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Ketoprofen-Induced Formation of Amino Acid Photoadducts: Possible Explanation for Photocontact Allergy to Ketoprofen

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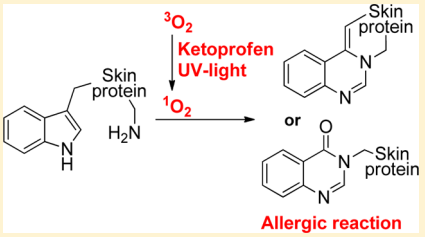
Supporting Information

ABSTRACT: Photocontact allergy is a well-known side effect of topical preparations of the nonsteroidal anti-inflammatory drug ketoprofen. Photocontact allergy to ketoprofen appears to induce a large number of photocross allergies to both structurally similar and structurally unrelated compounds. Contact and photocontact allergies are explained by structural modification of skin proteins by the allergen. This complex is recognized by the immune system, which initiates an immune response. We have studied ketoprofen's interaction with amino acids to better understand ketoprofen's photoallergenic ability. Irradiation of ketoprofen and amino acid analogues resulted in four different ketoprofen photodecarboxylation products (**6**–**9**) together with a fifth photoproduct (**5**). Dihydroquinazoline **5** was shown to be a reaction product between the indole moiety of 3-methylindole (Trp analogue) and the primary amine benzylamine (Lys analogue). In presence of air, dihydroquinazoline **5** quickly degrades into stable quinazolinone **12**. The corresponding quinazolinone (**17**) was formed upon irradiation of ketoprofen and the amino acids *N*-acetyl-L-Trp ethyl ester and L-Lys ethyl ester. The formation of these models of an immunogenic complex starts with the ketoprofen-sensitized formation of singlet oxygen, which reacts with the indole moiety of Trp. The formed intermediate subsequently reacts with the primary amino functionality of Lys, or its analogue, to form a Trp−Lys adduct or a mimic thereof. The formation of a specific immunogenic complex that does not contain the allergen but that can still induce photocontact allergy would explain the large number of photocross allergies with ketoprofen. These allergens do not have to be structurally similar as long as they can generate singlet oxygen. To the best of our knowledge, there is no other suggested explanation for ketoprofen's photoallergenic properties that can account for the observed photocross allergies. The formation of a specific immunogenic complex that does not contain the allergen is a novel hypothesis in the field of contact and photocontact allergy.



INTRODUCTION

2-(3-Benzoylphenyl)propionic acid (ketoprofen, **1**) (Figure 1) is a nonsteroidal anti-inflammatory drug (NSAID) that is commonly used both topically and systemically for treatment of musculoskeletal diseases because of its analgesic and anti-inflammatory effects. However, since 1985, a substantial number of cases of photoallergic contact dermatitis from topical formulations of ketoprofen have been reported.[1−11] According to an Italian multicenter study performed between 2004 and 2006, where 1082 patients with a history of photoallergic contact dermatitis were evaluated, as many as 10% reacted positively to ketoprofen, which by far made it into the photoallergen causing most reactions. Not only is ketoprofen one of the most common photoallergens but also the reactions from it are often both severe and persistent.[5,6,12] Furthermore, photocontact allergy to ketoprofen seems to induce a large number of other photocross allergies.[2−5,7,13,14] Some of these compounds, such as tiaprofenic acid and suprofen, are structurally similar to ketoprofen, whereas other compounds, like etofenamate and octocrylene, have quite different structures (Figure 1).

Contact allergy is caused by a wide range of chemicals that come in contact with the skin. However, the allergenic compounds themselves are too small to be recognized by the immune system. The widely accepted hypothesis is that they bind to a protein and this modified macromolecule is recognized by the immune system as nonself, thereby intitiating an immune response.[15] In photocontact allergy, light, usually UV radiation, is needed in combination with the allergen for an allergic reaction to occur.[16] The aim of this study was to investigate ketoprofen's photochemical reactivity toward amino acids to better understand the reason for its ability to induce photocontact allergy. As a simple model system of ketoprofen's interaction with skin proteins when irradiated (photolysis of ketoprofen in the presence of chemical analogues of the amino acids Trp (3-methylindole), Tyr (4-propylphenol), Cys (1-octanethiol), Lys (benzylamine), and His (4-methylimidazole)) was conducted (Figure 1). To corroborate the most important results from the amino acid analogues experiments, an

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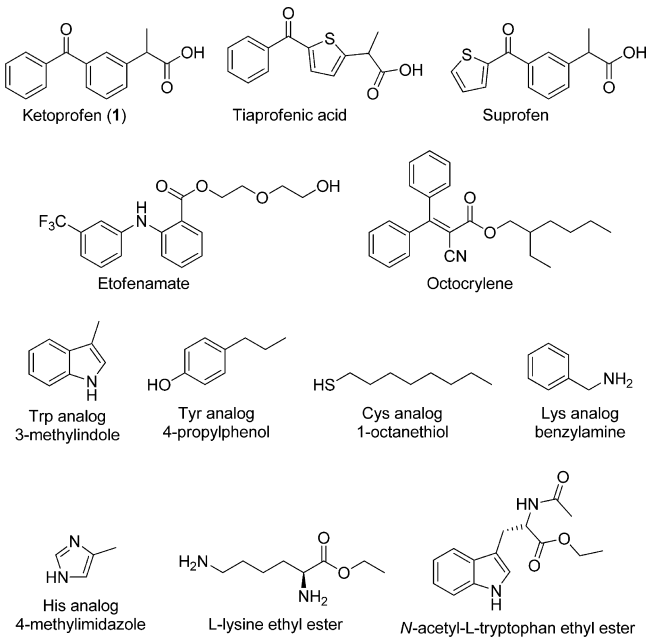

Figure 1. Structures of compounds discussed in this article.

irradiation experiment was also performed with ketoprofen and protected amino acids Trp (*N*-acetyl-L-Trp ethyl ester) and Lys (L-Lys ethyl ester).

EXPERIMENTAL PROCEDURES

Caution: *Skin contact with ketoprofen must be avoided. This is a skin-sensitizing substance in the presence of UV light and must be handled with care.*

Chemicals. Reagents were obtained from commercial suppliers and used without further purification. Benzylamine, anthranilic acid, formic acid, and potassium carbonate were purchased from Fluka, Sigma-Aldrich Chemie (Steinheim, Germany). Acetic anhydride, 2-amino-acetophenone, anhydrous *N*,*N*-dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA), hydroxybenzotriazole hydrate, ketoprofen (**1**), *S*-(+)-ketoprofen (*S*-(+)-**1**), L-Lys ethyl ester dihydrochloride, 4-methylimidazole, 3-methylindole, 1-octanethiol, potassium hydroxide, 4-propylphenol, and L-Trp ethyl ester hydrochloride were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Dicyclohexylcarbodiimide (DCC) was purchased from Merck Millipore (Darmstadt, Germany). Synthetic air (78% N_2, 22% O_2) was obtained from AGA (Sweden). *N*-(2-Acetylphenyl) formamide was synthesized as previously described.[17]

Instrumentation and Modes of Analyses. Flash chromatographic separations were performed using Merck silica gel Geduran Si 60 (0.063−0.200 mm) and Sigma-Aldrich hexane mixture of isomers (bp 68−70 °C). TLC was performed using silica gel plates (Merck, 60 F254).

Photolysis reactions were performed in a falling film photoreactor, according to the method of Professor de Meijere,[18] with forced liquid circulation (purchased from NORMAG Labor- and Prozesstechnik[19]). The photoreactor was equipped with a medium-pressure mercury UV lamp (700 W, Heraeus, TQ 718, Z4 doped) as the irradiation source. The total radiant power of the lamp in the wavelength interval from 200 to 600 nm is 389 W. Both the lamp and the reaction mixture were cooled with water. Synthetic air (78% N_2, 22% O_2) was continuously bubbled through the sample and allowed to pass through the reaction zone. More detailed information about the photoreactor is available in the Supporting Information.

GC/MS analyses were performed using electron impact ionization (70 eV) on a Hewlett-Packard model 5973 mass spectrometer (scanned *m*/*z* 50−500) connected to a gas chromatograph (Hewlett-Packard model 6890). The GC was equipped with an on-column inlet and an HP-5MSI fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness). Helium was used as carrier gas, the flow rate was 1.2 mL/min, and the injection volume was 1 μL. The column temperature was 100 °C at injection and was raised to 200 °C at a rate of 5 °C/min; it was then raised from 200 to 270 °C at a rate of 15 °C/min and finally held at 270 °C for 20 min.

HPLC/MS analyses were performed using electrospray ionization on a Hewlett-Packard 1100 HPLC/MS. The system included a vacuum degasser, a binary pump, an autoinjector, a column thermostat, a diode array detector, and a single quadrupole mass spectrometer. The electrospray interface was used with the following spray chamber settings: nebulizer pressure, 35 psig; capillary voltage, 3000 V; drying gas temperature, 350 °C; and drying gas flow rate, 12 L/min. For mass spectral analysis, the mass spectrometer was used in the scan mode detecting ions with *m*/*z* ranging from 50 to 1000. Mass spectral analysis was performed in positive ionization mode with fragmentor voltage of 70 V. A Zorbax SB-C18 column (150 mm × 2.1 mm, 5 μm particles, Agilent Technologies) or a Hypersil-Keystone HyPurity C18 column (150 mm × 3 mm, 3 μm particles, Thermo Scientific) was used, and the column temperature was set to 40 °C. Mobile phase A consisted of 0.1% formic acid in Milli-Q water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. Aliquots of 5 μL were injected onto the column and eluted with a gradient flow of 0.40 mL/min. A linear gradient from 10 to 100% B in 20 min was followed by 10 min of isocratic elution at 100% B. The column was equilibrated with 10% B for 10 min between each run.

Preparative HPLC was performed using a Gilson pump (model 305), a Gilson UV/vis detector (model 119), and a Zorbax Semi-Preparative column (250 mm × 9.4 mm, 5 μm particles, Agilent Technologies); the flow rate was 9.0 mL/min, and the compounds were monitored at 210 nm.

^{1}H and ^{13}C NMR spectra were recorded either on a JEOL eclipse+ 400 MHz spectrometer (100 MHz for ^{13}C) or an Agilent 400 MHz spectrometer (100 MHz for ^{13}C) using $CDCl_3$ or Me_2SO-d_6 as solvent. ^{1}H spectra were referenced to the residual solvent peaks: $CHCl_3$ (7.26 ppm) or Me_2SO (2.50 ppm). ^{13}C spectra were referenced to $CDCl_3$ (77.0 ppm) or Me_2SO (39.5 ppm).

HRMS was performed on a Waters Micromass Q-TOF micro instrument using electrosparay ionization and a time-of-flight mass analyzer.

General Procedure for Photolysis Experiments. Photolysis was performed on 10 mM solutions of each compound in ethanol (EtOH) at 20−30 °C, and samples of approximately 4 mL were withdrawn from the photoreactor at each specified time point. All samples were diluted 20 times with acetonitrile before analysis with HPLC/MS.

Photolysis of Ketoprofen (1) and Isolation of Photoproducts. Compound **1** was illuminated for 10 min, and samples were taken every minute. The 10 min photolysis mixture was concentrated under reduced pressure and fractionated by semi-preparative HPLC (isocratic, acetonitrile/water 2:3). Four fractions were isolated and characterized. Two fractions contained isomeric mixtures of compound **2**, and two fractions contained isomeric mixtures of compound **3**. Compound **2** was identified as 2-(3-(1,2-dihydroxy-1-phenylpropyl)phenyl)propanoic acid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.55 (1H, s), 7.48−7.38 (3H, m), 7.29−7.22 (3H, m), 7.19−7.12 (2H, m), 4.78 (1H, q, *J* = 6.2 Hz), 3.67 (1H, q, *J* = 7.1 Hz), 1.48−1.42 (3H, m), 1.09−1.02 (3H, m). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 179.4, 146.2, 143.9, 140.18, 140.15, 128.9, 128.2, 126.8, 126.4, 126.3, 125.9, 125.8, 125.6, 125.53, 125.49, 80.0, 72.0, 71.9, 45.5, 45.4, 18.2, 18.1, 16.7. ESI-MS *m*/*z* (%): 323.1 $[MNa]^+$ (25), 283.1 $[MH - H_2O]^+$ (7), 237.1 $[MH - H_2O\text{-}HCOOH]^+$ (100). Compound **3** was identified as 2,2′-((1,2-dihydroxy-1,2-diphenylethane-1,2-diyl)-bis(3,1-phenylene))dipropanoic acid, and the obtained NMR data was in agreement with the literature.[20]

Photolysis of *S*-(+)-1 and Isolation of Photoproduct. Illumination of *S*-(+)-**1** was performed for 3 min, and samples were taken every minute. The 3 min photolysis mixture was concentrated under reduced pressure and fractionated by semipreparative HPLC (isocratic, acetonitrile/water 1:3). Compound **4** was isolated and

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characterized as 2-(3-(4-(1-hydroxyethyl)benzoyl)phenyl)propanoic acid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.81−7.75 (3H, m), 7.68 (1H, d, J = 7.7 Hz), 7.56 (1H, d, J = 7.7 Hz), 7.50−7.43 (3H, m), 4.99 (1H, q, J = 6.6 Hz), 3.83 (1H, q, J = 7.3 Hz), 1.58−1.51 (6H, m). ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 196.1, 178.6, 150.5, 140.1, 138.0, 136.5, 131.6, 130.5, 129.3, 129.2, 128.6, 125.3, 70.0, 45.0, 25.3, 18.2. ESI-MS m/z (%): 321.0 $[MNa]^+$ (62), 299.0 $[MH]^+$ (95), 281.0 $[MH - H_2O]^+$ (100).

Photolysis of Amino Acid Analogues. Illumination of a mixture of the amino acid analogues benzylamine, 4-methylimidazole, 3-methylindole, 4-propylphenol, and 1-octanethiol was performed for 30 min, and samples were taken after 0, 5, 10, 15, and 30 min.

Photolysis of Ketoprofen (1) in the Presence of Amino Acid Analogues. Compound **1** and a mixture of the amino acid analogues benzylamine, 4-methylimidazole, 3-methylindole, 4-propylphenol, and 1-octanethiol were illuminated for 10 min. Samples were taken after 0, 1, 2, 3, 4, 5, and 10 min.

Photolysis of Ketoprofen (1) in the presence of Benzylamine and 3-Methylindole. Illumination of **1**, benzylamine, and 3-methylindole was performed for 4 min, and samples were taken every minute.

Photolysis of Benzylamine and 3-Methylindole Followed by Fractionation of the Mixture. Illumination of benzylamine and 3-methylindole was performed for 30 min, and samples were taken after 0, 5, 10, 15, and 30 min. After 30 min, the mixture was concentrated under reduced pressure and fractionated by flash chromatography on silica gel. Two pure fractions (compounds **10** and **11**) were obtained after elution with ethyl acetate/hexanes (1:4), and one additional fraction, containing a mixture of compounds **5** and **12**, was obtained after elution with ethyl acetate/hexanes (1:1). Compound **10** was characterized as *N*-(2-acetylphenyl) formamide, and the obtained ^{1}H NMR and ESI-MS spectra were in agreement with those from a previous report.[21] Compound **11** was characterized as 3-methyl-2-indolinone, and the obtained ^{1}H and ^{13}C NMR spectra were in accordance with those from previous reports.[22,23] During the analysis of the mixture of compounds **5** and **12**, compound **5** degraded in the NMR solvent ($CDCl_3$) so that, instead of a mixture of these two compounds, a rather pure sample of compound **12** was obtained. Compound **12** was characterized as 3-benzylquinazolin-4(3*H*)-one, and the obtained ^{1}H and ^{13}C NMR spectra were in agreement with the literature.[24] One more compound, compound **13**, was identified in the photolysis mixture. This compound was verified as 2-acetylaniline mixture by spiking with a reference compound. Compound **5** was identified by chemical synthesis as 3-benzyl-4-methylene-3,4-dihydroquinazoline; see below for details.

Photolysis of 3-Methylindole. 3-Methylindole was illuminated for 15 min, and samples were taken after 0, 5, 10, and 15 min. The major photoproduct from this experiment was *N*-(2-acetylphenyl) formamide **10**.

Synthesis of Compound 5 (3-Benzyl-4-methylene-3,4-dihydroquinazoline). All solvents used in the synthesis and purification were bubbled with nitrogen before usage. *N*-(2-Acetylphenyl) formamide (160 mg, 0.98 mmol) and benzylamine (105 μL, 0.96 mmol) were dissolved in EtOH (100 mL, 95%). The reaction mixture was stirred at room temperature for 20 h under an argon atmosphere and then refluxed for 10 h. The solvent was evaporated under reduced pressure, and the crude product was purified by flash chromatography (ethyl acetate/hexanes 1:4). Compound **5** was isolated and characterized as 3-benzyl-4-methylene-3,4-dihydroquinazoline, and the obtained ^{1}H and ^{13}C NMR spectra were in agreement with the literature.[25] Note that dihydroquinazoline **5** decomposes rapidly in the presence of oxygen.

Synthesis of *N*-Acetyl-L-Trp Ethyl Ester. A procedure was adapted from the literature as follows:[26] DIPEA (2.8 mL, 16 mmol) and acetic anhydride (0.76 mL, 8.0 mmol) were added dropwise to a stirred solution of L-Trp ethyl ester hydrochloride (2.15 g, 8.0 mmol) in dry dichloromethane (DCM) (40 mL) under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 4 h and then washed with H_2O (2 × 15 mL), saturated $NaHCO_3$ (2 × 15 mL), and HCl (1 M, 15 mL). When HCl was added, the product precipitated as a white solid. The precipitate was filtered and dried under vacuum to yield *N*-acetyl-L-Trp ethyl ester (1.19 g, 54%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.15 (bs, 1H), 7.53 (d, 1H, J = 8.0 Hz), 7.36 (d, 1H, J = 8.0 Hz), 7.19 (dd, 1H, J = 8.0, 7.1 Hz), 7.11 (dd, 1H, J = 8.0, 7.1 Hz), 6.97 (s, 1H), 6.02 (d, 1H, J = 7.9 Hz), 4.96−4.91 (m, 1H), 4.21−4.07 (m, 2H), 3.38−3.26 (m, 2H), 1.96 (s, 3H), 1.23 (t, 3H, J = 7.2 Hz) ^{13}C NMR ($CDCl_3$, 400 MHz): δ 172.1, 169.9, 136.2, 127.9, 122.7, 122.4, 119.8, 118.7, 111.3, 110.3, 61.6, 53.2, 27.7, 23.4, 14.2. ESI-MS m/z (%): 297.0 $[MNa]^+$ (28), 275.1 $[MH]^+$ (100), 229.0 $[M - OCHH_2CH_3]^+$ (50), 201.0 $[M - COOCH_2CH_3]^+$ (60).

Photolysis of Ketoprofen (1) in the Presence of Trp and Lys. Illumination of **1**, *N*-acetyl-L-Trp ethyl ester, L-Lys ethyl ester dihydrochloride, and KOH (20 mM) was performed for 15 min, and samples were taken after 0, 1, 2, 3, 4, 5, 10, and 15 min. Compound **17** was identified as ethyl 2-amino-6-(4-oxoquinazolin-3(4*H*)-yl)hexanoate by spiking with a synthetically prepared reference; see below for the synthetic procedure. The spiked chromatograms can be found in the Supporting Information.

Synthesis of Compound 17 (Ethyl 2-amino-6-(4-oxoquinazolin-3(4*H*)-yl)hexanoate). A slurry of anthranilic acid (1.30 g, 9.5 mmol) in formic acid (1 mL) was heated at 80 °C for 15 min, after which a solid product was formed. The solid still contained 15% anthranilic acid (quantified by NMR). Formic acid (8 mL) was added, and the obtained slurry was stirred for another 90 min at 80 °C. The slurry was concentrated under reduced pressure at 60 °C to a crystalline solid, which was dissolved in a mixture of acetone (100 mL) and acetonitrile (50 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure to a white crystalline solid (1.54 g) containing 2-formamidobenzoic acid (95%) and anthranilic acid (5%). The compound was used for the next synthetic procedure without any further purification.

A solution of DCC (138 mg, 0.67 mmol) in anhydrous DMF (0.8 mL) was added dropwise to a solution of 2-formamidobenzoic acid (100 mg, 0.61 mmol) and 1-hydroxybenzotriazole hydrate (89 mg, 0.66 mmol) in DMF (0.4 mL) under argon at 0 °C. The mixture was stirred for 10 min at 0 °C and 20 min at room temperature. A solution of K_2CO_3 (502 mg, 3.6 mmol) and L-Lys ethyl ester dihydrochloride (167 mg, 0.68 mmol) in H_2O (6.7 mL) was added dropwise, and the resulting mixture was diluted with additional H_2O (4.3 mL). The reaction was stirred for 20 h and then extracted with ethyl acetate (4 × 20 mL). The combined extracts were washed with brine (20 mL), dried over Na_2SO_4, filtered, and concentrated to a white solid. DCM (2 mL) was added to the solid, and the formed slurry was filtered to remove dicyclohexylurea. The filter cake was washed with additional DCM (3 mL), and the obtained filtrate was purified by flash chromatography (DCM/methanol 95:5 to 9:1) to afford ethyl 2-amino-6-(4-oxoquinazolin-3(4*H*)-yl)hexanoate (18 mg, 10% from anthranilic acid) as an uncolored oil. R_f = 0.2 (DCM/methanol 9:1). ^{1}H NMR (Me_2SO-d_6, 400 MHz): δ 8.39 (1H, s, H_2), 8.15 (1H, ddd, J = 8.0, 1.6, 0.6 Hz, H_5), 7.82 (1H, ddd, J = 8.2, 7.1, 1.6 Hz, H_7), 7.67 (1H, ddd, J = 8.2, 1.2, 0.6 Hz, H_8), 7.54 (1H, ddd, J = 8.2, 7.1, 1.2 Hz, H_6), 4.08−4.00 (2H, m, H_{15}), 3.97 (2H, t, J = 7.2 Hz, H_9), 3.37−3.22 (1H, m, H_{13}), 1.97 (2H, br s, NH_2), 1.74−1.63 (2H, m, H_{10}), 1.63−1.54 (1H, m, H_{12}), 1.53−1.42 (1H, m, $H_{12'}$), 1.40−1.29 (2H, m, H_{11}), 1.13 (3H, t, J = 7.1 Hz, H_{16}). ^{13}C NMR (Me_2SO-d_6, 400 MHz): δ 175.6 (C_{14}), 160.1 (C_4), 148.0 (C_2), 147.9 (C_{8a}), 134.2 (C_7), 127.1 (C_8), 127.0 (C_6), 126.0 (C_5), 121.5 (C_{5a}), 59.9 (C_{15}), 53.8 (C_{13}), 45.8 (C_9), 34.1 (C_{12}), 28.4 (C_{10}), 22.3 (C_{11}), 14.1 (C_{16}). HRMS (ES+TOF) calculated for $C_{16}H_{21}N_3O_3$ $[MH]^+$, 304.1661; found, 304.1662. ^{1}H NMR, ^{13}C NMR, and 2D NMR (COSY, HSQC, and HMBC) spectra used for assignment can be found in the Supporting Information.

RESULTS

Photolysis of Ketoprofen (1) in EtOH. When ketoprofen was photolyzed in EtOH, more than 50% was consumed after only 3 min (Figure 2). Five different peaks could be observed in the total ion count (TIC) chromatogram from the HPLC/MS analysis: two peaks had m/z 323 ($[MNa]^+$), and both

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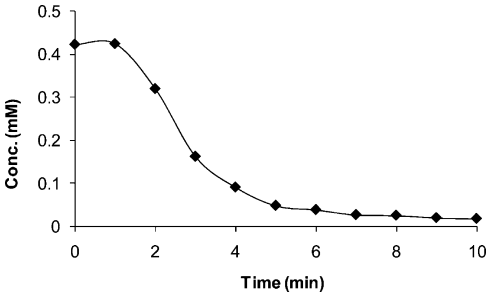

Figure 2. Depletion of ketoprofen during photolysis in ethanol.

correspond to different isomers of the ketoprofen ethylhydroxylated ketone (**2**), two peaks had m/z 533 ($[MNa]^+$), and both correspond to different isomers of the ketoprofen hydrodimer (**3**), and the fifth peak had m/z 299 ($[MH]^+$) and was characterized as the aromatic ethylhydroxylated ketoprofen (**4**); the TIC chromatogram from the HPLC/MS analysis is shown in Figure 3, and the structures of the compounds are shown in Figure 4. The ketoprofen hydrodimer (**3**) is known from a previous study by Bosca et al. in which **1** was irradiated in methanol.[20] Both the ketoprofen ethylhydroxylated ketone (**2**) and the aromatic ethylhydroxylated ketoprofen (**4**) are reaction products between ketoprofen and the solvent. In compound **2**, a 1-hydroxyethyl group has been added to the keto-carbonyl group, and in compound **4**, an aromatic hydrogen has been exchanged for the 1-hydroxyethyl group (Figure 4). To obtain enough pure material of compound **4** for characterization, a shorter photolysis experiment (3 min) had to be performed because **4** degraded during longer photolysis experiments. To further simplify the purification of the aromatic ethylhydroxylated ketoprofen (**4**), enantiomerically pure *S*-(+)-**1** was used in the shorter photolysis experiment to obtain a less complex mixture of diastereoisomers of this compound.

Photolysis of Ketoprofen (1) and Amino Acid Analogues. Ketoprofen together with a mixture of chemical analogues of the amino acids, 3-methylindole (Trp analogue), 4-propylphenol (Tyr analogue), 1-octanethiol (Cys analogue), benzylamine (Lys analogue), and 4-methylimidazole (His analogue), was photolyzed for 10 min in EtOH. Five new peaks in the TIC chromatogram from the HPLC/MS analysis (compounds **5**–**9**) could be detected after 2 min, and the amounts of these five compounds increased during the experiment; a representative chromatogram can be seen in

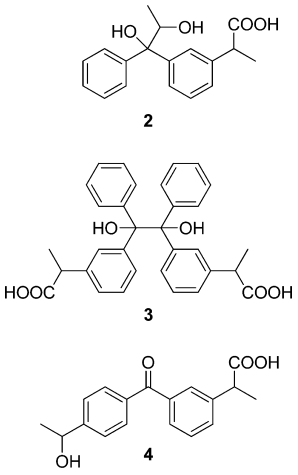

Figure 4. Characterized photoproducts from the UV illumination of ketoprofen in ethanol.

Figure 5. Compounds **6**–**9** are degradation products from ketoprofen and correspond to different photodecarboxylation

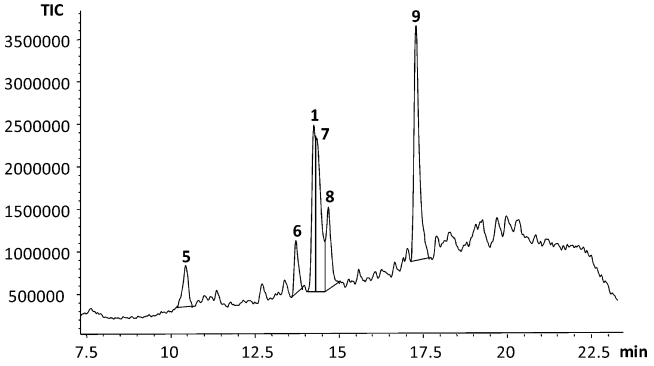

Figure 5. Total ion count chromatogram from the HPLC/MS analysis after 4 min photolysis of ketoprofen (**1**) in the presence of the chemical amino acid analogues.

products (Figure 6). All of these four products have been previously reported in studies where **1** was illuminated in buffered aqueous mediums.[20,27] Compound **5**, on the other hand, did not correspond to any of the ketoprofen degradation products previously reported in the literature. Furthermore, the fragmentation pattern did not agree with a compound containing a ketoprofen moiety or fragment (see Figure 7 for

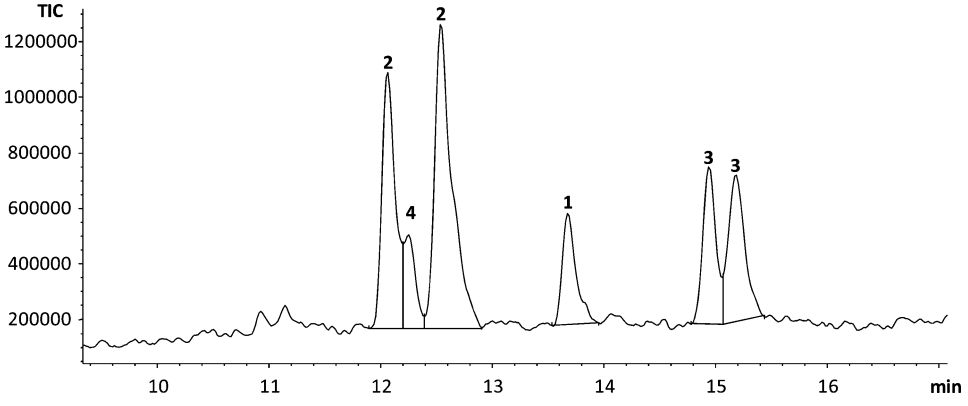

Figure 3. Total ion count chromatogram from the HPLC/MS analysis after 10 min photolysis of ketoprofen (**1**) in ethanol.

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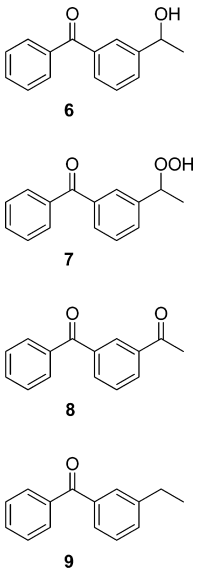

Figure 6. Photoproducts formed when ketoprofen was illuminated in the presence of amino acid analogues.

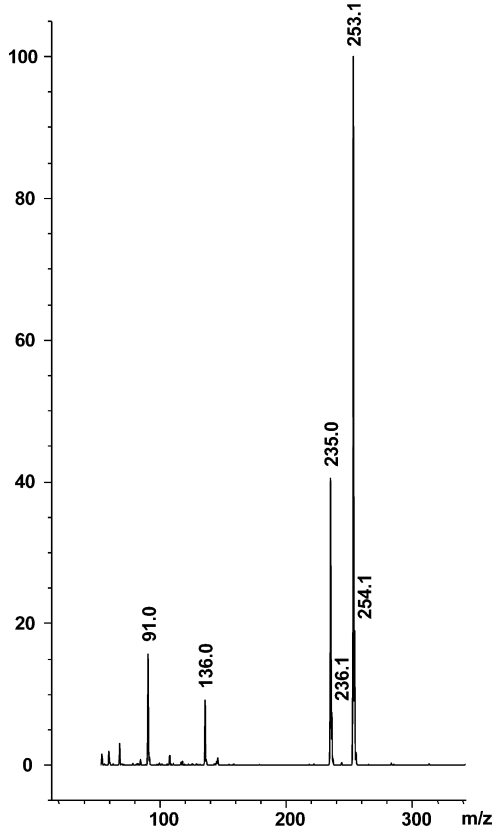

Figure 7. ESI-MS chromatogram for dihydroquinazoline **5**. The major peak is believed to be the H_2O adduct ($[MH + H_2O]^+$) with m/z 253, and the second largest peak, the $[MH]^+$ adduct with m/z 235.

the ESI-MS spectrum of **5**). Instead, it matched a reaction product derived from 3-methylindole and benzylamine. In addition, compound **5** could also be detected in the photolysis of the amino acid analogues in the absence of **1**, which proves that **5** does not contain a ketoprofen moiety. Although compound **5** is produced when **1** is not present, the formation rate is considerably slower. In EtOH, it takes approximately 30 min to give the same amount of compound **5** that is formed after only 3 min when **1** is present in the solution. It therefore seems as if ketoprofen promotes the formation of compound **5**. To establish whether compound **5** is actually a reaction product between the chemical analogues for the amino acids Lys and Trp, a photolysis experiment with only **1**, benzylamine, and 3-methylindole was conducted. Indeed, compound **5** was formed in approximately the same amount as in the photolysis experiment with **1** and all of the amino acid analogues. Although larger amounts of compound **5** were formed in the presence of **1**, a more complex mixture was also obtained because it contained all of the photodecarboxylation products (compounds **6**–**9**). Therefore, a mixture of only benzylamine and 3-methylindole was illuminated and fractionated in order to isolate compound **5**. Three different fractions were obtained, and two of them consisted of pure compounds: *N*-(2-acetylphenyl) formamide **10** and 3-methyl-2-indolinone **11** (Figure 8). One fraction contained compound **5**; however, it

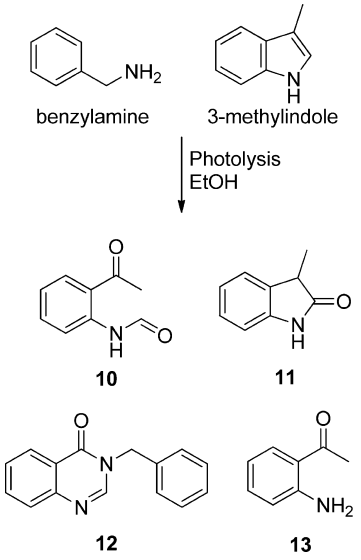

Figure 8. Identified products from the photolysis of benzylamine and 3-methylindole.

also contained another compound, quinazolinone **12**. After short time (10–15 min) in the NMR solvent ($CDCl_3$) at room temperature, compound **5** was completely degraded to quinazolinone **12** (Figure 8). In addition, one more compound, 2-acetylaniline **13** (Figure 8), was identified in the photolysis.

When irradiated alone, 3-methylindole (Trp analogue) gave *N*-(2-acetylphenyl) formamide **10** as the main photoproduct after 30 min. This verifies that **10** is formed from 3-methylindole alone in the presence of oxygen and that benzylamine is not involved in the formation of this compound. To better understand the mechanistic pathway that leads to compound **5**, synthesis of this compound was attempted by reacting *N*-(2-acetylphenyl) formamide **10** with benzylamine in EtOH (Figure 9). An inert atmosphere was used because oxygen was suspected to be the reason for the degradation of **5** to **12**. Indeed, small amounts of compound **5** were formed already at room temperature, and the amount increased during reflux. This result indicates that compound **5** is formed via a reaction of *N*-(2-acetylphenyl) formamide **10** and benzylamine in the photolysis experiments. By careful exclusion of oxygen during the reaction, workup, and purification, isolation of

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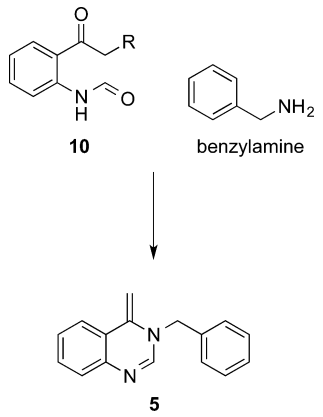

Figure 9. Synthetic procedure to obtain dihydroquinazoline **5**.

compound **5** from the synthetic mixture was successful. Compound **5** was characterized as 3-benzyl-4-methylene-3,4-dihydroquinazoline (Figure 10). The synthesis of this

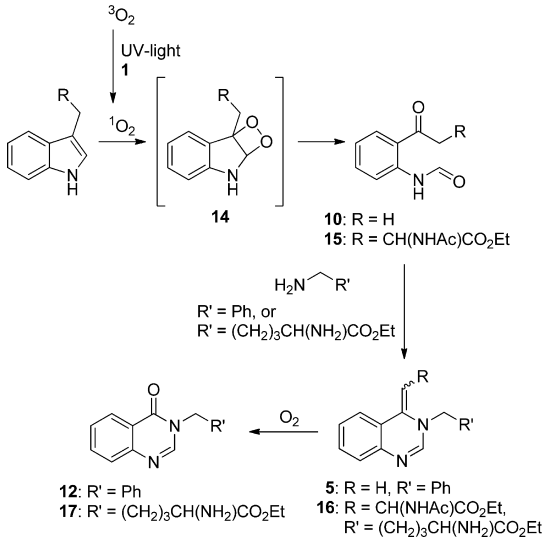

Figure 10. Tentative mechanism for the formation of dihydroquinazolines **5** and **16** as well as quinazolinones **12** and **17**. First, the indole derivative (3-methylindole or *N*-acetyl-L-Trp ethyl ester) reacts with singlet oxygen via a [2 + 2]-cycloaddition and forms an intermediate dioxethane (**14**). This unstable compound cleaves by ring-opening, and a dicarbonyl is formed (**10** or **15**). In the following step, the dicarbonyl (**10** or **15**) reacts with the amine (benzylamine or L-Lys ethyl ester) to form a dihydroquinazoline (**5** or **16**). In the presence of air, the dihydroquinazoline (**5** or **16**) readily oxidizes and forms a stable quinazolinone (**12** or **17**).

compound together with the observation that it readily decomposes to quinazolinone **12** in the presence of air was recently reported by Neue et al.[25] The major peak in the ESI-MS spectrum of dihydroquinazoline **5** is believed to be the H_2O adduct ($[MH + H_2O]^+$) with m/z 253, and the second largest peak is the $[MH]^+$ adduct with m/z 235 (Figure 7).

Photolysis of Ketoprofen (1) and the Amino Acids Lys and Trp. Ketoprofen and the amino acids Trp (*N*-acetyl L-Trp ethyl ester) and Lys (L-Lys ethyl ester) were irradiated for 15 min in EtOH to verify that the amino acids undergo the same reactions as their analogues (discussed above). A reaction scheme for the formation of the anticipated reaction product, dihydroquinazoline **16**, can be found in Figure 10. Two new peaks (t_R = 7–8.5 min), with the same MS-spectra, were observed already after 2 min, and they continued to increase during the first 5 min of irradiation of the sample. This corresponds to the point in time when **1** was consumed; see Figure 11a for a representative MS chromatogram of the photolysis mixture and Figure 11c for the ESI-MS spectrum for the new peaks. On the basis of the assumption that **16** would fragment in the same way as **5**, the m/z of these two new peaks matched the expected product, **16** (i.e., the $[MH + H_2O]^+$ adduct (m/z 463) is larger than the $[MH]^+$ adduct (m/z 445)) (Figure 11c). The two peaks that can be seen in the chromatogram are believed to be the two configurational isomers of the exocyclic double bond (i.e., the *E* and *Z* isomers). The HPLC/MS analysis of the 10 min photolysis sample showed a new peak (t_R = 10 min) that had not been present in the earlier samples; see Figure 11b for a representative MS chromatogram of the photolysis mixture and the ESI-MS spectrum for the new peak. The m/z of this peak matched the fragmentation of the expected quinazolinone, **17** (Figure 11d). This implies that dihydroquinazoline **16** degrades by the same route as dihydroquinazoline **5** (i.e., the exocyclic alkene is cleaved and a ketone is formed); see Figure 10. Quinazolinone **17** was synthesized to verify that this was in fact the observed compound (Figure 12). A two-step procedure was employed, starting with formylation of anthranilic acid, which produced 2-formamidobenzoic acid. In the following step, 2-formamidobenzoic acid was coupled with L-Lys ethyl ester by using DCC as a coupling reagent. This synthetic reference was used to spike the 15 min photolysis sample, which proved that it indeed was quinazolinone **17**; see Supporting Information for the spiked chromatograms.

DISCUSSION

Photolysis of Ketoprofen (1) in EtOH. The products found after the photolysis of **1** in EtOH are probably formed via a ketyl radical. No photodecarboxylation products were observed. Bosca et al.[20] performed photolysis experiments with **1** in methanol, and they report a clean and efficient transformation of **1** to its hydrodimer (compound **3**). This compound was also formed in substantial amounts in the photolysis experiment performed in this study. However, in our study, a large amount of **1** reacted with the solvent to give compounds **2** and **4**. These reaction products were not reported by Bosca et al.[20]

Photolysis of Ketoprofen (1) and Amino Acid Analogues. The result from the photolysis of **1** together with the chemical amino acid analogues 3-methylindole (Trp analogue), 4-propylphenol (Tyr analogue), 1-octanethiol (Cys analogue), benzylamine (Lys analogue), and 4-methylimidazole (His analogue) was completely different from that of the photolysis of **1** alone. In the presence of amino acid analogues, four different ketoprofen degradation products derived via photodecarboxylation[20,28] were identified (compounds **6**–**9**). None of these adducts were detected in the photolysis of **1** alone. Furthermore, none of the compounds that were formed during the illumination of **1** alone (compounds **2**–**4**) were detected when the amino acid analogues were present in the photolysis mixture.

Interestingly, one more compound, dihydroquinazoline **5**, was produced in the photolysis of **1** with the amino acid analogues. Much smaller amounts of this compound were also formed in the photolysis of the amino acid analogues in the absence of **1**, proving that this compound does not contain a

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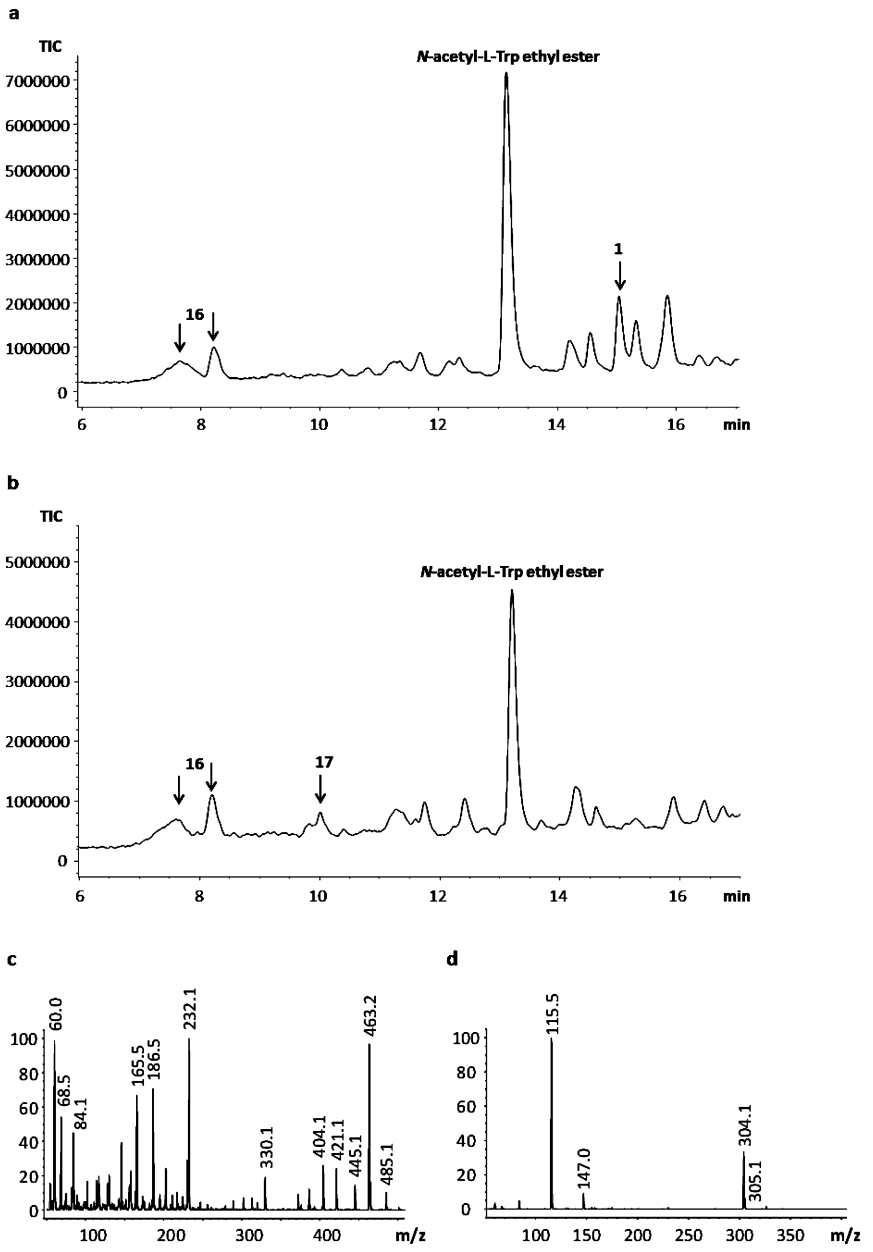

Figure 11. (a) Total ion count chromatogram from the HPLC/MS analysis after 4 min photolysis of ketoprofen (**1**) and the protected amino acids *N*-acetyl L-Trp ethyl ester and L-Lys ethyl ester. (b) Total ion count chromatogram from the HPLC/MS analysis after 15 min photolysis of ketoprofen (**1**) and the protected amino acids *N*-acetyl L-Trp ethyl ester and L-Lys ethyl ester. (c) ESI-MS spectrum for compound **16** (i.e., the two peaks with t_R = 7−8.5 min). (d) ESI-MS spectrum for compound **17** (i.e., the new peak in panel b with t_R = 10 min).

ketoprofen moiety. Photolysis experiments excluding the chemical analogues for Tyr (4-propylphenol), Cys (1-octanethiol), and His (4-methylimidazole) verified that dihydroquinazoline **5** was a reaction product between the analogues of Lys (benzylamine) and Trp (3-methylindole). However, it was not possible to isolate dihydroquinazoline **5** because it rapidly degraded into quinazolinone **12**. The instability of **5** toward oxygen has been noted earlier in the literature.[25] In addition to quinazolinone **12**, three other compounds, *N*-(2-acetylphenyl) formamide **10**, 3-methyl-2-indolinone **11**, and 2-acetylaniline **13**, were identified in the photolysis mixture (Figure 8). For quinazolinone **12** to be formed from benzylamine and 3-methylindole, one carbon atom has to be lost from 3-methylindole. First, *N*-(2-acetylphenyl) formamide **10** is most likely formed via a [2 + 2]-cycloaddition of singlet oxygen to 3-methylindole, giving dioxethane **14**. This labile compound then readily cleaves and forms compound **10**; see Figure 10. The same type of reaction has been reported previously in the literature for different substrates,[29,30] and this mechanistic pathway is further supported by the formation of *N*-(2-acetylphenyl) formamide **10** in the photolysis of 3-methylindole alone. Second, *N*-(2-acetylphenyl) formamide **10** reacts with the primary amine and forms dihydroquinazoline **5**. Finally, this adduct loses one carbon in an oxidation process, forming quinazolinone **12** (Figure 10). The suggested pathway for the formation of the dihydroquinazoline **5** and quinazolinone **12** was verified by mixing preformed *N*-(2-acetylphenyl) formamide **10** with benzylamine, which indeed gave dihydroquinazoline **5**. In solution and in the presence of air, **5** rapidly degrades to

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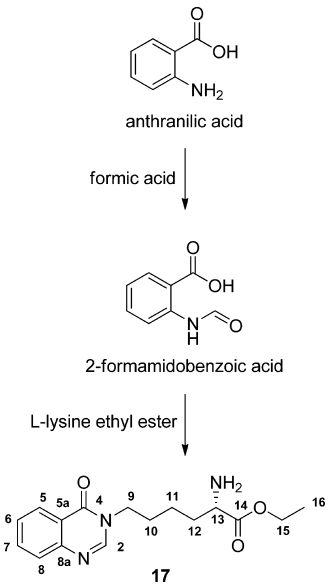

Figure 12. Synthetic procedure to obtain compound **17**.

quinazolinone **12**. The conversion of the exomethylene group in dihydroquinazoline **5** into a carbonyl group in the presence of oxygen has been recently reported.[25]

Photolysis of Ketoprofen (1) and Amino Acids Lys and Trp. To establish that the corresponding reaction would indeed take place between the amino acids themselves, a photolysis experiment was performed with ketoprofen, L-Lys ethyl ester, and *N*-acetyl-L-Trp ethyl ester. After only 2 min of irradiation, two new peaks with the expected *m*/*z* of compound **16** could be seen (Figures 10 and 11). These peaks kept growing until ketoprofen was consumed (around 5 min of irradiation). Interestingly, after 10 min of irradiation, when all ketoprofen was consumed, a new peak started to appear (Figure 11), and by the use of a synthetic reference, this compound was identified as quinazolinone **17** (Figures 10). This implies that dihydroquinazoline **16**, although it is not a terminal alkene, degrades in the presence of air in a similar mode as that of dihydroquinazoline **5** (Figure 10). Excellent experimental overlap between amino acid analogues (3-methylindole and benzylamine) and the actual amino acids (Trp and Lys) was observed.

Formation of a Specific Immunogenic Complex. The clinical observations often associated with photocontact allergy to ketoprofen may be explained by the fact that it forms a specific immunogenic complex that does not include ketoprofen itself. The general belief is that for allergenic compounds to cross-react they have to form the same or very similar immunogenic complexes, especially in terms of their three-dimensional structure.[31] In this case, a specific protein modification would result from the requirements of the described reaction (i.e., to enable the adduct formation the two amino acid residues (Lys and Trp) have to be placed in close proximity and in a specific relative orientation). Because the positioning in space of amino acid residues is determined by the amino acid sequence and the folding pattern of the protein, the requirements of the reaction limits the number of possible target proteins and directs the chemical modification to certain positions on a specific protein.

Relevance to Clinical Findings. The reports from the clinic that ketoprofen appears to photo-cross-react with compounds like etofenamate and octocrylene (Figure 1) is in marked contrast to other cross-reactivity studies,[32−34] where only structurally very similar compounds have had the ability to cross-react. However, if the immunogenic complex does not include the allergen itself, but its formation is only induced by the allergen, then these allergens can have rather different structures but still cross-react. For ketoprofen, the formation of the hypothetical immunogenic complex starts with ketoprofen-induced generation of singlet oxygen, and it is singlet oxygen that subsequently reacts with a Trp residue (Figure 10). It is therefore possible that compounds able to generate singlet oxygen will photo-cross-react with ketoprofen. This theory is supported by a published study that showed that octocrylene, one of the compounds associated with photo-cross-reactivity to ketoprofen, generates singlet oxygen upon radiation.[35] It is still uncertain whether all of these other cross-reacting compounds induce high enough concentrations of the suggested immunogenic complex itself to sensitize humans. However, among those who are already sensitized to this immunogenic complex, via the use of topical ketoprofen, the generated singlet oxygen concentrations may be high enough to cause an elicitation. This reasoning is supported by a photo-cross-reactivity study in guinea pigs by Sugiura et al.[36] in which they showed that animals photosensitized to ketoprofen also displayed a photoinduced reaction to benzophenone, but animals photosensitized to benzophenone did not react to ketoprofen. This is also in line with articles suggesting that photosensitization to ketoprofen leads to hyper-photosusceptibility to nonrelated allergens.[4,5,14] Because there is no curative therapy for contact or photocontact allergy, exposure to the allergenic compound has to be avoided.[15] For patients that have been photosensitized to ketoprofen, this means that it will not be enough to simply avoid ketoprofen but that they also have to avoid all other compounds that may generate singlet oxygen. This will obviously be extremely hard, and it is likely that many of those patients will often suffer from adverse skin reactions after being exposed to solar radiation.

CONCLUSIONS

We have shown that ketoprofen degrades rapidly when irradiated in ethanol. When no other compounds were in the solution, ketoprofen probably degraded via a ketyl radical, forming a ketoprofen hydrodimer (**3**) and two different solvent adducts (**2** and **4**). In the presence of chemical amino acid analogues for Tyr, Trp, Lys, Cys, and His, ketoprofen degraded via photodecarboxylation, and the same degradation products (**6**−**9**) were formed as those from previously reported photolysis experiments of ketoprofen in buffered aqueous medium.[20,27] However, more importantly, the formation of a reaction product (**5**) between the analogues of Trp and Lys was significantly enhanced in the presence of ketoprofen compared to that when the amino acid analogues were irradiated without ketoprofen. This unstable dihydroquinazoline, **5**, spontaneously degrades in the presence of air and forms quinazolinone **12**. The corresponding compounds dihydroquinazoline **16** and quinazolinone **17** were also detected in the experiment with actual amino acids (Lys and Trp) instead of analogues. A synthetic reference was used to establish the authenticity of quinazolinone **17**. The formation of a specific immunogenic complex that does not contain a ketoprofen moiety could explain the clinical observations associated with photoallergy to ketoprofen, such as photo-cross-allergy to a number of structurally different compounds. To the best of our knowl-

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edge, this is the first time that a specific immunogenic complex not containing the allergen has been suggested as the reason for contact or photocontact allergy.

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of the photoreactor equipment, standard curve for ketoprofen, identfication of quinazolinone **17** in the photolysis mixture, and NMR spectra for quinazolinone **17**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

NSAID, nonsteroidal anti-inflammatory drug; DMF, *N*,*N*-dimethylformamide; DIPEA, *N*,*N*-diisopropylethylamine; DCC, dicyclohexylcarbodiimide; EtOH, ethanol; DCM, dichloromethane; TIC, total ion count

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