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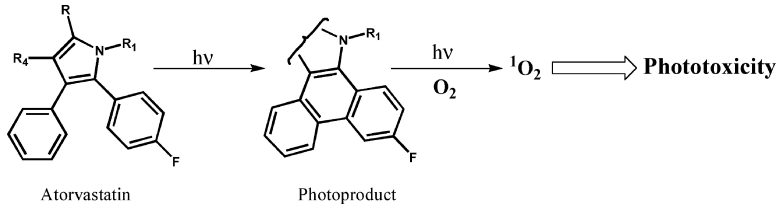

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A Mechanistic Study on the Phototoxicity of Atorvastatin: Singlet Oxygen Generation by a Phenanthrene-like Photoproduct

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Atorvastatin calcium (ATV) is one of the most frequently prescribed drugs worldwide. Among the adverse effects observed for this lipid-lowering agent, clinical cases of cutaneous adverse reactions have been reported and associated with photosensitivity disorders. Previous work dealing with ATV photochemistry has shown that exposure to natural sunlight in aqueous solution leads to photoproducts resulting from oxidation of the pyrrole ring and from cyclization to a phenanthrene derivative. Laser flash photolysis of ATV, at both 266 and 308 nm, led to a transient spectrum with two maxima at $\lambda =$ 360 and $\lambda = 580$ nm ($\tau = 41$ μs), which was assigned to the primary intermediate of the stilbene-like photocyclization. On the basis of the absence of a triplet−triplet absorption, the role of the parent drug as singlet oxygen photosensitizer can be discarded. By contrast, a stable phenanthrene-like photoproduct would be a good candidate to play this role. Laser flash photolysis of this compound showed a triplet−triplet transient absorption at $\lambda_{max} = 460$ nm with a lifetime of 26 μs, which was efficiently quenched by oxygen ($k_q = 3$ (± 0.2) $\times$ 10^9 M^{-1} s^{-1}). Its potential to photosensitize formation of singlet oxygen was confirmed by spin trapping experiments, through conversion of TEMP to the stable free radical TEMPO. The photoreactivity of the phenanthrene-like photoproduct was investigated using Trp as a marker. The disappearance of the amino acid fluorescence ($\lambda_{max} = 340$ nm) after increasing irradiation times at 355 nm was taken as a measurement of photodynamic oxidation. To confirm the involvement of a type II mechanism, the same experiment was also performed in D_2O; this resulted in a significant enhancement of the reaction rate. On the basis of the obtained photophysical and photochemical results, the phototoxicity of atorvastatin can be attributed to singlet oxygen formation with the phenanthrene-like photoproduct as a photosensitizer.

Introduction

Atorvastatin calcium (ATV, Chart 1), a statin drug of the second generation, is one of the most frequently prescribed drugs in the United States and in Europe (*1*). This synthetic lipid-lowering agent is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme involved in cholesterol biosynthesis that catalyzes the early and rate limiting step of HMG-CoA conversion to mevalonate. Recently, a new application dealing with treatment of Alzheimer's disease has also been described for statin drugs (*2*). The most common adverse effects observed for ATV have been related with gastrointestinal disturbances. Nevertheless, clinical cases of cutaneous adverse reactions have been reported and associated with photosensitivity disorders for statin drugs (*3*−*8*); in particular, a recent study of ATV has established its UVB phototoxicity by systemic phototests (*9*).

To evaluate the photobiological risk of a drug, it is necessary to understand the involved mechanism. This requires determination of the excited states generated after light absorption, as well as any other drug-derived short-lived intermediates and/or reactive oxygen species like singlet oxygen. Thus, photophysical and photochemical studies are important for understanding the key early events resulting in phototoxicity.

Previous work dealing with atorvastatin photochemistry has focused the attention on photodegradation in the environmental framework (*10, 11*). Thus, natural sunlight irradiation of ATV aqueous solution leads mainly to photoproducts resulting from oxidation of the pyrrole ring (Chart 1). Another reaction pathway has been associated with the stilbene-like structure of ATV that is in the origin of a cyclization process leading to formation of the phenanthrene derivative **4** (Chart 1). It has been proposed that the photolysis mechanism involves singlet oxygen production from the ATV triplet excited state; nevertheless, no studies have been reported dealing with atorvastatin photophysical properties. In this work, steady-state and time-resolved spectroscopic experiments have been performed to establish the generation and behavior of ATV-related excited states, to shed some light on the photolysis mechanism and to evaluate the photobiological risk of ATV toward biomolecules.

Materials and Methods

Chemicals. Atorvastatin calcium was obtained from KEMPROTEC Limited (Middlesbrough, United Kingdom) and was used without further treatments. TEMP and β-carotene were purchased from Fluka (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Perinaphthenone, TEMPO, (*S*)-naproxen and L-tryptophan were from Sigma-Aldrich. Water was Milli-Q grade; all other solvents were of HPLC grade.

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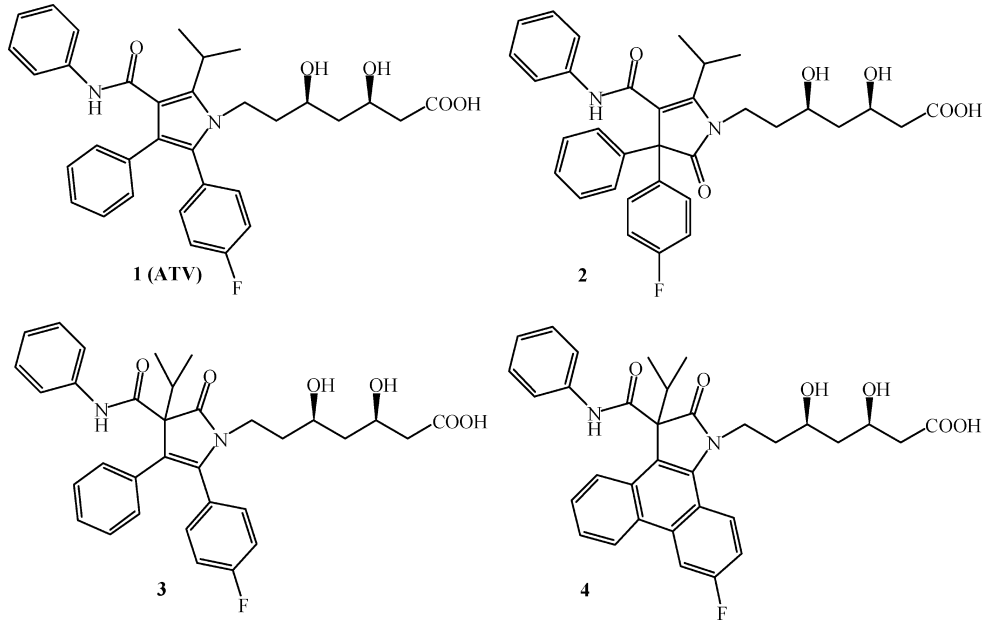
Chart 1. Stuctures of ATV and Its Photoproducts



Photoreactor. Irradiations were performed by means of a multilamp photoreactor equipped with lamps with a maximal output at ca. 300 nm (Hitachi, F15T8/BL) or at ca. 355 nm (Hitachi, F15T8/BLB).

Laser Flash Photolysis. For 266 nm excitation, experiments were carried out using the fourth harmonic ($\lambda_{exc} = 266$ nm) of a Quantel pulsed Nd:YAG spectrum laser system instrument. The single pulses were ca. 10 ns duration, and the energy was ca. 15 mJ/pulse. A pulsed Excimer Laser Systems with Xe/HCl/Ne mixture was used for excitation at 308 nm. The single pulses were ~17 ns duration, and the energy was ≪100 mJ/pulse. In both cases, a Xenon lamp was employed as the detecting light source. The laser flash photolysis apparatus consisted of the pulsed laser, the Xe lamp, a monochromator, and a photomultiplier (PMT) system made up of side-on PMT, PMT housing, and a PMT power supply. The output signal from the Tektronix oscilloscope was transferred to a personal computer for study. All transient spectra were recorded using 10 × 10 mm^2 quartz cells with 4 mL capacity and were bubbled for 10 min with N_2, air, or O_2, before acquisition. The absorbance of the samples was kept in the range 0.30−0.40 at the laser wavelength. Stock solutions of quenchers were prepared so that it was only necessary to add microliter volumes to the sample cell to obtain appropriate concentrations of the quencher. A linear quenching plot following eq 1 was obtained, and the resulting rate constant was calculated from the slope of the Stern−Volmer plot (*12*)

$$k_{obs} = k_o + k_q[Q] \quad (1)$$

where k_o is the triplet decay rate constant in the absence of quencher, k_q is the triplet decay rate constant in the presence of the quencher, and [Q] is the quencher concentration in mol L^{-1}.

Fluorescence. The steady-state fluorescence experiments were carried out on a Photon Technology International (PTI) LPS-220B spectrofluorometer. Fluorescence quantum yields measurements were performed with nitrogen-, air-, and oxygen-bubbled methanol solutions; the absorbance was adjusted at the excitation wavelength ($\lambda = 308$ nm), and naproxen was used as standard ($\phi_f = 0.53$) (*13*). Lifetimes were measured with a Time Master fluorescence lifetime spectrometer TM-2/2003 from PTI by means of the stroboscopic technique, which is a variation of the boxcar technique. A hydrogen/nitrogen flash lamp was used as the excitation source. The kinetic traces were fitted by monoexponential functions using a deconvolution procedure to separate from the lamp pulse profile. All measures were recorded using 10 × 10 mm^2 quartz cells with 4 mL capacity. The absorbance of the samples was kept in the range 0.10−0.20 at the excitation wavelength. All of the experiments were carried out at room temperature.

Singlet Oxygen. The luminescence (1270 nm) from singlet oxygen was detected by means of an Oriel 71614 germanium photodiode (5 mm^2) coupled to the laser flash photolysis cell in right angle geometry. The pulsed Excimer Laser Systems with Xe/HCl/Ne mixture was used for the excitation at 308 nm. A 5 mm thick (5 cm diameter) 1050 nm cutoff silicon filter and a 1270 interference filter were placed between the diode and the cell. The photodiode output current was amplified and fed into a TDS-640A Tektronix oscilloscope via a colinear 150 MHz 20 dB amplifier. The output signal from the oscilloscope was transferred to a personal computer for study.

All measurements were made at room temperature using acetonitrile as solvent in 10 × 10 mm^2 quartz cells with 4 mL capacity and were bubbled for 10 min with oxygen before acquisition. Singlet oxygen was generated by means of perinaphthenone ($\phi_\Delta \sim 1$) (*14, 15*), and the absorbance of the samples was at 0.40 at the laser wavelength.

For quenching, stock solutions of atorvastatin (10^{-3} M) in acetonitrile were used, and singlet oxygen lifetimes were recorded at increasing concentration of the quencher. The rate constant was calculated from the slope of the Stern−Volmer plot.

EPR Spin Trapping Experiments. The measurements were performed in a flat cell using a Bruker EMX 10/12 EPR spectrometer. Used were the following parameters: microwave power, 20 mW; modulation amplitude, 1.0 G; and modulation frequency, 100 kHz. Analysis was performed recording the EPR signal of the free radical TEMPO generated by reaction of singlet oxygen with the spin trap TEMP (*16*); methanolic solutions of 10 mM TEMP containing ATV or photoproduct **4**, with an absorbance of ~0.3 at wavelength 300 nm, were irradiated in a photoreactor (maximum output 300 nm); EPR spectra were recorded at different irradiation times.

Production of Phenanthrene 4. Atorvastatin solutions (8×10^{-4} M) were prepared dissolving 40 mg of the commercial product in 80 mL of MeOH/H_2O (1:8) and were irradiated for 5 h; irradiation was performed in Pyrex tubes using the described photoreactor equipped with six lamps with maximal output at 300 nm. All of the experiments were conducted at room temperature and under air atmosphere.

After irradiation, the solutions were concentrated under vacuum and separated by column chromatography on Lichroprep RP-18, 40−63 μm (Merck, Damstadt, Germany), (50:50) water/acetonitrile (50:50) (v/v) as eluent. In this way, 6 mg of pure photoproduct

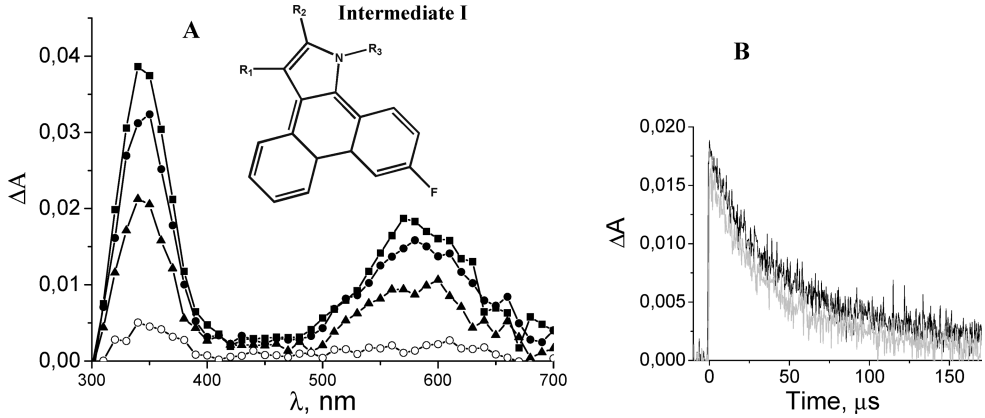


Figure 1. (A) Transient absorption spectra of a nitrogen-purged methanolic solution of ATV (4.8 μs, ■; 10.1 μs, ●; 28.2 μs, ▲; or 150 μs, ○, after the laser pulse at 266 nm). (B) Decay monitored at 590 nm of the transient obtained with nitrogen-bubbled (black line) or aerated (gray line) solutions of ATV.

was obtained. Its ^{1}H and ^{13}C NMR spectral data were identical to those previously reported in the literature (*10*). Although the two diastereoisomers obtained can be chromatographically separated as their methyl esters (*10*), the photophysical and photobiological experiments were performed with the diastereoisomeric mixture.

Formation of Intermediate II. A methanolic solution of ATV (10^{-3} M), containing an equimolar amount of molecular iodine, was bubbled for 15 min under N_2 in a 10 × 10 mm^2 quartz cell and irradiated at 300 nm for 45 min. The obtained solution was analyzed by HPLC-MS without further treatments [solid phase, spherisorb RP-18 ODS2 column (4.6 mm × 250 mm) (Waters, Dublin, Ireland); liquid phase, (50:50:0.5) water−acetonitrile−trifluoroacetic acid; and flux, 1 mL/min].

Tryptophan Photodegradation. Water solutions of photoproduct **4** (10^{-6} M) containing an equimolar concentration of tryptophan were irradiated at 355 nm. Tryptophan fluorescence emission intensity (λ_{exc}= 340 nm) was registered at increasing irradiation times (0, 10, 20, 30, 60, 90, 135, 180, 240, and 300 min).

A parallel experiment was performed using deuterium oxide as the solvent, under otherwise the same conditions. A solution of tryptophan in water (10^{-6} M) was used as the control.

Results and Discussion

Photophysics of Atorvastatin. Steady-state fluorescence was performed for methanolic solutions of ATV, and no emission from the singlet excited state was observed. Likewise, time-resolved laser flash photolysis experiments were performed in nitrogen-saturated methanolic solution, at both 266 and 308 nm. The same transient spectrum (Figure 1A) was obtained with both excitation wavelengths; it exhibited two maxima at λ = 360 and λ = 580 nm, sharing the same monoexponential decay (τ = 41 μs). As shown in Figure 1B, oxygen had no significant influence on the decay of this long-lived intermediate. As all triplet excited states are quenched by molecular oxygen, it was concluded that the detected species does not correspond to the triplet−triplet transition of ATV.

Photochemistry of ATV was considered to assign this transient. The drug contains a stilbene-like moiety and can in principle undergo the typical 6π-electrocyclization, leading to formation of a *trans*-4a,4b-dihydrophenanthrene, followed by an easy oxidation to phenanthrene (*17*). Indeed, such a photoreactivity has been previously evidenced during the steady-state photolysis of aerated aqueous solution of ATV (*10*) where photoproduct **4**, containing a phenanthrene moiety (Chart 1), has been isolated and characterized.

Hence, a possible candidate for the detected transient is the expected *trans*-dihydrophenanthrene (intermediate I) that corresponds to the primary photoproduct of the stilbene-like photocyclization of ATV. In the literature, absorption spectra of related species show two maxima in the region 300−360 and 440−640 nm (*18*−*20*).

A mechanism involving singlet oxygenation has previously been proposed to explain photoproducts obtained by steady-state photolysis of ATV (*10*). Nevertheless, in view of the obtained photophysical results, the role of the parent drug as singlet oxygen photosensitizer can be discarded. The lack of singlet- and triplet-state detection at room temperature is a common property of stilbene derivatives (*21*) attributed to their efficient *cis*/*trans* isomerization. In the case of ATV, photoisomerization is blocked by the pyrrole moiety; thus, the main process should be the stilbene-like photocyclization with formation of an unstable dihydrophenanthrene (see the structure in Figure 1A). It has been described that such intermediates suffer cycloreversion back to the starting reactants under anaerobic conditions, while they aromatize in the presence of oxygen (*17*). In the particular case of ATV, the only phenanthrene-like compound obtained during its photolysis is photoproduct **4** that corresponds to oxidation both at the dihydrophenanthrene moiety and at the pyrrole ring.

Photochemical Formation and Behavior of Phenanthrene 4. Photolysis of ATV was performed in deaerated methanol, in the presence of iodine as oxidant, to obtain the purported intermediate II, where the pyrrole moiety remains intact (*22*). Under these conditions, intermediate II was actually obtained in small quantity; although its isolation and characterization were very difficult due to its instability under air, HPLC-MS analysis allowed its detection as a major peak with m/z = 557 (Figure 2).

This photoproduct could be a real (first stage) singlet oxygen sensitizer; nevertheless, after self-sensitization, intermediate II would be oxygenated to photoproduct **4**, preventing its isolation during the aerobic photolysis. Thus, although intermediate II is likely to be the first stage photoproduct, its nonpersistence renders it a poor photosensitizer to account for the oxygenated photoproducts. By contrast, the stable phenanthrene-like compound **4** appears a good candidate as a singlet oxygen mediator. In addition, the absorption spectrum of **4** shows a long-wavelength UVA band with maximum at 330 nm (Figure 3).

Steady-state fluorescence of a methanolic solution of compound **4** showed an emission spectrum with λ_{max} at 405 nm and a fluorescence quantum yield of 0.35, obtained by using naproxen as standard. A singlet-state energy of 318 kJ mol^{-1} was determined by considering the intersection between the normalized emission and excitation spectra. Fluorescence emis-

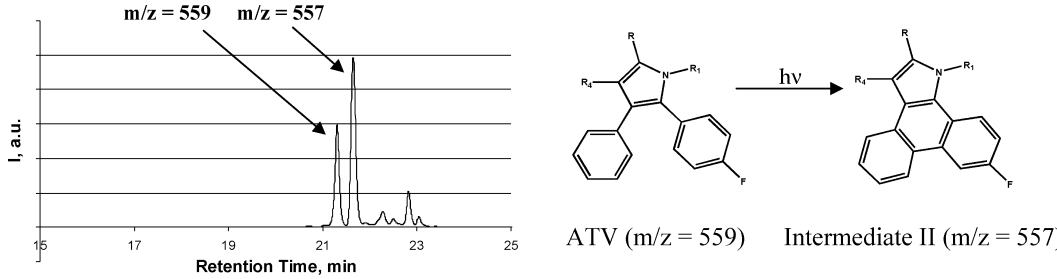


Figure 2. HPLC chromatogram obtained after UVA irradiation of a deaerated methanolic solution of ATV in the presence of iodine.

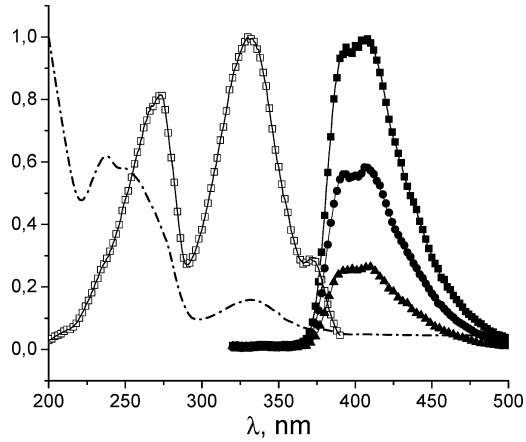


Figure 3. Normalized emission (closed symbols), excitation (open symbols), and absorption (dashed−dotted line) spectra of a methanolic solution of photoproduct **4** under deaerated (■), aerated (●), or oxygenated (▲) conditions.

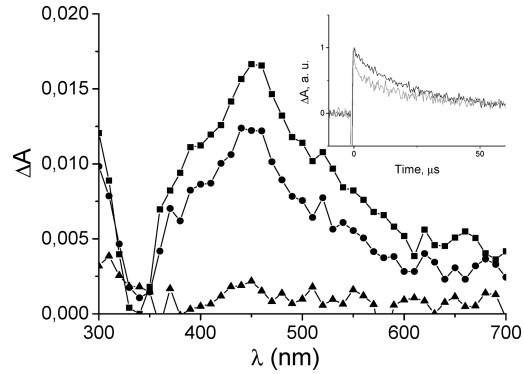


Figure 4. Transient absorption spectrum of a deaerated methanolic solution of **4** at 9 (■), 18 (●), and 150 μs (▲) after the laser pulse at 266 nm. Inset: Normalized decay monitored at 450 nm of the transient obtained with nitrogen-bubbled (black line) or aerated (gray line) solutions of photoproduct **4**.

sion was quenched by oxygen (Figure 3); hence, to investigate the nature of this quenching, the emission lifetime of photoproduct **4** was measured by time-resolved fluorescence, obtaining values of 15 ns under nitrogen, $\tau = 9$ ns under air, and $\tau =$ 3.9 ns under oxygen. On the basis of these data, a singlet quenching rate constant of 1.8 ($\pm$0.1) $\times$ 10^{10} M^{-1} s^{-1} was determined from the slope of the Stern−Volmer plot.

Laser flash photolysis of **4** showed a transient at $\lambda_{max} = 460$ nm with a lifetime of 26 μs (Figure 4). This transient was assigned to the triplet excited state, based on its diffusion-controlled quenching by β-carotene (E_T ca. 20 kJ mol^{-1}), which resulted in the concomitant build up of a new band peaking at 520 nm. This band corresponds to the characteristic T−T absorption of β-carotene, which can only be formed via energy transfer, due to inefficient intersystem crossing.

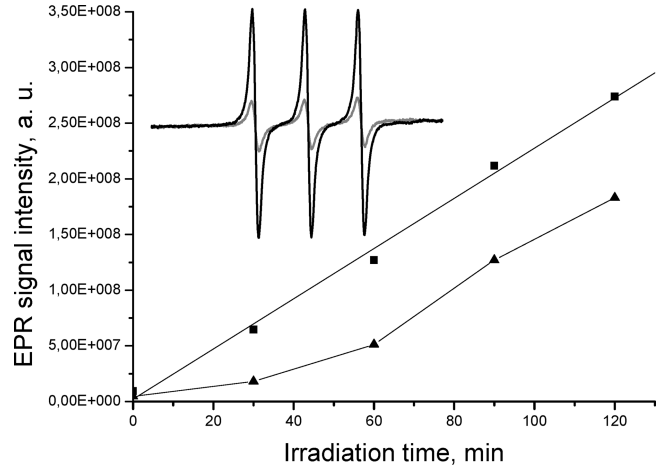


Figure 5. Time-dependent formation of TEMPO upon irradiation of aerated solutions of TEMP in the presence of ATV (▲) or photoproduct **4** (■). Inset: TEMPO signals obtained after 30 min of irradiation of a solution of ATV (gray line) and photoproduct **4** (black line).

Moreover, the triplet state of photoproduct **4** was efficiently quenched by oxygen [k_q= 3 ($\pm$0.2) $\times$ 10^9 M^{-1} s^{-1}] to give singlet oxygen with a quantum yield $\Phi_\Delta = 0.3$. These results strongly support the potential of photoproduct **4** to photosensitize singlet oxygen formation.

Singlet Oxygen Generation. To confirm the direct involvement of photoproduct **4** in singlet oxygen production, spin trapping experiments were conducted. In this framework, the well-known conversion of TEMP to the stable free radical TEMPO, detectable by EPR analysis, was of diagnostic value. A methanolic solution of TEMP (10 mM) was irradiated employing ATV and photoproduct **4** as potential singlet oxygen sensitizers (*16*). As shown in the inset of Figure 5, the typical TEMPO spectrum was observed after 30 min of irradiation in the presence of photoproduct **4**, while a much lower signal was obtained for ATV. The degree of conversion of TEMP into TEMPO in the presence of sensitizer, as a function of irradiation time (Figure 5), clearly evidenced the contribution of photoproduct **4** in singlet oxygen production. The data also showed that, in the case of ATV, an induction time was necessary to induce TEMPO formation, in agreement with the inability of ATV itself to act as singlet oxygen sensitizer. Indeed, at longer irradiation times, the increase of TEMPO signal can be explained by the photochemical conversion of the drug into the phenanthrene-like photoproduct.

Reaction between Atorvastatin and $^1O_2$. In the next step, to evaluate the reactivity of ATV with $^1O_2$, the influence of statin concentration on the singlet oxygen lifetime was studied. For this purpose, $^1O_2$ was photogenerated by perinaphthenone (ϕ_Δ of unity in acetonitrile), and near-IR emission decay was monitored at 1270 nm. As shown in Figure 6, singlet oxygen lifetime (initial value ca. 90 μs) (*23*) was shortened in the presence of ATV. A linear quenching plot was obtained by

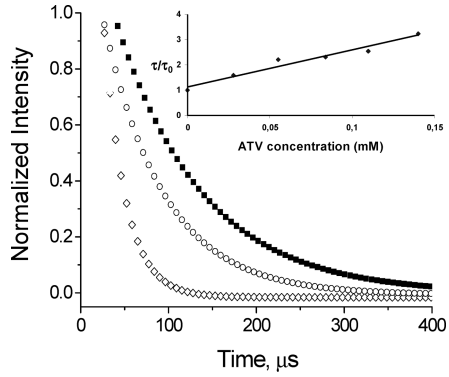


Figure 6. Decay monitored at 1270 nm of an oxygenated acetonitrile solution of perinaphthenone alone (■), or in the presence of increasing amounts of ATV: 2.8×10^{-5} M (○), and 1.4×10^{-4} M (◇). Inset: Stern-Volmer plot.

representing the reciprocal lifetime as a function of ATV concentration (inset Figure 6), and a rate constant of 1.5 (±0.2) $\times$ 10^8 M^{-1} s^{-1} was determined. This high rate constant is comparable with the reactivity of pyrrole with $^1O_2$ ca. 1.2 $\times$ 10^8 M^{-1} s^{-1} (*24*), in agreement with the nature of the steady-state photolysis products, resulting from photooxidation of the pyrrole moiety.

Tryptophan Photodegradation. Singlet oxygen is one of the main reactive oxygen species involved in the oxidative damage to living systems. It is now well-established that $^1O_2$, photogenerated by endogenous or exogenous compounds, is able to react with a large number of target biomolecules, including unsaturated lipids, proteins, and nucleic acids (*25, 26*). Indeed, some of the key biomolecule building blocks can be used as probes to anticipate the photodynamic potential of xenobiotics and to establish the mechanism of photosensitized oxygenation reactions.

In this context, tryptophan is a very convenient probe, as it can be oxidized by both type I and type II mechanisms; in combination with additional information (i.e., the effect of D_2O, radical scavengers, singlet oxygen quenchers, etc.), the photooxygenation kinetics may be used to discriminate between the possible mechanistic pathways. Thus, photoproduct **4** photoreactivity was also investigated using Trp as a photooxidative damage marker; the experiments were conducted irradiating photoproduct **4** at 355 nm in presence of an equimolar concentration of Trp, and the disappearance of the amino acid fluorescence emission (λ_{max}= 340 nm) at increasing irradiation times was taken as measurement of occurred oxidation.

To evidence the involvement of type II mechanism, the same experiment was also performed in D_2O, where $^1O_2$ presents a very longer lifetime in comparison with water. This resulted in a significant enhancement of the reaction rate, as shown in Figure 7.

Conclusion

On the basis of the obtained photophysical and photochemical results, the phototoxicity of atorvastatin can be attributed to singlet oxygen formation with the phenanthrene-like photoproduct **4** as the photosensitizer. Thus, laser flash photolysis studies of ATV have allowed us to understand the formation of the products obtained during its steady-state photolysis. Transient absorption spectra of the parent drug did not show any triplet−triplet absorption spectrum, ruling out its direct involvement in $^1O_2$ formation. By contrast, an intermediate of cyclization (intermediate I) was detected; oxidation of its dihydrophenanthrene moiety leads to the formation of an

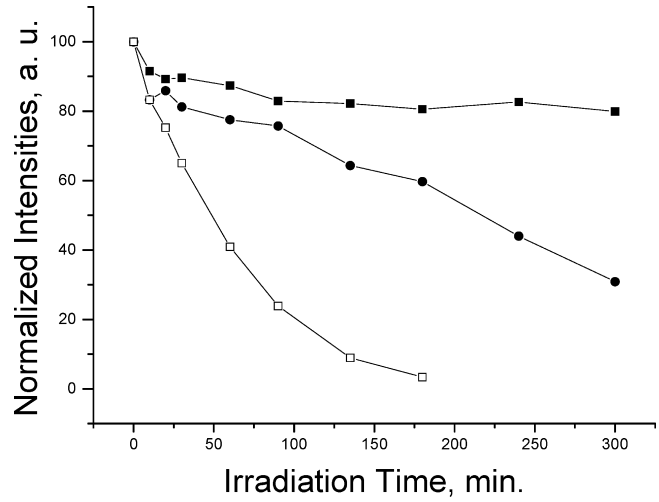
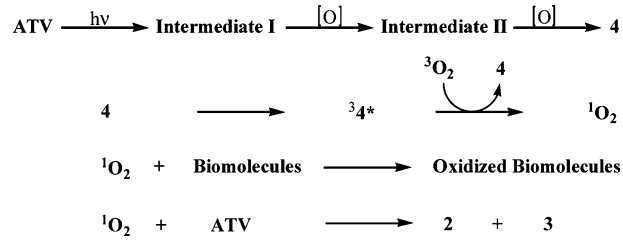


Figure 7. Tryptophan photodegradation monitored as function of irradiation time: tryptophan alone (■), tryptophan and photoproduct **4** in water (●), and tryptophan and photoproduct **4** in deuterium oxide as solvent (□).

Scheme 1. Phototoxicity Mechanism of Atorvastatin



unstable phenanthrene-like product (intermediate II) and, subsequently, to the stable compound **4**. The ability of this last compound to sensitize singlet oxygen was proven by detection of a triplet−triplet transition efficiently quenched by oxygen, together with TEMPO formation upon photolysis in the presence of TEMP. Singlet oxygen generated by **4** would then be in the origin of the photobiological damage to biomolecules associated with atorvastatin (Scheme 1). Hence, any structural change preventing photocyclization to a phenanthrene derivative (for instance, by exchanging the substituents at positions 3 and 4 of the pyrrole ring) would result in an increased photostability and a reduced photosensitizing potential.

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