nm) light. Since in vivo photoisomerization could also result from the sensitization of UA by the endogenous chromophores,[9] and there are contradictory reports[8,9] on the UVA-induced in vitro isomerization (perhaps due to broadband light sources used in these studies), we demonstrate here unequivocally that excitation of UA with the 355 and 340 nm monochromatic laser light leads to isomerization of (*E*)-UA to (*Z*)-UA.



(E)-Urocanic Acid [(E)-UA] (Z)-Urocanic Acid [(Z)-UA]

(1)

Recently, Hanson and Simon observed for the first time that 351 nm laser-excitation of UA leads to the sensitization of singlet oxygen via triplet energy transfer mechanism.[10] They have used sensitive techniques of

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emission spectroscopy and photoacoustic calorimetry to establish the production of singlet oxygen and excited triplet state of UA, respectively. However, it was not established if the excited triplet state arrives via intersystem crossing from the excited singlet state or it is directly populated from the ground singlet state.[10] We present here chemical evidence in favor of the former scenario. This is reminiscent that often times the chemical methodology has proven an invaluable tool to distinguish between such dichotomy.[11]

The electronic absorption spectrum of a 49.2 μM solution of (*E*)-UA in 50 mM phosphate buffer (pH 7.0) is presented in Fig. 1. The structureless absorption maximum in the UVB region centers at 277 nm and there is negligible absorbance in the UVA region. For comparison the unpublished absorption spectrum of (*Z*)-UA[12] in the UVA region at equal molar concentration is also included in Fig. 1, and it has absorption band similar to the (*E*) isomer, but is accompanied by 5 nm hypsochromic shift with reduced absorptivity. The absorption spectra of UA isomers in concentrated solutions (3.0 mM) used in the photoisomerization studies are presented as inset in Fig. 1. Both the spectra are remarkably similar and are associated with tailing in the UVA region. The absorbance values at the excitation wavelength of 355 nm are 0.014 and 0.012 for (*E*)- and (*Z*)-UA, respectively. The absorbance value for (*E*)-UA is comparable with the reported value in the this region.[10]

Early experiments for UA photoisomerization with the UVA light[13] employed 366 nm radiation from a medium-pressure mercury vapor lamp. Degassed duplicate solution of (*E*)-UA was irradiated at 8°C for 24 h. The reversed-phase high-performance liquid chromatography (RP-HPLC) analysis[14] indicated a 10.9% (±1.8%) formation of (*Z*)-UA. A second set of duplicate solution was irradiated under identical conditions but saturated with oxygen, when a 10.5% (±1.9%) formation of (*Z*)-UA was observed.[15] The lack of quenching of isomerization by oxygen suggests the involvement of excited singlet state in this process since oxygen quenches the UA triplet state via energy transfer mechanism.[10] The amount of (*Z*)-UA increased linearly with the light dose concomitant to the disappearance of the (*E*) isomer in a time-dependent manner. The mass balance during photoisomerization was excellent since no new product besides (*Z*)-UA was observed. No isomerization was observed in the dark when analyzed after 24, 48 and 72 h (data not shown).

Since the medium-pressure mercury vapor lamp has a continuum of wavelengths ranging from UV into IR, the 366-nm light tested in the UA photoisomerization could be contaminated with other wavelengths. To rule out the possibility for the leakage of some short wavelength(s), we utilized the monochromatic 355-nm line from the Nd:YAG laser to confirm UA isomerization. Indeed, irradiation of a magnetically stirred and undegassed (*E*)-UA solution at ambient temperature with the third harmonic of a Continuum Nd:YAG laser ($\lambda_{irr}=355$ nm) produced a 3.6% (*Z*)-UA after 60 min. As observed above in the 366-nm experiment, the photoisomerization with the laser radiation was also dose dependent, and data for a time course irradiation are presented in Fig. 2. There is a linear formation of

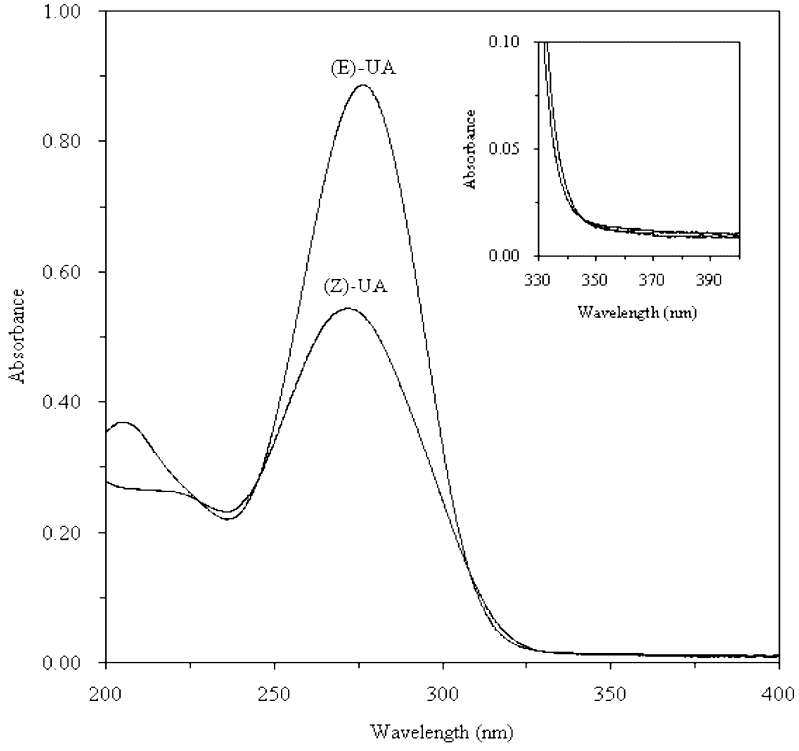


Figure 1. Electronic absorption spectrum of 49.2 μM (*E*)-UA and (*Z*)-UA in 50 mM sodium phosphate buffer (pH 7.0) at room temperature. The corresponding spectra of concentrated solutions (3.0 mM) in the UVA region are depicted in the inset.

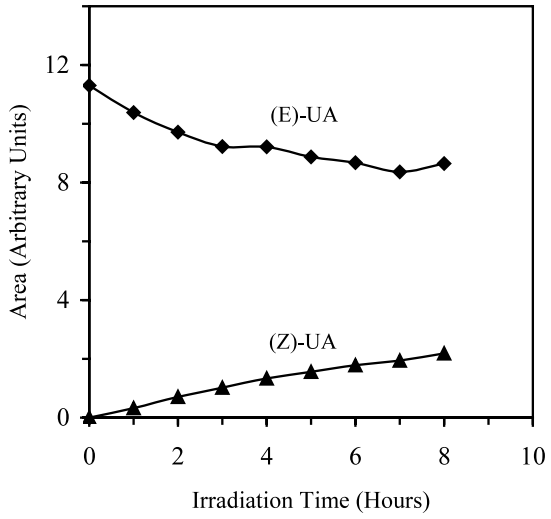


Figure 2. A time course photoisomerization of (*E*)-UA solution with the 355-nm laser radiation at room temperature. At specified intervals, the photolysates were analyzed by RP-HPLC.

(*Z*)-UA concomitant to the disappearance of (*E*)-UA with the light dose. Thus, despite the low extinction coefficient of UA in the UVA region, we have confirmed that UA isomerizes in vitro in the absence of a photosensitizer.

The issue of the nature of the excited state responsible for the UA chemistry in the UVA region was intriguing, especially the excited state that produces reactive oxygen species.[10] Hanson and Simon have observed the population of excited triplet state of UA upon excitation with the 351-nm laser radiation. The excited triplet state was quenched by ground triplet state molecular oxygen leading to the generation of singlet oxygen ($^1O_2$, $^1\Delta_g$). The authors confirmed this by observing singlet oxygen emission due to $^1\Delta_g \rightarrow {}^3\Sigma_g$ decay. We have studied simultaneously the effect of argon and oxygen on the rate of UA photoisomerization. The (*E*)-UA solutions were irradiated with the 355-nm laser line for 4 h. The RP-HPLC results are presented in Fig. 3, and indicate comparable formation of (*Z*)-UA, i.e. 11.9 and 13.0% under argon and oxygen, respectively. Since oxygen did not inhibit UA isomerization, supporting that the excitation of UA initially populates the singlet state that undergoes rapid isomerization (in competition with intersystem crossing to the excited triplet state). The action spectrum for the formation of UA triplet has been observed at 340 nm,[10] we employed this wavelength to test the UA isomerization. The data from irradiation of an oxygen-saturated solution of (*E*)-UA with this wavelength are included in Fig. 3. One notes that this wavelength is equally effective in converting (*E*)-UA to (*Z*)-UA, and in reality it seems more efficient (38.6% isomerization after 2.5 h) than 355-nm light because of the increased absorption of UA at shorter wavelengths. Since UA triplet state is quenched by oxygen[10] and in our hands oxygen did not intercept isomerization, we conclude that the unimolecular UA reaction comes from the excited singlet state. These

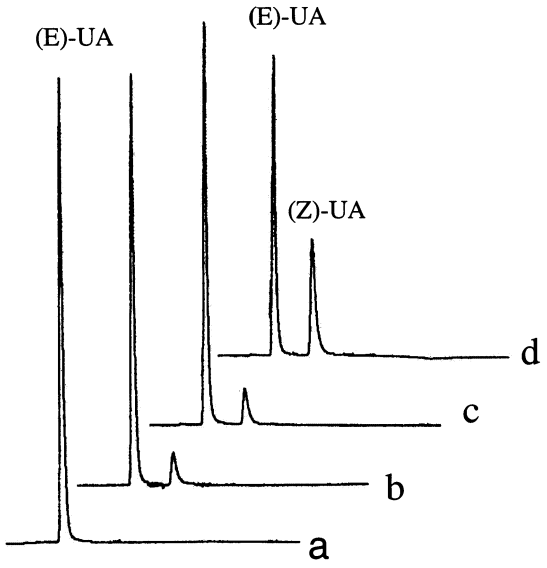
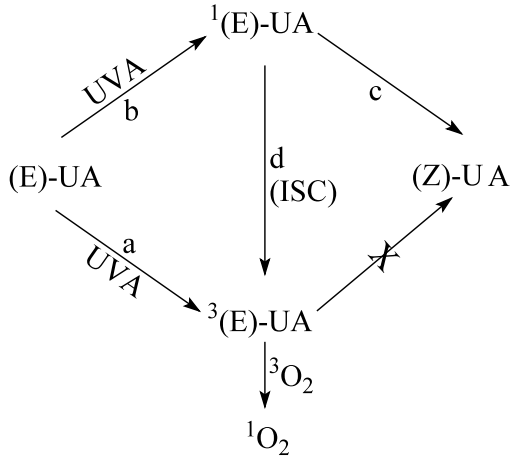


Figure 3. Effect of argon and oxygen on irradiation of (*E*)-UA with the 355 and 340 nm laser radiation at room temperature; (a) unirradiated, (b) irradiation at 355 nm under argon, (c) irradiation at 355 nm under oxygen, and (d) irradiation at 340 nm under oxygen. The run time was 25 min for each chromatogram.

arguments and conclusions are depicted in Scheme 1. If path 'a' for direct population of ^{3}(*E*)-UA is valid and responsible for singlet oxygen sensitization, we should not have observed formation of (*Z*)-UA since ^{3}(*E*)-UA does not isomerize.[1] This is supported from our data that oxygen does not quench UA isomerization, albeit quenches the excited triplet state. Alternatively, the (*E*)-UA is excited to the singlet manifold (path 'b'), which can decay either via isomerization (path 'c') or crosses over to the previously observed triplet state (path 'd'). We have observed both reactions corresponding to path 'c' and 'd',[15] thus unequivocally proving that UA is initially populated to the excited singlet state which is responsible for the population of previously observed UA excited triplet state.[10] These conclusions are in good agreement with the most recent femtosecond time-resolved spectroscopic data identifying a short-lived (ca. 1.0 ps) excited singlet state of UA,



Scheme 1.

which populates excited triplet state via intersystem crossing.[16] Theoretical calculations on the UA conformers also support the presence of such excited state transitions in the UVA region.[17]

In conclusion, we have established that the monochromatic 355 and 340 nm laser radiation of the UVA region are effective in the in vitro UA isomerization, and this reaction initiates from the excited singlet manifold. The UA triplet state responsible for the sensitization of singlet oxygen is derived from the excited singlet state. Future studies will be focused on the chemical reactivity of these excited states with biological molecules in understanding the consequences of UA/UVA chemistry in photocarcinogenesis and photoaging of the skin.

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13. For the photoisomerization experiments, a 2.0 mL solution of (*E*)-UA (3.0 mM) in 50 mM phosphate buffer (pH 7.0) was irradiated with one of the following light sources: (a) Canrad-Hanovia 450 W medium-pressure mercury vapor lamp surrounded by a cylindrical uranium yellow glass filter that transmits wavelengths >330 nm ($\lambda_{irr}=366$ nm); (b) third harmonic of a Continuum Nd:YAG laser ($\lambda_{irr}=355$ nm) operating at 10 Hz with a Q-switch delay of 265 μs with an output power of 90 mW; (c) dye-pumped excimer laser ($\lambda_{irr}=340$ nm) operating at 10 Hz with an output power of 50 mW. Prior to irradiation, the solutions were either degassed with argon or saturated with oxygen for 15 min, and sealed with septum and parafilm.
14. The analysis and separation of the UA isomers were carried on a Rainin Microsorb-MV C8 analytical column (4.6 mm i.d.×250 mm, 5 μm, 100 Å) using isocratic elution with 50 mM ammonium acetate (pH 5.0) at a flow-rate of 1.0 mL/min. Under these conditions, the retention time for (*E*)-UA and (*Z*)-UA was ca. 5.0 and 8.0 min, respectively.
15. Irradiation of UA in deuterium oxide saturated with oxygen leads to extensive degradation of UA via sensitization of and reaction with singlet oxygen. The original data were reproduced from the research notebooks, see: Menon, E. L.; Morrison, H. *Photochem. Photobiol.* **2002**, *75*, 565–569.
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