Photodegradation of Dibenzoylmethanes: Potential Cause of Photocontact Allergy to Sunscreens

Isabella Karlsson,[†] Lisa Hillerström,[†] Anna-Lena Stenfeldt,[†] Jerker Mårtensson,[‡] and Anna Börje*,[†]

Dermatochemistry and Skin Allergy, Department of Chemistry, University of Gothenburg, SE-412 96 Gothenburg, Sweden, and Department of Chemical and Biological Engineering/Organic Chemistry, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden

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One of the most frequently observed photoallergens today is the sunscreen agent 4-*tert*-butyl-4′-methoxy dibenzoylmethane (**1a**). The structurally similar compound, 4-isopropyldibenzoylmethane (**1b**), was a common cause of sunscreen allergy in the eighties and early nineties but was removed from the market in 1993 and replaced with dibenzoylmethane **1a**. We have studied the photodegradation of the dibenzoylmethane **1a**, to better understand how these substances cause an immune reaction. Several expected degradation products were formed and identified. Of these, arylglyoxals and benzils were of particular interest because they were unexplored as potential contact allergens. The allergenic potential of photodegraded **1a** was evaluated by screening the formed arylglyoxals and benzils for their sensitizing capacity in the murine local lymph node assay. The arylglyoxals were found to be strong sensitizers. They were also found to be highly reactive toward the nucleophile arginine, which indicates that the immunogenic hapten−protein complex could be formed via an electrophilic−nucleophilic pathway. By varying the electron-withdrawing or -donating capacity of the substituent in the *para* position of the arylglyoxal, the electronic effects were shown to have no significant impact on either the sensitizing or the electrophilic power of arylglyoxals. Thus, a change in the substitution pattern of the parent dibenzoylmethane will not influence the sensitizing capacity of the products formed from them upon photodegradation. Furthermore, the combined studies of benzils, using the local lymph node assay and a cell proliferation assay, indicate that the benzils are cytotoxic rather than allergenic. Taken together, this study presents strong indication that photocontact allergy to dibenzoylmethanes is caused by the arylglyoxals that are formed upon photodegradation.

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Introduction

The increasing awareness, by the general public, of the carcinogenic and photoaging effects of UV radiation has resulted in an increase in the use of sunscreens. Chemical UV filters are frequently incorporated not only into sunscreens but also into facial cosmetics and toiletries (*1, 2*). Although photocontact allergy is an uncommon disorder, the frequency of reports concerning adverse reactions from UV filters are steadily increasing. Today, UV filters are suggested to be the most frequent cause of photocontact allergy (*1*−*6*). Contact allergy is caused by a wide range of chemicals upon skin contact. Its clinical manifestation, allergic contact dermatitis, is developed upon repeated contact with the allergen. The compounds that cause contact allergy are called haptens and are reactive chemicals of low molecular weight and appropriate lipofilicity. For a hapten to be recognized by the immune system and cause an allergic reaction, it has to react with a macromolecule (usually considered to be proteins) in the skin, thus forming an immunogenic hapten−protein complex (*7*). Photoallergic contact dermatitis arises when a compound subsequent absorption of light, usually UV radiation, forms a hapten or an immunogenic complex that causes an allergic reaction (*6, 8*). Compounds forming haptens once activated by light are called photoallergens.

4-*tert*-Butyl-4′-methoxy dibenzoylmethane (**1a**) is a widely used chemical UVA filter despite the fact that it is one of the most frequently observed photoallergens (*1, 5, 6*). 4-Isopropyldibenzoylmethane (**1b**) was a common cause of sunscreen allergy in the eighties and early nineties (*9, 10*) and was voluntarily removed from the market in 1993 (*11*) and replaced with dibenzoylmethane **1a**. Thus, it would be interesting to investigate if a change in the substitution pattern of the parent compound and its photodegradation products has any impact on the sensitizing capacity of these compounds. Both of these dibenzoylmethanes have been proven to photodegrade via a Norris type I radical mechanism and form stable electrophilic photoproducts such as benzoic acids, benzaldehydes, acetophenones, benzils, and arylglyoxals (*12*). However, to the best of our knowledge, the allergenic potential of the photodegradation products of dibenzoylmethanes has not been studied. Therefore, the following was set out to be studied, first to identify and quantify the photodegradation products of **1a** and, second, to investigate if the degradation products arylglyoxals and benzils might contribute to the photoallergenic potency of dibenzoylmethanes. To study the photodegradation of **1a**, a test series of three different dibenzoylmethanes was designed (**1a**,**c**,**d**, Figure 1). Photolysis reactions of these compounds were performed in a falling film photoreactor. Also, two dibenzoylmethanes (**1a**,**b**, Figure 1), four arylglyoxals (**2a**−**d**, Figure 1), and four benzils (benzil and **3a**,**c**,**d**, Figure 1) were screened for sensitizing

* To whom correspondence should be addressed. Tel: +46 31 772 4725. Fax: +46 31 772 3840. E-mail: aborje@chem.gu.se.
[†] University of Gothenburg.
[‡] Chalmers University of Technology.

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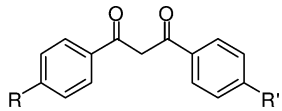
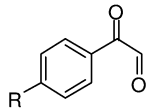
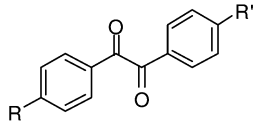


1a: R=C(CH$_3$)$_3$, R'=OCH$_3$
1b: R=CH(CH$_3$)$_2$, R'= H
1c: R=C(CH$_3$)$_3$, R'=C(CH$_3$)$_3$
1d: R=OCH$_3$, R'=OCH$_3$



2a: R=C(CH$_3$)$_3$
2b: R=OCH$_3$
2c: R=CH(CH$_3$)$_2$
2d: R=NO$_2$



Benzil: R=H, R'=H
3a: R=C(CH$_3$)$_3$, R'=OCH$_3$
3c: R=C(CH$_3$)$_3$, R'=C(CH$_3$)$_3$
3d: R=OCH$_3$, R'=OCH$_3$

Figure 1. Chemical structures of compounds studied in this paper.

capacity using the local lymph node assay (LLNA)[1] (*13*). The cytotoxic capacity of the benzils (benzil and **3a**,**c**,**d**) was investigated, as well as the chemical reactivity of the arylglyoxals **2b**−**d** toward the acetyl-protected amino acid arginine.

Experimental Procedures

Caution: *Skin contact with arylglyoxals* **2a**−**d** *must be avoided. As skin-sensitizing substances, these compounds must be handled with care.*

Chemicals. Unless otherwise indicated, reagents were obtained from commercial suppliers and used without further purification. Compound **1a** (purity 98%) was purchased from AK Scientific, Inc. (Mountain View, CA). Benzil (purity 98%), 4,4′-dimethoxybenzil (**3d**) (purity 98%), RPMI-1640 cell culture medium without phenol red (R7509), L-glutamine, sodium pyruvate, HEPES, and glucose were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone was purchased from Merck (Darmstadt, Germany), and olive oil was from Apoteket AB (Göteborg, Sweden). The CellTiter96AQ$_{ueous}$ nonradioactive cell proliferation assay was purchased from Promega (Madison, WI). Penicillin and streptomycin were obtained from Fisher Scientific AB (Göteborg, Sweden). 4-*tert*-Butyl-4′-metoxy diphenylacetylene (*14*), 4-*tert*-butylbenzoic acid (**4a**) (*15*), and 4-methoxybenzoic acid (**4b**) (*15*) were synthesized as previously described. THF was dried by distillation from sodium/benzophenone. The purity of test compounds for LLNA and cell viability was determined to be ≥98% (GC/MS) before testing.

Instrumentation and Mode of Analysis. 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 Professor de Meijere (*16*) with forced liquid circulation, purchased from NORMAG Labor- und Prozesstechnik. The photoreactor was equipped with a medium pressure mercury UV lamp (700 W, Heraeus, TQ 718, Z4 doped) as an irradiation source. The total radiant power of the lamp in the wavelength interval 200−600 nm was 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. (For a more detailed description of the photoreactor equipment, see the Supporting Information.)

Electron ionization mass spectral analysis (70 eV) was performed 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 a flame ionization detector, an HP-5MSI fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness), and helium as the carrier gas.

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 a fragmentor voltage of 70 V. A HyPURITY C18 column (150 mm × 3 mm, 3 μm particles, Thermo Hypersil-Keystone, Bellafonte, PA) 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.

^{1}H and ^{13}C NMR spectra were recorded on a JEOL eclipse+ 400 MHz spectrometer using Me_2SO-d_6 or $CDCl_3$ as the solvent. Chemical shifts (δ) are reported in ppm either relative to Me_2SO at δ 2.50 and δ 39.5 for ^{1}H and ^{13}C, respectively, or to $CHCl_3$ at δ 7.25 and δ 77.0 for ^{1}H and ^{13}C, respectively.

General Procedure for Preparation of Dibenzoylmethanes. A procedure was adapted from the literature (*17*) as follows: Sodium hydride (60% in mineral oil, 2.5 equiv) and dry THF (3.5 mL/mmol acetophenone) were added to an oven-dried flask. The mixture was cooled to 0 °C, and acetophenone (1.0 equiv) and ethylbenzoate (1.1 equiv) were added dropwise. The suspension was heated to reflux under N_2 for 16 h. The reaction mixture was allowed to reach room temperature and was filtered through Celite and washed once with ethanol (EtOH). The filtrate was treated with a mixture of ether and aqueous HCl (2 M). The organic phase was separated and washed three times with brine, dried over $MgSO_4$, and concentrated under reduced pressure.

4-Isopropyldibenzoylmethane (1b). Compound **1b** was prepared from 0.33 g of 4-isopropylacetophenone (2.0 mmol) and 0.32 g of ethylbenzoate (2.2 mmol) according to the general procedure. The crude product was purified by column chromatography on silica gel (5% ethyl acetate in hexanes) affording the product as a yellow oil (0.39 g, 73%). ^{1}H NMR ($CDCl_3$) δ: 1.30 (d, 6H, J = 6.9), 2.99 (septet, 1H, J = 6.9), 6.88 (s, 1H), 7.36 (d, 2H, J = 8.3), 7.46−7.59 (m, 3H), 7.96 (d, 2H, J = 8.3), 8.02 (d, 2H, J = 7.1). ^{13}C NMR ($CDCl_3$) δ: 23.9, 34.4, 93.0, 127.0, 127.2, 127.5, 128.8, 132.5, 133.3, 135.7, 154.2, 185.4, 186.1.

4,4′-Di-*tert*-butyldibenzoylmethane (1c). 4,4′-Di-*tert*-butyldibenzoylmethane was prepared from 0.35 g of 4-*tert*-butylacetophenone (2.0 mmol) and 0.45 g of 4-*tert*-butylethylbenzoate (2.2 mmol) according to the general procedure. The crude product was purified by column chromatography on silica gel (5% ethyl acetate in hexanes) affording the product as a pale orange solid (0.31 g, 47%). The obtained NMR was according to the literature (*18*).

4,4′-Dimethoxydibenzoylmethane (1d). 4,4′-Dimethoxydibenzoylmethane was prepared from 0.38 g of 4-methoxyacetophenone (2.5 mmol) and 0.46 g of 4-methoxyethylbenzoate (2.75 mmol) according to the general procedure. The crude product was purified

[1] Abbreviations: LLNA, local lymph node assay; EtOH, ethanol; SI, stimulation index; EC3, the estimated concentration required to induce an SI of 3; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium inner salt; PMS, phenazine methosulfate.

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by recrystallization in EtOH affording the product as a pale yellow solid (0.29 g, 51%). The obtained NMR was according to the literature (*19*).

General Procedure for Preparation of Arylglyoxal Hemiacetal Dimers (2a″ and 2b″) and Arlylglyoxal Hydrates (2c′ and 2d′). Arylglyoxal hemiacetal dimers (**2a″** and **2b″**) and arylglyoxal hydrates (**2c′** and **2d′**) were synthesized using the literature procedure described by Floyd et al. (*20*). Aqueous HBr (48%, 3.4 mL, 30 mmol) was added dropwise to a stirred solution of acetophenone (10 mmol) in Me_2SO (15 mL) at room temperature. The mixture was heated to 55 °C, and the reaction was stirred until TLC showed that all starting material was consumed. After 19 h, the solution was poured onto ice.

4-*tert*-Butylphenylglyoxal Hemiacetal Dimer (2a″). 4-*tert*-Butylphenylglyoxal hemiacetal dimer was prepared from 1.76 g (10 mmol) of 4-*tert*-butylacetophenone according to the general procedure, affording a yellow oil. The mixture was extracted with chloroform and concentrated under reduced pressure. The product was purified with column chromatography on silica gel (ethyl acetate/hexanes 1:4) affording the product as a yellow oil. The product was then precipitated from cold hexanes, which afforded the product as a white powder (0.76 g, 38%). ^{1}H NMR (Me_2SO-d_6) δ: 1.29 (s, 9H), 6.00 (d, 1H, J = 9.5), 7.38 (d, 1H, J = 9.5, *OH*), 7.50 (d, 2H, J = 8.4), 8.0 (d, 2H, J = 8.4). ^{13}C NMR (Me_2SO-d_6) δ: 31.3, 35.5, 91.2, 125.9, 130.1, 131.3, 157.3, 193.8. Anal. calcd for $C_{24}H_{30}O_5 \cdot 0.17C_6H_{14}$: C, 72.80; H, 7.95; O, 19.24. Found: C, 72.17; H, 7.91; O, 19.03.

4-Methoxyphenylglyoxal Hemiacetal Dimer (2b″). 4-Methoxyphenylglyoxal hemiacetal dimer was prepared from 1.50 g (10 mmol) of 4-methoxyacetophenone according to the general procedure, affording a yellow oil. The mixture was extracted with dichloromethane and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (ethyl acetate/hexanes 1:1) affording the product as a pale yellow powder (1.27 g, 73%). ^{1}H NMR (Me_2SO-d_6) δ: 3.85 (s, 3H), 5.98 (d, 1H, J = 9.7), 7.02 (d, 2H, J = 8.9), 7.32 (d, 1H, J = 9.7, O*H*), 8.06 (d, 2H, J = 8.9). ^{13}C NMR δ: (Me_2SO-d_6) 56.1, 91.1, 114.4, 126.7, 132.5, 164.1, 192.6. Anal. calcd for $C_{18}H_{18}O_7$: C, 62.42; H, 5.24; O, 32.34. Found: C, 62.26; H, 5.23; O, 32.28.

4-Isopropylphenylglyoxal Hydrate (2c′). 4-Isopropylphenylglyoxal hydrate was prepared from 1.62 g (10 mmol) of 4-isopropylacetophenone according to the general procedure, affording a light yellow precipitate. Recrystallization from acetone−pentane gave the product as a white solid (0.975 g, 50%). ^{1}H NMR (Me_2SO-d_6) δ: 1.23 (d, 6H, J = 6.9), 2.96 (septet, 1H, J = 6.9), 5.66 (t, 1H, J = 7.3), 6.68 (d, 2H, J = 7.3, O*H*), 7.39 (d, 2H, J = 8.1), 8.00 (d, 2H, J = 8.1). ^{13}C NMR (Me_2SO-d_6) δ: 24.1, 32.0, 89.6, 127.0, 130.2, 132.1, 154.8, 196.3. Anal. calcd for $C_{11}H_{14}O_3$: C, 68.02; H, 7.27; O, 24.71. Found: C, 68.12; H, 7.19; O, 24.53.

4-Nitrophenylglyoxal Hydrate (2d′). 4-Nitrophenylglyoxal hydrate was prepared from 1.50 g (10 mmol) of 4-nitroacetophenone according to the general procedure, affording a yellow oil. The mixture was extracted with ethyl acetate and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (ethyl acetate/hexanes 1:1) affording the product as a pale yellow powder (0.875 g, 44%). ^{1}H NMR (Me_2SO-d_6) δ: 5.66 (t, 1H, J = 6.8), 7.06 (d, 2H, J = 6.8, O*H*), 8.29 (d, 2H, J = 8.5), 8.35 (d, 2H, J = 8.5). ^{13}C NMR (Me_2SO-d_6) δ: 90.6, 124.09, 131.4, 139.1, 150.4, 195.9.

4-*tert*-Butyl-4′-metoxybenzil (3a). 4-*tert*-Butyl-4′-metoxybenzil was synthesized using the literature procedure described by Mousset et al. (*21*). 4-*tert*-Butyl-4′-metoxy-diphenylacetylene (0.48 g, 1.8 mmol) and PdI_2 (19 mg, 0.09 mmol) in Me_2SO (13 mL) were stirred at 140 °C for 2 h. After the mixture was cooled to room temperature, H_2O (30 mL) was added, and the mixture was extracted three times with ethyl acetate (30 mL). The organic layers were then combined and washed with aqueous saturated NH_4Cl solution, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (10% ethyl acetate in hexanes) affording the product as a yellow oil (0.49 g, 92%). ^{1}H NMR ($CDCl_3$) δ: 1.33 (s, 9H), 3.87 (s, 3H), 6.96 (d, 2H, J = 9.0), 7.50 (d, 2H, J = 8.7), 7.89 (d, 2H, J = 8.7), 7.94 (d, 2H, J = 9.0). ^{13}C NMR ($CDCl_3$) δ: 31.0, 35.4, 55.7, 114.4, 126.1, 126.3, 130.0, 130.8, 132.5, 158.9, 165.0, 193.5, 194.7. Anal. calcd for $C_{19}H_{20}O_3$: C, 77.00; H, 6.80; O, 16.20. Found: C, 76.41; H, 6.73; O, 16.17.

4,4′-Di-*tert*-butylbenzil (3c). A procedure adapted from the literature (*22*) was used as follows: EtOH (6 mL) and 4-*tert*-butylbensaldehyde (3.34 mL, 20 mmol) were added to a solution of potassium cyanide (260 mg, 4 mmol) in water (4 mL). The mixture was heated to reflux for 18 h. After it was cooled, the reaction mixture was poured on to ice, and the crude benzoin derivative was percipitated. The solid was collected and washed with water and pentane. The crude di-*tert*-butylbenzoin was transferred to a 25 mL round-bottomed flask, and concentrated nitric acid (5 mL) was added. The mixture was heated to reflux for 90 min and was then poured into ice water. The mixture was extracted with chloroform, washed twice with water, dried over Na_2SO_4, and concentrated under reduced pressure. The product was afforded as a pale yellow powder (1.34 g, 42%). ^{1}H NMR ($CDCl_3$) δ: 1.33 (s, 18H), 7.51 (d, 4H, J = 8.7), 7.90 (d, 4H, J = 8.7). ^{13}C NMR ($CDCl_3$) δ: 31.1, 35.5, 126.1, 130.0, 130.7, 159.0, 194.6.

GC/MS Method for Determination of the Purity of Test Compounds for LLNA. The GC was equipped with an on-column injector. The column temperature was 35 °C at injection, held isothermally for 1 min, raised to 250 °C at a rate of 10 °C/min, and finally held at 250 °C for 5 min.

General Procedure for Photolysis of Dibenzoylmethanes. Photolysis was performed on 350 mL, 2 mM solutions of dibenzoylmethanes in both cyclohexane and EtOH. The samples were illuminated for 60 min at 35−40 °C. After 5, 15, 30, and 60 min, samples of 4 mL were withdrawn from the photoreactor and analyzed with HPLC/MS and GC/MS. The reaction time for the photolysis experiment with **1a** was extended to 90 or 210 min when performed in cyclohexane or EtOH, respectively. Samples were collected at 5, 15, and 30 min and thereafter every 30 min.

GC/MS Analysis of Photodegradation Products from Photolysis of Dibenzoylmethanes. The GC was equipped with a split/splitless inlet and an injection port temperature of 270 °C. The column temperature was 100 °C at injection and raised to 200 °C at a rate of 5 °C/min, then raised from 200 to 270 °C at a rate of 15 °C/min, and finally held at 270 °C for 20 min. Each sample was diluted with dichloromethane (1:10) prior to injection of 1 μL.

HPLC/MS Analysis of Photodegradation Products from Photolysis of Dibenzoylmethanes. For each sample, 5 μL aliquots were injected onto the column and eluated with a gradient flow of 0.40 mL/min. A linear gradient from 5 to 100% B in 20 min was followed by 10 min of isocratic eluation at 100% B. The column was equilibrated with 5% B for 10 min between each run. Photoreaction products were detected with a diode array detector at 265.4 and 280.4 nm. Quantification of photoreaction products was performed using standard curves for each product.

Sensitization Experiments in Mice. The sensitizing potency of the dibenzoylmethanes **1a** and **1b**, arylglyoxals **2a−d**, and benzils benzil and **3a**,**c**,**d** was investigated using the LLNA (*23*). All animal procedures were approved by the local ethics committee. Each compound was tested in five different concentrations using mice in groups of three. Compounds **2a″**, **2b″**, **2c′**, and **2d′** below form in solution the corresponding glyoxals **2a−d** (vide infra). Used were the following concentrations (% w/v): **1a**, 0.032 (1%) to 2.6 M (80%); **1b**, 0.038 (1%) to 3.0 M (80%); **2a″**, 2.5×10^{-4} (0.01%) to 0.25 M (10%); **2b″**, 2.9×10^{-4} (0.01%) to 0.29 M (10%); **2c′**, 0.0026 (0.05%) to 1.5 M (30%); **2d′**, 5.1×10^{-4} (0.01%) to 0.51 M (10%); benzil, 0.0048 (0.1%) to 1.2 M (25%); **3a**, 0.0034 (0.1%) to 0.34 M (10%); **3c**, 0.0031 (0.1%) to 0.31 M (10%); and **3d**, 0.0037 (0.1%) to 0.092 M (2.5%). Exact figures for each concentration are given in the Supporting Information. Briefly, groups of female CBA/CA mice received 25 μL of a solution of test compound, dissolved in the vehicle acetone/olive oil (4:1 v/v), on the dorsum of the ears daily for three consecutive days. Control animals were treated in the same way with vehicle alone. All mice were injected intravenously 5 days after the first treatment, with

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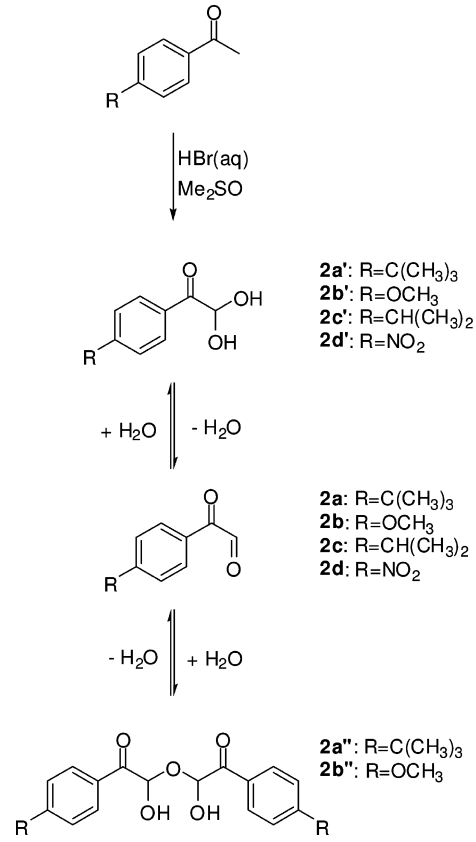
250 μL of PBS (pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer solution) containing 20 μCi of [^{3}H]-thymidine. Five hours later, the draining lymph nodes were excised and pooled for each group, and a single cell suspension of lymph node cells was prepared. The thymidine incorporation was measured by β-scintillation counting. Results are expressed as the mean dpm/lymph node for each experimental group and as stimulation index (SI). The SI is defined as the ratio between dpm/lymph node for the test group and the control group. Test materials that at one or more concentrations caused an SI greater than 3 were considered to be positive in the LLNA. EC3 values (the estimated concentration required to induce an SI of 3) were calculated by linear interpolation (*24*). The sensitizing potency of the test compounds was classified according to the following: <0.1%, extreme; ≥0.1 to <1%, strong; ≥1 to <10% moderate; and ≥10 to <100%, weak (*25*).

Cell Culture. The human U937 monoblastoid cell line originally from American Type Culture Collection (Rockville, MD) was purchased through LGC Standards AB (Borås, Sweden). The cells were cultured at 37 °C with 5% CO_2 in phenol red-free RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 14 mM glucose, 100 U/mL penicillin, 100 μg streptomycin, and 10% fetal bovine serum. Cells were harvested at the ninth passage and used in the cytotoxic assay.

Cytotoxic Studies. The CellTiter96AQ$_{ueous}$ nonradioactive cell proliferation assay was used to study the cytotoxicity of the compounds benzil and **3a**,**c**,**d**. The cytotoxic assay was adopted from previously published papers (*26, 27*). In this assay, solutions of a tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt (MTS)] and an electron-coupling reagent [phenazine methosulfate (PMS)] are used. The MTS assay is based on the ability of viable cells to metabolize the tetrazolium salt into a colored formazan product. The absorbance of the formazan at 492 nm is directly proportional to the number of living cells. Cells were seeded into 96-well plates at a density of 5000 cells/well in 0.1 mL of RPMI-1640 medium with all of the additives (see cell culture) except for the antibiotics and cultured at 37 °C with 5% CO_2 for 3 h. Stock solutions of 100 mM benzil and **3a** were prepared in EtOH and diluted to 25 and 12.5 mM with EtOH. One microliter of each solution was added to the wells, giving final concentrations of 1, 0.25, and 0.125 mM benzil and **3a** and 1% EtOH. Because of solubility problems, **3c** was prepared as a 50 mM stock solution and diluted to 12.5 and 6.25 mM with EtOH. Two microliters of each solution was added to the wells, giving final concentrations of 1, 0.25, and 0.125 mM **3c** and 2% EtOH. A mixture of Me_2SO/EtOH 1:10 had to be used to solvate **3d**, and even then, no more than 25 mM could be dissolved. The stock solution was diluted to 12.5 and 6.25 mM with EtOH, and 2 μL was added to the wells giving the following final concentrations of **3d**: 0.5 (0.2% Me_2SO and 1.8% EtOH), 0.25 (0.1% Me_2SO and 1.9% EtOH), and 0.125 mM (0.05% Me_2SO and 1.95% EtOH). Copper sulfate (0.2 and 0.02 mM) was used as a positive control. Medium with solvents and cells alone served as negative controls. All test compounds and controls were added to the 96-well plate in triplicates. The plate was incubated with the test compounds at 37 °C with 5% CO_2 for 24 h. MTS/PMS reagents were mixed in a 20:1 ratio, 20 μL was added to each well, and the cells were incubated for another 2 h, after which the absorbance was measured at 492 nm. After the measurement, tryphan blue was added, and the cell status was investigated visually by light microscopy.

Reactivity Experiments of Arylglyoxals toward N_α-Acetyl-L-Arg. A solution of arylglyoxal equivalents (vide infra) (0.02 mmol) in Me_2SO (200 μL) and phosphate buffer, pH 7.0 (800 μL, 26.0 mM potassium dihydrogen phosphate and 103.6 mM disodium hydrogen phosphate), was added dropwise to a stirred solution of N_α-acetyl-L-Arg (4.3 mg, 0.02 mmol) and 2-nitrotoluene (1.78 μL, 0.02 mmol) in Me_2SO (200 μL) and phosphate buffer, pH 7.0 (800 μL, 26.0 mM potassium dihydrogen phosphate and 103.6 mM disodium hydrogen phosphate), at room temperature. At specific intervals (15, 45, 75, 105, 135, 165, and 205 min), aliquots of 100 μL were removed and diluted with milli-Q water to 1000 μL prior to HPLC/MS analysis.

Scheme 1. Synthetic Route to the Dimeric Hemiacetals and the Hydrates of Studied Arylglyoxals



HPLC/MS Analysis of N_α-Acetyl-L-Arg Conjugates. For each sample, 2 μL aliquots were injected onto the column and eluted with a gradient flow of 0.40 mL/min. A linear gradient from 5 to 100% B in 20 min was used. The column was equilibrated with 5% B for 10 min between each run. Arylglyoxals were detected with a diode array detector at their respective absorption maximum, that is, 264 nm for compounds **2c** and **2d** and 284 nm for compound **2b**. Quantification of the arylglyoxals with HPLC/UV was performed using standard curves for the arylglyoxals and 2-nitrotoluene as an internal standard.

Results and Discussion

Compound **1a** is frequently used as a UVA filter in commercial sunscreen formulations, to protect the skin from the deep-reaching UVA radiation. To date, two dibenzoylmethanes have been used commercially, the dibenzoylmethane **1a** and **1b**. In the early 1990s, **1b** was removed from commercial formulations as it caused many cases of photoallergy and was replaced by the structurally similar compound **1a**. However, the only difference between the two compounds is the substituent in the *para* positions (Figure 1). Both dibenzoylmethanes have been shown to photodegrade and form electrophilic products, such as arylglyoxals and benzils (*12*). Furthermore, both of the latter classes of compounds possess the structural motives usually considered as structural alerts for alkylating agents and potential allergens (*28*). Thus, we were interested in investigating the sensitizing capacity of these compounds and to investigate if a change in the substitution pattern of the parent compounds and their photodegradation products could modulate this capacity. In the remainder of this paper, results from such investigations are presented and discussed. The presentation commences

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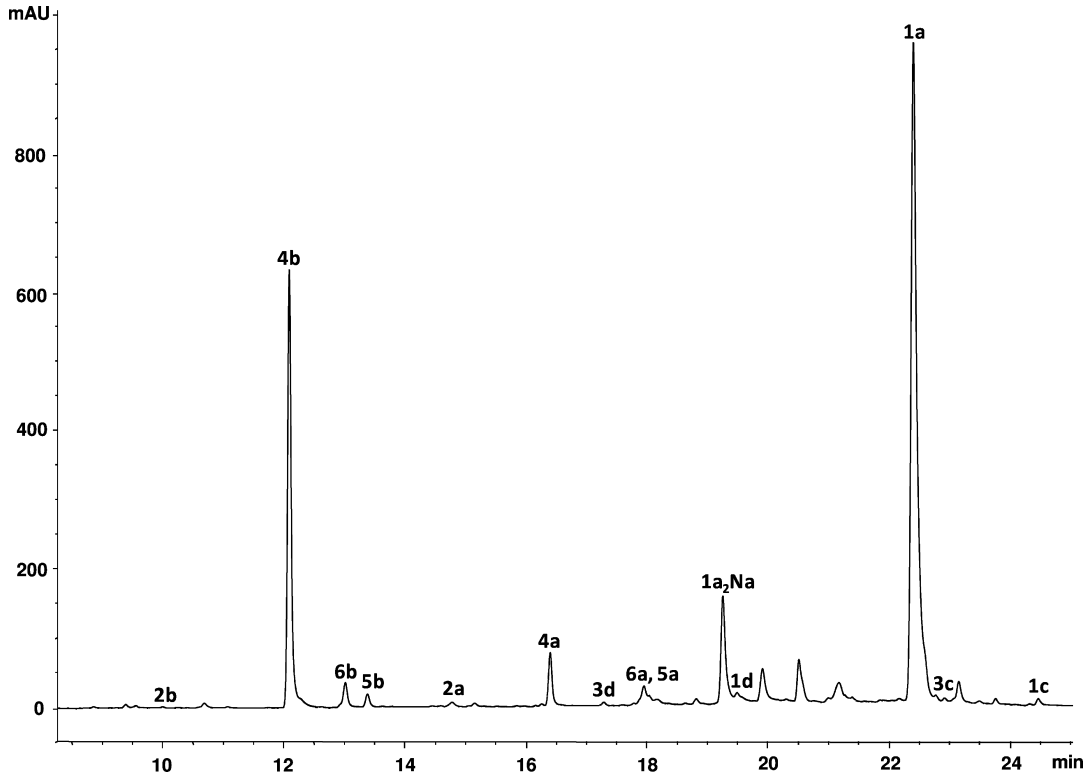

Figure 2. HPLC chromatogram recorded at 265.4 nm from analysis of the photodegradation mixture of **1a** after 90 min. Photolysis was performed on 2 mM solutions of dibenzoylmethane **1a** in cyclohexane. The observed peaks correspond to dibenzoylmethanes: 4,4′-di-*tert*-butyldibenzoylmethane (**1c**), 4,4′-dimethoxydibenzoylmethane (**1d**), and a parent dimeric sodium adduct (**1a**$_2$Na); arylglyoxals: 4-*tert*-butylphenylglyoxal (**2a**) and 4-methoxyphenylglyoxal (**2b**); benzils: 4,4′-di-*tert*-butylbenzil (**3c**) and 4,4′-dimetoxybenzil (**3d**); benzoic acids: 4-*tert*-butylbenzoic acid (**4a**) and 4-methoxybenzoic acid (**4b**); benzophenones: 4-*tert*-butylacetophenone (**5a**) and 4-methoxyacetophenone (**5b**); and benzaldehydes: 4-*tert*-butylbenzaldehyde (**6a**) and 4-methoxybenzaldehyde (**6b**).

with a short account of the preparation of the necessary compounds. It is followed by a discussion of the photodegradation of dibenzoylmethanes **1a** and the identification of degradation products as well as the measurement of their relative amounts. Sensitization and cytotoxicity experiments are then presented that show that the arylglyoxals and benzils formed in the photodegradation are strong sensitizers and cytotoxic, respectively. It is finally argued, on the basis of comparative reactivity studies of arylglyoxals capability as electrophiles, that the substituents on the parent dibenzoylmethanes have no significant influence on the sensitizing potency of the arylglyoxals formed in the photodegradation.

Synthesis. The symmetrical dibenzoylmethanes **1c** and **1d** as well as the unsymmetrical monoisopropyl analogue **1b** were all obtained in high purity according to literature (*17*), via a mixed Claisen condensation between their corresponding acetophenones and ethylbenzoates. The arylglyoxals **2a**−**d** were synthesized as their hemiacetal dimers (**2a**″ and **2b**″) or hydrates (**2c**′ and **2d**′) in high purity but in moderate yields from their corresponding acetophenones via an oxidation with aqueous hydrobromic acid in dimethyl sulfoxide (Scheme 1) (*20*). In the prescence of water, an equilibrium between the glyoxal and its hydrate will rapidly be established (Scheme 1) (*29, 30*). Thus, because the hydrates have been found to be more stable than the corresponding glyoxals (*29*), the arylglyoxal hydrates were the prime targets for the synthesis. However, we were not able to crystallize the arylglyoxals **2a** and **2b** in their hydrate form. Instead, these were found to crystallize in a dimeric hemiacetal form. In addition, the *tert*-butyl analogue cocrystallized with a small fraction of solvent molecules. Such crystallization behavior has been reported earlier for other arylglyoxals (*31, 32*). However, similarly to the hydrate, the dimeric hemiacetal form is in solution in equilibrium with the corresponding glyoxal (Scheme 1) (*33*). This was confirmed by HPLC/MS (not shown) and NMR studies. The dimers are perfectly stable in Me_2SO-d_6 but hydrolyze in D_2O/Me_2SO-d_6 (1:1) to the respective hydrates on a shorter time scale than the time course of preparation and insertion of the sample into the NMR spectrometer (≪5 min). This is also in accordance with the results obtained from the comparative study of the reactivity for the hydrates **2c**′ and **2d**′ and hemiacetals **2a**″ and **2b**″ toward argine (vide infra), which indicate identical reactivities for the dimers as for the hydrates. Thus, both the hydrate form and the hemiacetal dimer can be considered to be arylglyoxal equivalents. (The hydrate corresponds to one unit of arylglyoxal and the hemiacetal dimer to two units.) From here on, these equilibrium mixtures will always be referred to as arylgloxals **2a**−**d**.

The unsymmetrical 4-*tert*-butyl-4′-methoxybenzil **3a** was synthesized as previously described (*21*), via an oxidation of the corresponding alkyne in a yield of 92%. The symmetrical di-*tert*-butylbenzil **3c** was synthesized in two steps according to the literature (*22*). Accordingly, the corresponding benzoin was synthesized from 4-*tert*-butylbenzaldehyde and subsequently oxidized to the benzil in a total yield of 42%.

Photodegradation of Dibenzoylmethanes. It has previously been shown that the dibenzoylmethanes **1a** and **1b** photodegrade via a Norris type I radical mechanism (*12*). The photodegradation products were identified using mass spectrometry, but they were not quantified. To establish the relative amounts of the different degradation products formed in the photodegradation, we repeated the experiment with **1a** in a falling film photoreactor. The outcome of the photochemical degradation reactions was analyzed by HPLC- and GC/MS. Reference compounds were used to identify the different degradation

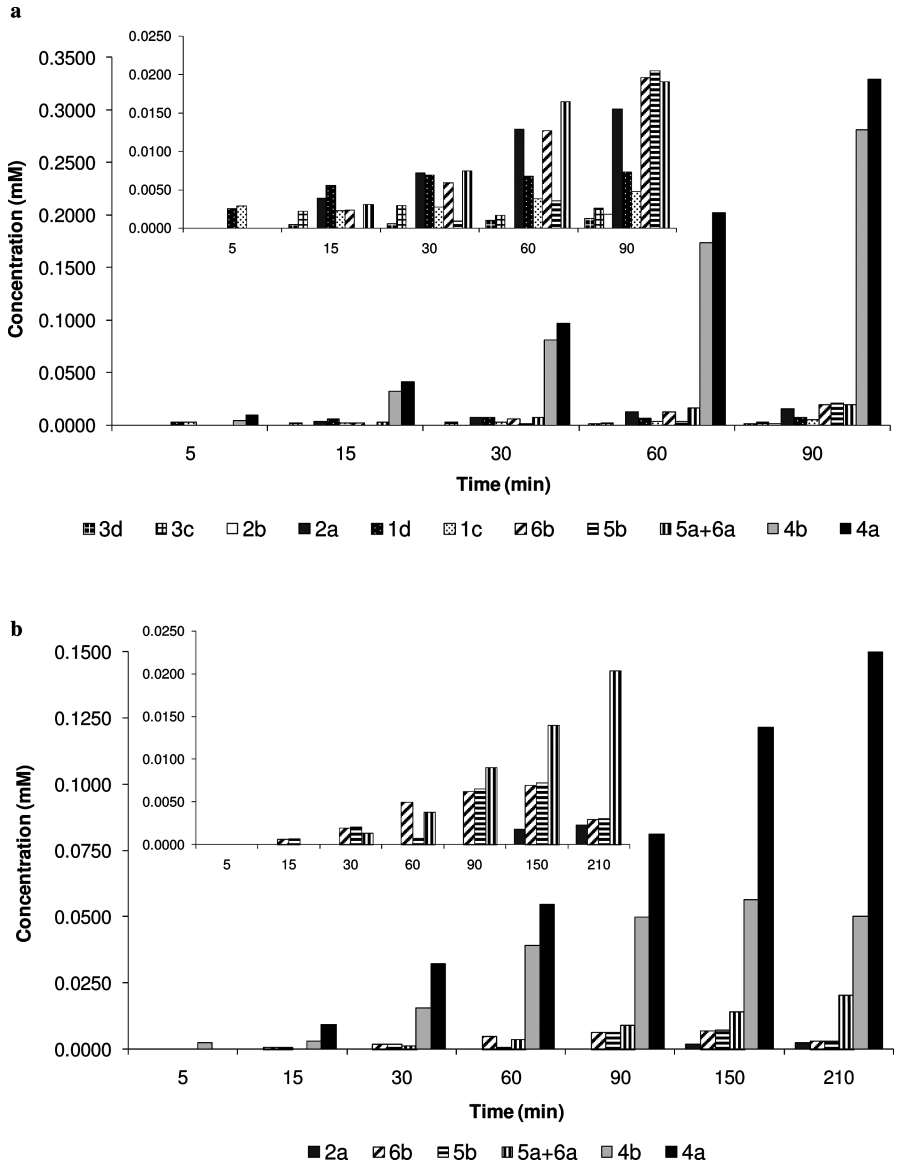

Figure 3. Photoproduct distribution in photolysis of the dibenzoylmethane **1a**. Photolysis was performed on 350 mL, 2 mM solutions of dibenzoylmethane **1a** in both cyclohexane and EtOH. (a) Photolysis performed in cyclohexane for 90 min. The inset is an enhancement of the concentrations of photoproducts when the benzoic acids are excluded. (b) Photolysis performed in EtOH for 210 min. The inset is an enhancement of the concentrations of photoproducts when the benzoic acids are excluded. Dibenzoylmethanes: 4,4′-di-*tert*-butyldibenzoylmethane (**1c**) and 4,4′-dimethoxydibenzoylmethane (**1d**); arylglyoxals: 4-*tert*-butylphenylglyoxal (**2a**) and 4-methoxyphenylglyoxal (**2b**); benzils: 4,4′-di-*tert*-butylbenzil (**3c**) and 4,4′-dimetoxybenzil (**3d**); benzoic acids: 4-*tert*-butylbenzoic acid (**4a**) and 4-methoxybenzoic acid (**4b**); benzophenones: 4-*tert*-butylacetophenone (**5a**) and 4-methoxyacetophenone (**5b**); and benzaldehydes: 4-*tert*-butylbenzaldehyde (**6a**) and 4-methoxybenzaldehyde (**6b**).

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products, and standard curves were used for quantification. Because **1a** is an unsymmetrical dibenzoylmethane, it can give rise to a large number of degradation products. Therefore, to facilitate the analysis of the degradation outcome, the corresponding two symmetrical dibenzoylmethanes (**1c** and **1d**) were also studied. In addition, to receive amounts large enough to quantify, the reaction time for the unsymmetrical dibenzoylmethane **1a** was extended as compared to that for the symmetrical dibenzoylmethanes.

For all three compounds (**1a**, **1c**, and **1d**), photodegradation could be observed in both cyclohexane and EtOH; however, in EtOH, the degradation was much slower. GC/MS analysis of all three photolysis reactions in cyclohexane showed the occurrence of pronounced benzoic acid as well as the formation of solvent adducts and extensive formation of solvent oxidation products, which is in accordance with the observations reported by Roscher et al. (*34*). Rigorous analysis of the degradation products formed in smaller amounts was not possible with the GC/MS system at hand, because of intervening peaks from solvent adducts and solvent oxidation products. Using our HPLC/MS system, these peaks were far less pronounced; on the other hand, this system enabled a successful analysis of the other expected main degradation products. For all three test compounds, the results from the HPLC/MS analysis were mutually very similar. Two of the major peaks in the HPLC/UV chromatogram correspond to the parent dibenzoylmethane and its dimeric sodium adduct (e.g., $\mathbf{1a}_2$Na in Figure 2). A representative chromatogram for compound **1a** is presented in Figure 2. The observed degradation product outcome from photodegradation of **1a** in cyclohexane is shown in Figure 3a, and the results from the degradation of **1a** in EtOH can be seen in Figure 3b. The corresponding data for the two symmetrical dibenzoylmethanes (**1c** and **1d**) can be found in the Supporting Information. The most abundant degradation products were the corresponding benzoic acids, which after 90 min in cyclohexane constituted approximately 30% of the initial concentration of

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Table 1. Product Concentrations after 90 min of Irradiation of Dibenzoylmethane 1a[a]

photodegradation product	concentration (mM)	% of initial concentration of **1a**
4a	0.3287	16.43
4b	0.2812	14.06
5a + 6a	0.0191	0.95
5b	0.0204	1.02
6b	0.0196	0.98
1c	0.0048	0.24
1d	0.0073	0.37
2a	0.0155	0.78
2b	0.0018	0.09
3c	0.0026	0.13
3d	0.0012	0.06

[a] The photodegradation experiments were performed as described in the Experimental Procedures. The initial concentration of **1a** was 2 mM in cyclohexane. Dibenzoylmethanes: 4,4′-di-*tert*-butyldibenzoylmethane (**1c**) and 4,4′-dimethoxydibenzoylmethane (**1d**); arylglyoxals: 4-*tert*-butylphenylglyoxal (**2a**) and 4-methoxyphenylglyoxal (**2b**); benzils: 4,4′-di-*tert*-butylbenzil (**3c**) and 4,4′-dimetoxybenzil (**3d**); benzoic acids: 4-*tert*-butylbenzoic acid (**4a**) and 4-methoxybenzoic acid (**4b**); benzophenones: 4-*tert*-butylacetophenone (**5a**) and 4-methoxyacetophenone (**5b**); and benzaldehydes: 4-*tert*-butylbenzaldehyde (**6a**) and 4-methoxybenzaldehyde (**6b**).

1a (Table 1). Other degradation products obtained in fair amounts were benzaldehydes and benzophenones. Arylglyoxals were detected in all photodegradation reactions, and after 90 min of photolysis of the unsymmetrical dibenzoylmethane **1a** in cyclohexane, arylglyoxal **2a** corresponded to 0.8%, and **2b** corresponded to 0.1% of the initial amount of **1a** (Table 1). However, for **1a** in EtOH, the only detected arylglyoxal was **2a**; the other possible arylglyoxal degradation product **2b** was not detected. For all three dibenzoylmethanes, the corresponding benzils could be detected in cyclohexane but not in EtOH. For **1a** in cyclohexane, the two possible symmetrical benzils (**3c** and **3d**) could be quantified. However, after optimization of the chromatographic separation conditions to obtain good separation of the arylglyoxals, the unsymmetrical benzil (**3a**) coeluated with other compounds and could therefore not be quantified.

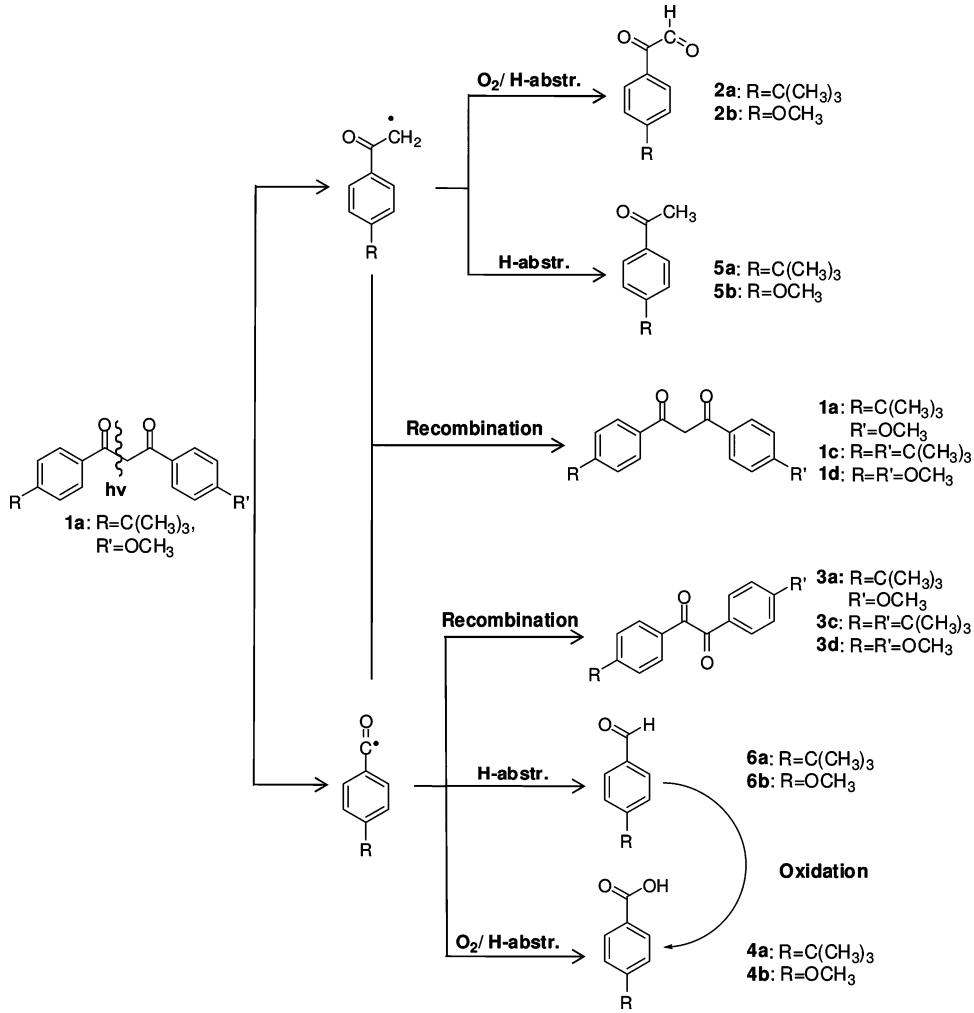
The results from the degradation studies in cyclohexane (Figure 3a) were consistent with the results reported by Schwack and Rudolph (*12*), except that no dibenzoylethanes were detected; see Scheme 2 for proposed degradation scheme. However, contrary to these earlier findings, our results showed that **1a** photodegrades in a polar solvent like EtOH. Even if the degradation of **1a** is much slower in EtOH than in cyclohexane, it does degrade, and the degradation products are mainly the same in EtOH as in cyclohexane (Figure 3a,b). It is noteworthy that the methoxy-substituted degradation products (**4b**, **5b**, and **6b**) formed in the EtOH experiments are unstable and continue to degrade over time (Figure 3b). The observed discrepancies between these two studies are likely due to differences in the reaction conditions used. Most notably are the differences in oxygen supply, irradiation time, and irradiation source. The combined effect of oxygen depletion and the triplet quenching ability of oxygen were put forward by Schwack and Rudolph as a possible cause for the observed increase in degradation rate with increasing irradiation time. Therefore, in the present study, the samples were aerated by continuous bubbling of air to prevent oxygen depletion. Furthermore, as a consequence of the combined use of a falling film reactor and short reaction times, the overall irradiation exposure times were much shorter in the present study. Finally, the irradiance of the light source used was higher, and the full spectral width was utilized, that is, principally $\lambda > 240$ nm, which comprises somewhat shorter wavelengths than applied in the previous study ($\lambda > 260$ nm). It is worth noting that the purpose of the present experiment was not primarily to simulate the conditions under natural sunlight but to establish which products are formed during photochemical degradation, to give an estimate of their relative amounts, and to reveal potential differences in the time evolution of their formation and consumption.

Skin-Sensitizing Potency. The degradation products that could be detected were benzoic acids, benzaldehydes, benzophenones, arylglyoxals, and benzils. Among these, benzoic acids (*35*), benzaldehydes (*36*), and benzophenones (*37*) have previously been tested in the LLNA, and they were all classified as nonsensitizers. Another study (*38*) has shown that the parent structures glyoxal and methylglyoxal are strong allergens in the LLNA; therefore, we believed that the arylglyoxals that are formed when dibenzoylmethane degrades may contribute to the overall photosensitizing potency of these UV filters. Benzils, on the other hand, have, to our knowledge, not been studied in the LLNA. However, other diketones such as 2,3-butadione, furil, and 1-phenyl-1,2-propanedione have been tested with results ranging from potent sensitizer (EC3 = 1.3%) for 1-phenyl-1,2-propanedione to nonsensitizer for furil (*39*). Therefore, we wanted to test arylglyoxals and benzils in the LLNA to establish whether any of these might contribute to the photoallergenic potency of dibenzoylmethanes.

The sensitizing potencies of compounds **1a**,**b**, **2a**−**d**, benzil, and **3a**,**c**,**d** as assessed in the murine LLNA are shown in Figure 4. The UVA filter **1b** was shown to be a weak sensitizer, whereas the UVA filter **1a** was a nonsensitizer (Table 2 and Figure 4a). In marked contrast, the arylglyoxals, **2a**−**d**, were all found to be strong allergens with EC3 values of 0.011, 0.0092, 0.016, and 0.012 M, respectively (Table 2 and Figure 4a). Interestingly, the LLNA response was, within experimental variations, the same for all tested arylglyoxals in spite of the large difference in electronic effects of the *para* substituents. The reason for this was explored further by a reactivity study of the arylglyoxals (vide infra). The LLNA result for the benzils varied from nonsensitizers (**3a** and **3d**) to a response not corresponding to a dose−response behavior (benzil and **3c**) (Figure 4b).

Benzils' Allergenic and Cytotoxic Capacity. The two benzils, **3a** and **3d**, did not give any response in the LLNA and may be considered as nonsensitizers. Because of low solubility in the vehicle, **3d** was only tested up to 2%. Benzil and its derivative **3c** on the other hand gave an LLNA response but not the expected dose−response curve (Figure 4b). Other benzils have previously been studied for their cytotoxicity and antiproliferative activity (*27, 40*). Thus, it seemed likely that the observed LLNA response could be explained by the cytotoxicity of the benzils. According to the cell proliferation assay, all of the benzils (benzil and **3a**,**c**,**d**) were cytotoxic in the tested concentrations 0.125 and 0.25 mM (Figure 5). At the highest concentration tested (1 mM for benzil, **3a**, and **3c** and 0.5 mM for **3d**), all substances formed crystals or oil droplets in the water-based medium (Figure 6a). The light scattering caused by the crystals formed at highest test concentrations had a detrimental effect on the absorption measurements; therefore, these results were excluded. At lower concentrations (0.125 and 0.25 mM), at which no crystal formation was observed, the three compounds benzil, **3a**, and **3c** were all shown to be highly cytotoxic (Figure 5). They all gave rise to a lower light absorbance in the test than **3d**, which should correspond to a higher toxicity. However, the higher absorption for cells treated with **3d** is more likely a consequence of the light scattering caused by the crystals formed in the cell medium. This latter

Scheme 2. Proposed Photodegradation Pathways and Degradation Products for Dibenzoylmethanes, Shown for 1a (*12*)[a]



[a] H-abstr. stands for H-abstraction, and in this experiment, the hydrogen is most likely abstracted from the solvent.

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compound formed crystals at all test concentrations. Therefore, it was not possible to accurately determine the cytotoxicity of **3d**. It can still be concluded that this compound is cytotoxic.

After the final reading, the cells were stained with trypan blue and examined visually using a light microscope. All of the negative controls looked healthy with a characteristic round shape (Figure 6b), whereas cells treated with benzils were deformed and had turned into fragments or flattened out (Figure 6c,d). We therefore conclude that the response and its deviation from the normal dose−response relationship observed in the LLNA studies for benzil and **3c** are due to their cytotoxic properties and do not indicate a sensitizing ability.

Arylglyoxals' Sensitizing Potency and Reactivity. The dibenzoylmethane **1a** degrades to *p-tert*-butylphenylglyoxal (**2a**) and *p*-methoxyphenylglyoxal (**2b**) (Scheme 2), whereas the dibenzoylmethane **1b** degrades to *p*-isopropylphenylglyoxal (**2c**) and phenylglyoxal (*12*). To establish whether the *para* substituent on the arylglyoxals has any influence on the allergenic activity, four different arylglyoxals were investigated. The *p*-methoxyphenylglyoxal (**2b**) has a strong electron-donating *para* substituent and was therefore included in the test series. The substituents *tert*-butyl, isopropyl, and hydrogen are all modest or nonelectron donating; therefore, they were considered to be chemically similar. Accordingly, only **2a** and **2c** were included in the LLNA series to keep the number of test animals to a minimum. To fully explore the influence of the electronic factors *p*-nitrophenylglyoxal (**2d**), which has a strong electron-withdrawing *para* substituent, was synthesized and added as the fourth arylglyoxal in the series.

The compounds **2a**−**d** were all classified as strong allergens based on the LLNA experiments (Table 1 and Figure 4a). Despite the fact that they have substituents with very different chemical properties in the *para* position, no significant difference in sensitizing capacity could be seen. The sensitizing capacity could be expected to follow the electrophilicity of the arylglyoxals, which is expected to increase for arylglyoxals with increasing electron-withdrawing ability of the *para* substituent. The electrophilic nature of a molecule is considered to be good predictor for its sensitizing capacity. The rationale behind this is that formation of the immunogenic hapten−protein complex often involves an electrophilic−nucleophilic interaction between the hapten and the nucleophilic moieties of the amino acid side chains in skin proteins (*41*). Therefore, the chemical reactivity of **2b**−**d** toward the nucleophilic acetyl-protected arginine was also investigated. Compound **2a** was not included in the reactivity assay since it is considered to be chemically similar to **2c**. Arginine was chosen as the nucleophile because phenylglyoxal has proven to be specific for this amino acid (*42*). The reactions were performed in 20% Me_2SO in phosphate buffer, pH 7.0, at room temperature. The reaction rates were measured as the depletion of the individual arylglyoxals. The reactions were analyzed with HPLC/MS, and the depletion was monitored by HPLC/UV using standard curves for each arylglyoxal as well as an internal standard. Note that the arylglyoxal

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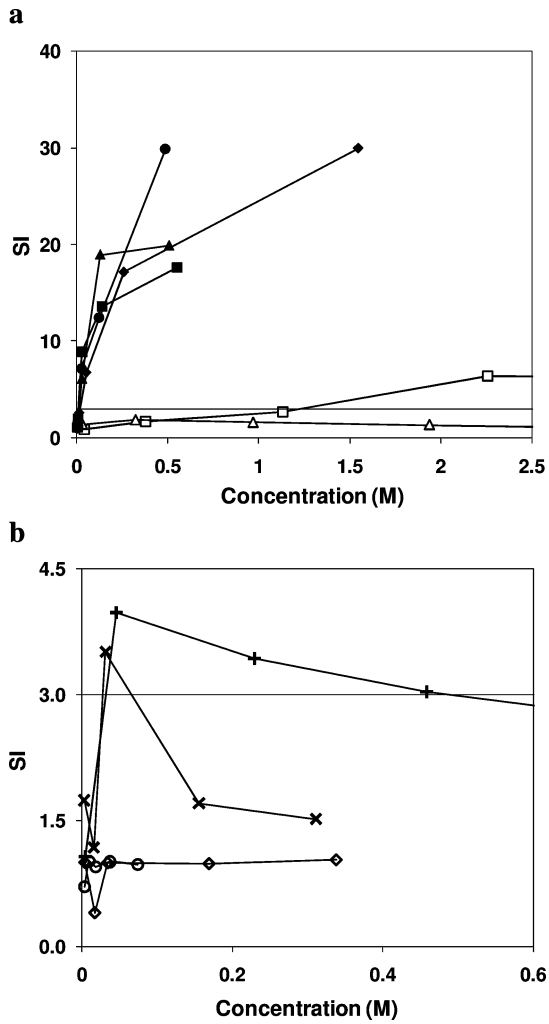


Figure 4. Dose−response curves for the compounds tested in LLNA. The concentrations are given in molar. The horizontal line marks an SI of 3, the cutoff limit for a compound to be considered a sensitizer. (a) Dibenzoylmethanes: **1a** (△) and **1b** (□); arylglyoxals: **2a** (●), **2b** (■), **2c** (♦), and **2d** (▲). (b) Benzils: benzil (+), **3a** (◇), **3c** (×), and **3d** (○).

Table 2. LLNA[a] Responses

test compound	EC3 value % w/v	EC3 value M	classification
1a			nonsensitizing
1b	32.5	1.2	weak
2a	0.22	0.011	strong
2b	0.16	0.0092	strong
2c	0.32	0.016	strong
2d	0.23	0.012	strong

[a] The sensitization experiments were performed as described in the Experimental Procedures. EC3 values were calculated using linear prediction. EC3 < 0.1%, extreme; EC3 ≥ 0.1 to <1%, strong; EC3 ≥ 1 to <10%, moderate; and EC3 ≥ 10 to <100%, weak.

and its corresponding hydrate are in rapid equilibrium at the reaction conditions used and in the eluents used for HPLC. In effect, the rapid equilibrium prevents the two forms to be separable by HPLC and their concentrations to be measured individually. Instead, a measure of the total concentration of glyoxal and its hydrate is obtained in the HPLC analysis. Furthermore, the arylglyoxals exist mainly as their hydrates at the reaction conditions used (at equilibrium). However, a small concentration of the reactive glyoxal form is present at all times thanks to the rapid equilibrium between glyoxal and its hydrate. Thus, the hydrate is, under the reaction conditions used, funneled

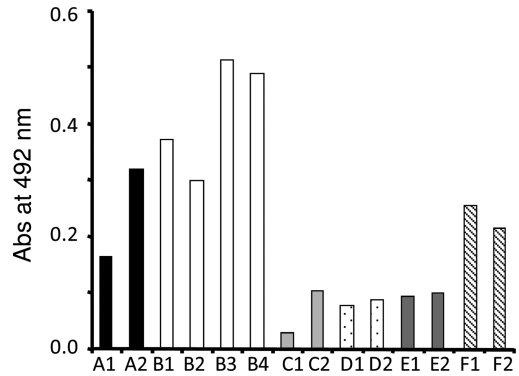


Figure 5. Cytotoxicity of the benzils was evaluated with a cell proliferation assay. The displayed results are the mean absorbance of triplicates for each benzil or control at 492 nm after 24 h of incubation followed by another 2 h of incubation after the addition of MTS/PMS. Positive controls: A1, 0.25 mM $CuSO_4$; and A2, 0.125 mM $CuSO_4$. Negative controls: B1, medium only; B2, medium with 1.0% EtOH; B3, medium with 2.0% EtOH; and B4, medium with 1.8% EtOH and 0.20% Me_2SO. Benzils: C1, 0.25 mM benzil; and C2, 0.125 mM benzil; D1, 0.25 mM **3a**; and D2, 0.125 mM **3a**; E1, 0.25 mM **3c**; and E2, 0.125 mM **3c**; and F1, 0.25 mM; and F2, 0.125 mM **3d**.

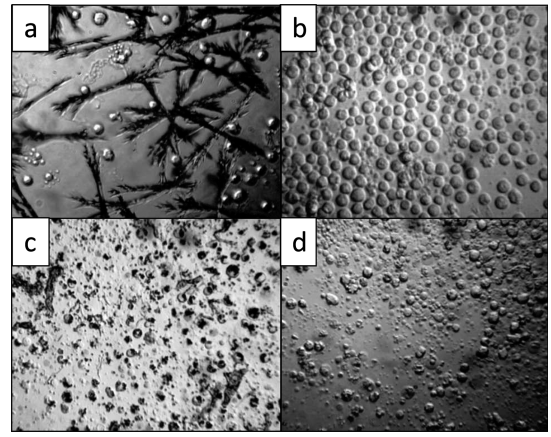
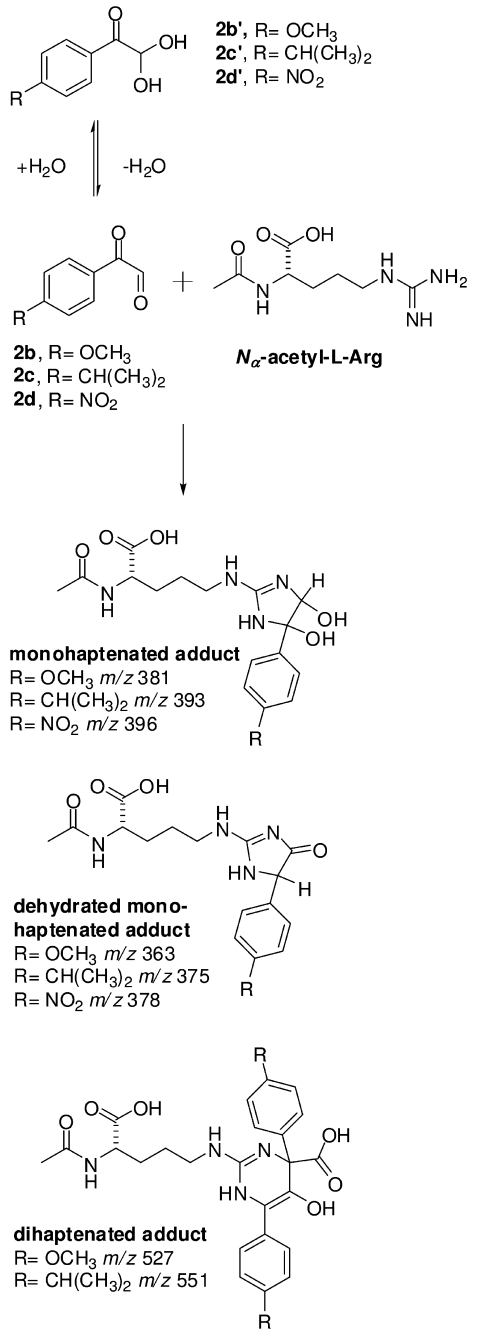


Figure 6. Benzils cytotoxicity was evaluated with a cell proliferation assay. After 26 h of incubation (24 h of incubation followed by another 2 h of incubation after the addition of MTS/PMS) with benzil or dilution medium alone, the cells were examined visually. (a) Crystals formed at 1 mM concentration of benzil in medium. (b) Cells incubated with dilution medium (RPMI-1640 with 1% EtOH) only, with a healthy characteristic round shape. Deformed cells after incubation with (c) 0.25 mM benzil **3a** and (d) 0.25 mM benzil.

through the glyoxal form to several related arginine−arylglyoxal adducts; see Scheme 3. For arylglyoxals **2b** and **2c**, quantification was done by measurement of the peak area and calculation of the concentration from standard curves. For compound **2d**, the peak was very broad, probably due to the equilibrium with its hydrate. As a result, other adducts coeluted with the tail of **2d**, making quantification by peak area difficult. Hence, a more accurate result was obtained by using the peak height instead of the peak area (see the Supporting Information for standard curves). All three arylglyoxals reacted at approximately the same rate with N_α-acetyl-L-Arg (Figure 7). In all of the three reaction systems studied, four adducts with *m/z* corresponding to monoconjugates of arylglyoxal and arginine were detected, that is, ions at 381, 393, and 396 for reaction systems **2b**, **2c**, and **2d**, respectively (see the Supporting Information). This seems to be consistent with the fact that four different diastereoisomers are possible; see Scheme 3 for pinacole structure elements of haptenated adducts. Also, in all three systems, one or two signals were observed with *m/z* corresponding to monoconjugates of arylglyoxal and arginine minus water, which also agrees with

Scheme 3. Reaction System, N_α-Acetyl-L-Arg and Arylglyoxals 2b−d and Possible Adducts (*43*)



the fact that two isomers are possible. For reaction systems **2b** and **2c**, two adducts with *m/z* corresponding to two arylglyoxals and one arginine minus water could also be seen, that is, ions at 527 and 551, respectively (see the Supporting Information). Proposed structures, presented in Scheme 3, for the arylglyoxal−arginine adducts have been adopted from the work by Saraiva et al. (*43*). In addition, all systems displayed signals with *m/z* corresponding to monoconjugates of arylglyoxal and arginine minus two, minus 19, and minus 20. The structures of these adducts are not known. The overall results agree well with earlier studies of the reactivity of phenylglyoxal toward arginine (*42, 43*).

No difference in reactivity toward arginine could be observed between the three arylglyoxals (Figure 7). A reason for this may be that for a more reactive arylglyoxal, the equilibrium is shifted more toward the hydrate, which is nonelectrophilic and thus

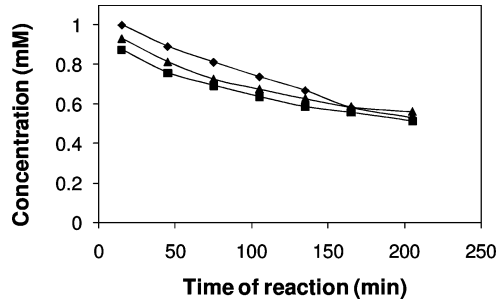


Figure 7. Depletion of arylglyoxals **2b** (■), **2c** (♦), and **2d** (▲), upon reaction with N_α-acetyl-L-Arg. Reactions were preformed in 20% Me_2SO in phosphate buffer, pH 7.0, at room temperature with equimolar amounts of arylglyoxal and arginine. Reactions were analyzed with HPLC/MS and quantified with HPLC/UV by using standard curves for each arylglyoxal as well as an internal standard.

unreactive toward arginine. The higher reactivity is therefore moderated by a lower available concentration of the reactive glyoxal form. Such a shift in the equilibrium composition could also explain the similarity in sensitizing potency of these compounds (Table 1 and Figure 4a). Thus, the strategy of changing the *para* substituent of the dibenzoylmethanes will not affect the allergenic activity of their degradation products arylglyoxals; they will still all be considered strong sensitizers.

The exact mechanisms for the formation of immunogenic hapten−protein complexes in photoallergic contact dermatitis on a molecular level are not known. Two pathways have been suggested for the immunogenic complex formation, the first way involves immunogenic complex formation with stable photoproducts, whereas the other pathway involves an exited state molecule (*44*). Our results suggest that the immunogenic hapten−protein complexes in photoallergic reactions to dibenzoylmethanes could be formed via an electrophilic−nucleophilic pathway from stable photooxidation products such as the arylglyoxals. Although only a small percentage of the dibenzoylmethane is converted to these strong allergens, that is, arylglyoxals, the practice of repeated application of a sun lotion can cause an adverse effect (*45, 46*). This is supported by our previous work, which shows that the primary oxidation products of limonene, that is, limonene hydroperoxides, although formed in small amounts (1−5%), are the principal allergens in contact allergy to oxidized limonene (*47, 48*).

Conclusion

4-*tert*-Butyl-4′-methoxy dibenzoylmethane (**1a**), which is a frequently used chemical UVA filter, photodegrades, and potential health hazardous arylglyoxals and benzils are formed. The benzils formed from the dibenzoylmethane **1a** are cytotoxic rather than allergenic, whereas the arylglyoxals are strong skin sensitizers based on the LLNA. Thus, it is very likely that the photocontact allergy to dibenzoylmethanes is caused by the arylglyoxals. In addition, we have shown that the *para* substituent does not affect the reactivity of the arylglyoxals toward the nucleophile arginine, nor does it significantly influence the sensitizing potency of the arylglyoxals. Thus, it is not possible to lower the allergenic potency of photodegraded dibenzoylmethanes by changing the *para* substituents.

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of the platform Göteborg Science Centre for Skin Research at the Faculty of Science, University of Gothenburg.

Supporting Information Available: A more detailed description of the photoreactor equipment, photolysis results for dibenzoylmethanes **1c**,**d** in cyclohexane and EtOH, characteristic standard curves for compounds used for quantification of photodegradation products, [^{3}H]-thymidine incorporation (dpm/lymph node) and SI values for all compounds tested in the LLNA, standard curves for compounds **2b**−**d**, and characteristic HPLC/MS spectra from the reactivity study of **2b**−**d** toward N_α-acetyl-L-Arg. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting information for:
Photodegradation of Dibenzoylmethanes: Potential cause of Photocontact Allergy to Sunscreens

Isabella Karlsson,[†] Lisa Hillerström, [†] Anna-Lena Stenfeldt, [†] Jerker Mårtensson,[‡] and Anna Börje[*, †]
[†] Dermatochemistry and Skin Allergy, Department of Chemistry, University of Gothenburg, SE-412 96 Göteborg, Sweden
[‡] Department of Chemical and Biological Engineering/Organic Chemistry, Chalmers University of Technology, SE-412 96 Göteborg, Sweden

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[*] To whom correspondence should be addressed. E-mail: aborje@chem.gu.se; Tel: +46 31 772 4725; Fax: +46 31 772 3840

Falling film photoreactor

Detailed description of the photoreactor equipment

In the photoreactor, the reaction mixture is forced up through a riser pipe by a pump and allowed to flow evenly over the edge of a wide vertical glass tube. The liquid then flows downwards forming a thin film on the inside wall of the tube. The wall is cooled by a stream of tap water to maintain the reaction mixture at a constant temperature, 35°C to 40°C at steady state. As the film flows downwards it is irradiated by a water cooled immersion lamp positioned in the center of the tube. The distance between the radiation source and the thin film varies from approximately 0.04 m at the same level as the lamp to 0.1 m at the top edge of the tube where the reaction mixture enters the reaction zone and at the bottom where the mixture leaves. All glass details between the irradiation source and the thin film is made of quartz. The reaction mixture is collected below the reaction zone in a reservoir containing the impeller that forces the liquid up to the overflow at the top of the tube. In the reservoir, synthetic air is bubbled through the reaction mixture and allowed to pass over the thin film in the reaction zone.
More detailed information about the design of the photoreactor is available on the homepage (NORMAG Labor- und Prozesstechnik, October 7, 2009, < http://www.laboratory-glassware.com/index.php>) of NORMAG Labor- und Prozesstechnik Gmbh, Ilmenau, Germany.

Irradiation source characteristics

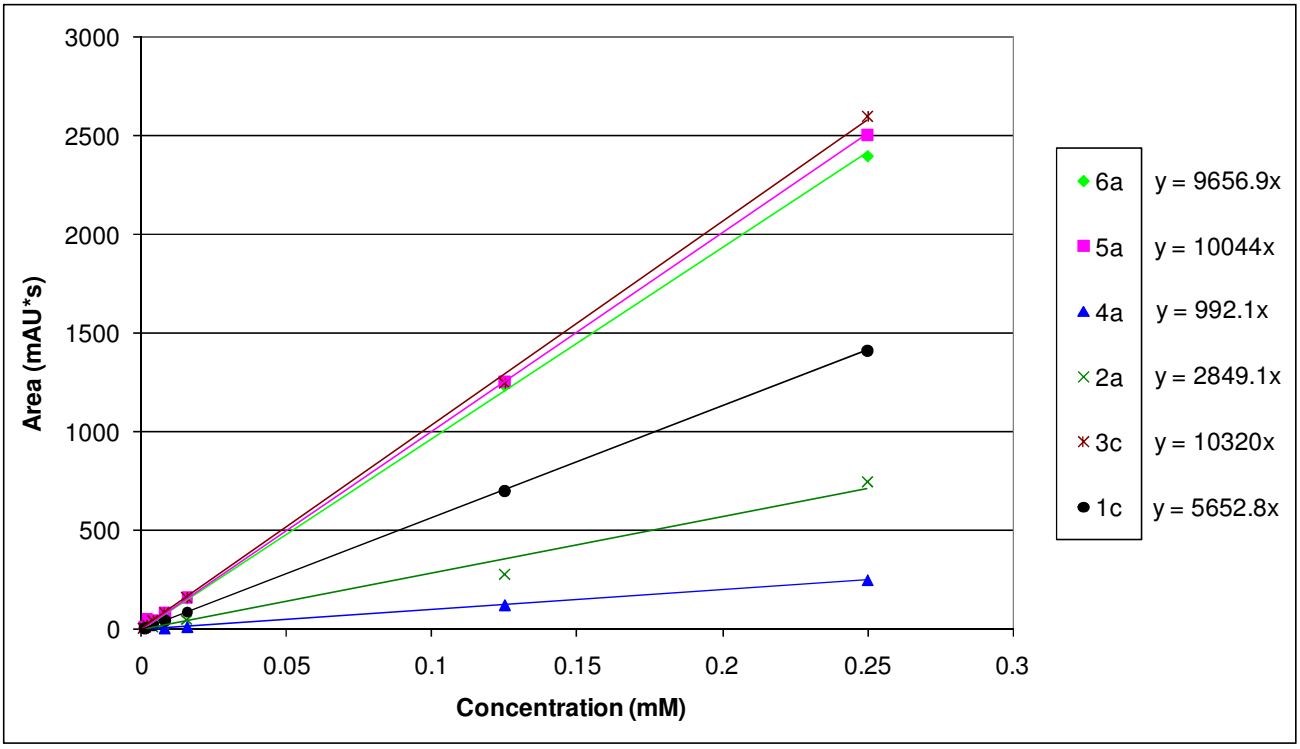
The photoreactor is equipped with a 700 W TQ 718, Z4 doped, medium pressure mercury lamp (Heraeus Noblelight Gmbh). The lamp is operated by a VG TQ 718 power supply (Heraeus Noblelight Gmbh).

Radiant power (Φ) at 200-600 nm and 700 W

λ (nm)	Φ (W) at 700 W
200-280	75
280-315	45
315-400	147
400-700	122
Total radiant power	389

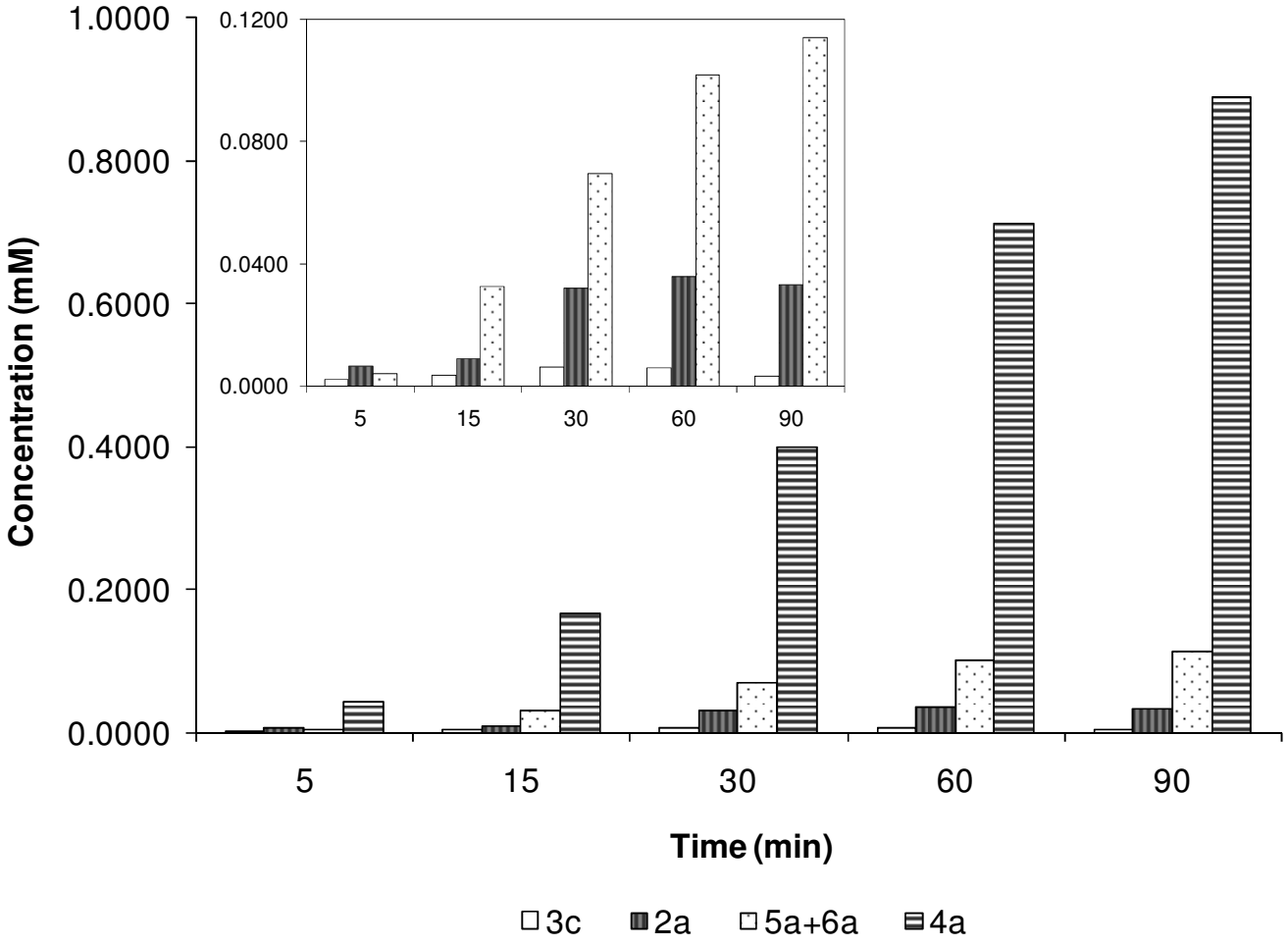
Photolysis results for the symmetrical dibenzoylmethanes 1c and 1d

Characteristic standard curves for compounds used for quantification of photodegradation products[a]



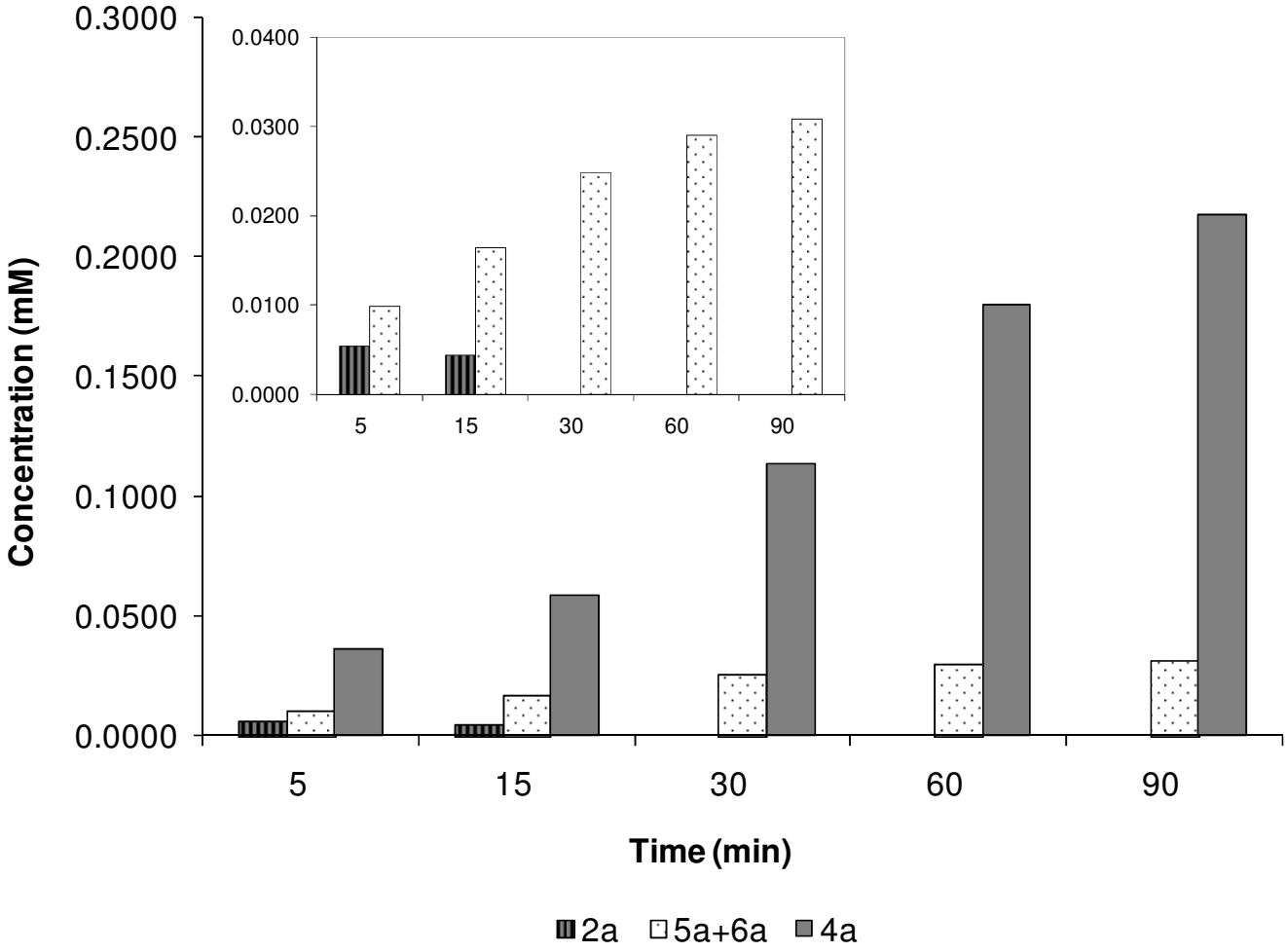
[a]Standard curves for compounds with a metoxy group in *para* position instead of a *tert*-butyl group were obtained in the same way.

Photolysis of 1c in cyclohexane[a]



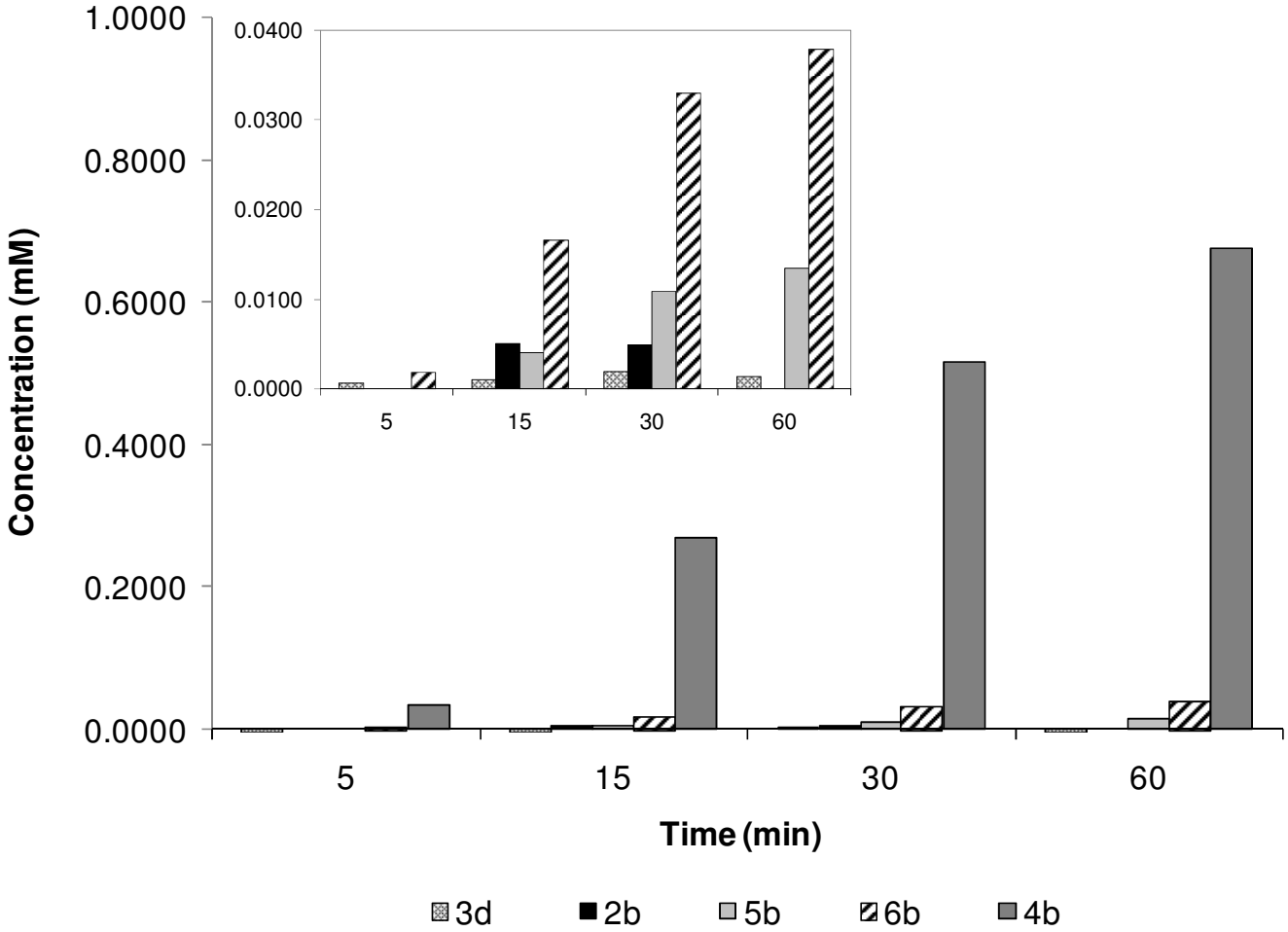
[a] Photoproduct distribution in photolysis of the dibenzoylmethane **1c**. Photolysis was performed on 350 mL, 2 mM solutions of dibenzoylmethane **1c** in cyclohexane for 60 min. The detected degradation products were: 4-*tert*-butylphenylglyoxal (**2a**), 4,4'-Di-*tert*-butylbenzil (**3c**), 4-*tert*-butylbenzoic acid (**4a**), 4-*tert*-butylacetophenone (**5a**) and, 4-*tert*-butylbenzaldehyde (**6a**).The inset is an enhancement of the concentrations of photoproducts when the benzoic acid is excluded.

Photolysis of 1c in ethanol[a]



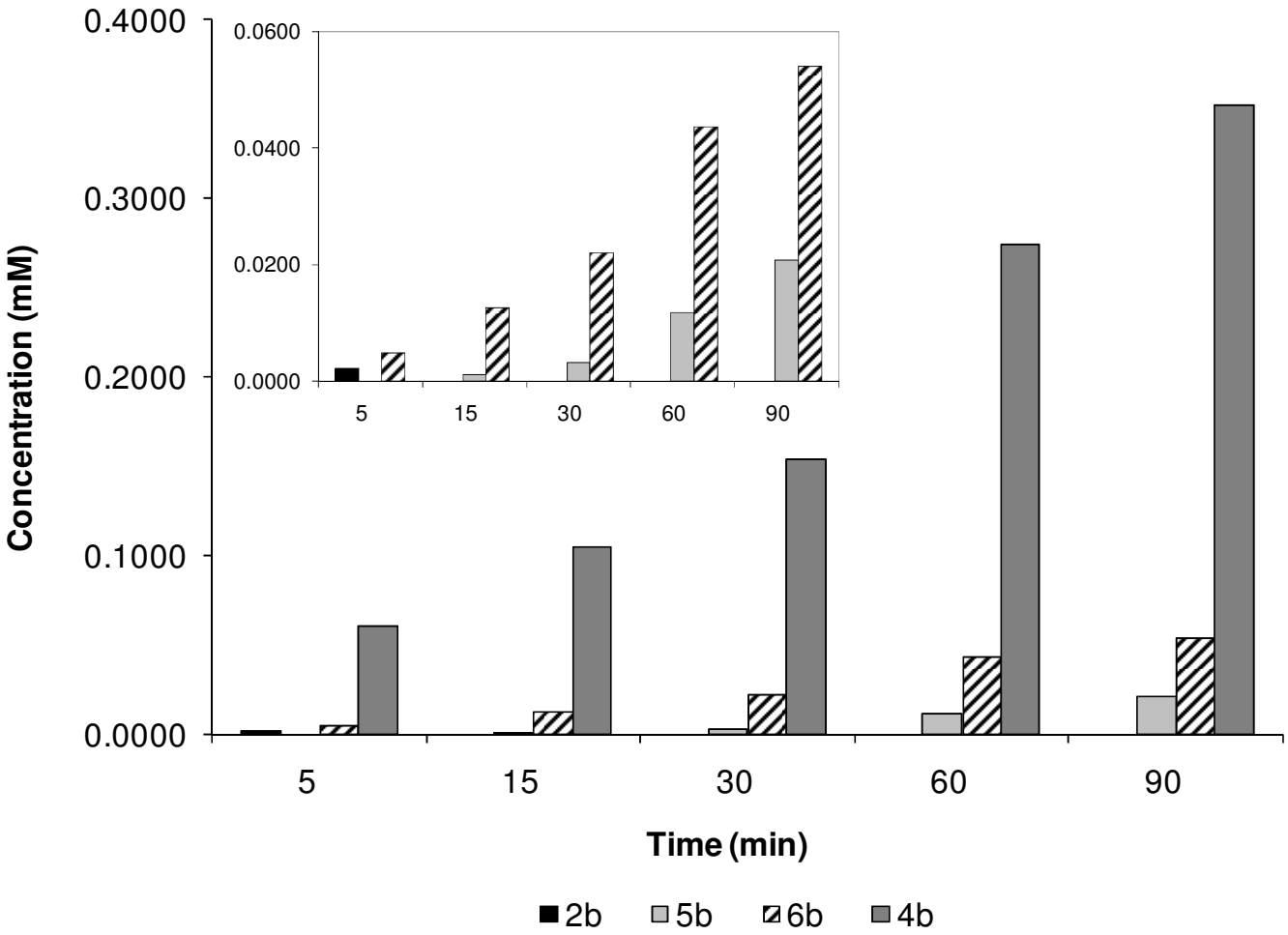
[a] Photoproduct distribution in photolysis of the dibenzoylmethane **1c**. Photolysis was performed on 350 mL, 2 mM solutions of dibenzoylmethane **1c** in ethanol for 90 min. The detected degradation products were: 4-*tert*-butylphenylglyoxal (**2a**), 4-*tert*-butylbenzoic acid (**4a**), 4-*tert*-butylacetophenone (**5a**), and 4-*tert*-butylbenzaldehyde (**6a**). The inset is an enhancement of the concentrations of photoproducts when the benzoic acid is excluded.

Photolysis of 1d in cyclohexane[a]



[a] Photoproduct distribution in photolysis of the dibenzoylmethane **1d**. Photolysis was performed on 350 mL, 2 mM solutions of dibenzoylmethane **1d** in cyclohexane for 60 min. The detected degradation products were: 4-methoxyphenylglyoxal (**2b**), 4,4'-dimetoxybenzil (**3d**), 4-methoxybenzoic acid (**4b**), 4-methoxyacetophenone (**5b**), and 4-methoxybenzaldehyde (**6b**). The inset is an enhancement of the concentrations of photoproducts when the benzoic acid is excluded.

Photolysis of 1d in ethanol[a]



[a] Photoproduct distribution in photolysis of the dibenzoylmethane **1d**. Photolysis was performed on 350 mL, 2 mM solutions of dibenzoylmethane **1d** in ethanol for 90 min. The detected degradation products were: 4-methoxyphenylglyoxal (**2b**), 4-methoxybenzoic acid (**4b**), 4-methoxyacetophenone (**5b**), and 4-methoxybenzaldehyde (**6b**). The inset is an enhancement of the concentrations of photoproducts when the benzoic acid is excluded.

[^{3}H]-thymidine incorporation (dpm/lymph node) and SI values for compounds tested in the LLNA

Table 1. LLNA[a] responses for compounds 1a, b and 2a-d

test material and concentration (% w/v)	[^{3}H]thymidine incorporation (DPM/lymph node)	SI
1a		
control	788	
1% (0.032 M)	1083	1.4
10% (0.32 M)	1448	1.8
30% (0.97 M)	1243	1.6
60% (1.9 M)	1059	1.3
80% (2.6 M)	902	1.2
1b		
control	887	
1% (0.038 M)	787	0.9
10% (0.38 M)	1481	1.7
30% (1.1 M)	2396	2.7
60% (2.3 M)	5649	6.4
80% (3.0 M)	5587	6.3
2a		
control	481	
0.01% (2.5×10^{-4} M)	474	1.0
0.1% (0.0025 M)	573	1.2
0.5% (0.013 M)	3518	7.3
2.5% (0.063 M)	6137	12.8
10% (0.25 M)	14758	30.7
2b		
control	920	
0.01% (2.9×10^{-4} M)	1010	1.1
0.1% (0.0029 M)	1844	2.0
0.5% (0.014 M)	8162	8.9
2.5% (0.072 M)	12477	13.6
10% (0.29 M)	16196	17.6
2c		
control	656	
0.05% (0.0026 M)	1166	1.8
0.25% (0.013 M)	1723	2.6
1% (0.051 M)	4452	6.8
5% (0.26 M)	11248	17.2
30% (1.5 M)	19616	29.9
2d		
control	660	
0.01% (5.1×10^{-4} M)	824	1.3
0.1% (0.0051 M)	998	1.5
0.5% (0.025 M)	4040	6.1
2.5% (0.013 M)	12505	19.0
10% (0.51 M)	13133	19.9

[a] The sensitization experiments were performed as described in Experimental Procedures. The SIs correspond to the increase in the thymidine incorporation of treated groups relative to vehicle-treated controls.

Table 2. LLNA[a] responses for benzils (benzil, 3a, c, d)

test material and concentration (% w/v)	[^{3}H]thymidine incorporation (DPM/lymph node)	SI
benzil		
control	361	
0.1% (0.0048 M)	386	1.1
1% (0.048 M)	1436	4.0
5% (0.24 M)	1239	3.4
10% (0.48 M)	1094	3.0
25% (1.2 M)	814	2.3
3a		
control	548	
0.1% (0.0034 M)	549	1.0
0.5% (0.017 M)	221	0.4
1% (0.034 M)	547	1.0
5% (0.17 M)	540	1.0
10 (0.34 M))	565	1.0
3c		
control	304	
0.1% (0.0031 M)	529	1.7
0.5% (0.016 M)	358	1.2
1% (0.031 M)	1067	3.5
5% (0.16 M)	519	1.7
10% (0.31 M)	461	1.5
3d		
control	396	
0.1% (0.0037 M)	282	0.7
0.25% (0.0092 M)	401	1.0
0.5% (0.018 M)	377	1.0
1% (0.037 M)	400	1.0
2.5% (0.092 M)	387	1.0

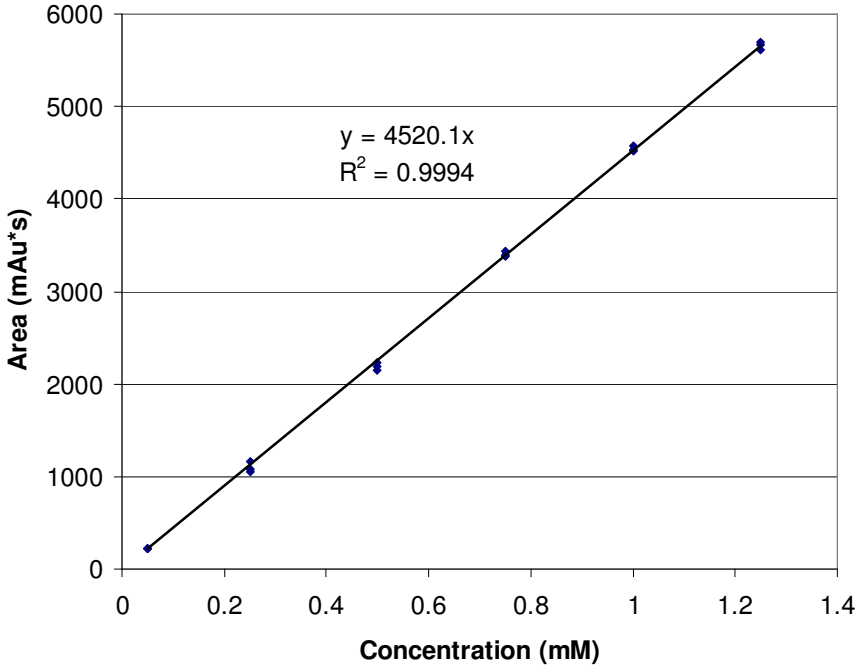
[a] The sensitization experiments were performed as described in Experimental Procedures. The SIs correspond to the increase in the thymidine incorporation of treated groups relative to vehicle-treated controls.

Reactivity of compounds 2b-d toward N_α-acetyl-L-Arg

Standard curves for compounds 2b-d

Standard curve for compound 2b at 280.4 nm

concentration (mM)	area (mAu*s)
0.05	225.57625
0.05	219.58344
0.05	217.322
0.25	1078.7146
0.25	1162.68286
0.25	1047.6781
0.50	2231.75366
0.50	2184.08496
0.50	2150.86987
0.75	3440.24463
0.75	3384.32275
0.75	3388.61401
1.00	4535.79395
1.00	4575.91406
1.00	4514.08838
1.25	5664.83301
1.25	5699.38477
1.25	5614.24512



Standard curve for compound 2c at 265.4 nm

concentration (mM)	area (mAu*s)
0.05	200.57097
0.05	200.28395
0.05	203.08937
0.25	1127.6073
0.25	1063.9187
0.25	1129.69006
0.50	2132.84668
0.50	2147.99023
0.50	2193.01074
0.75	3222.58228
0.75	3167.40063
0.75	3436.70728
1.0	4299.03906
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1.25	5290.95215
1.25	5383.1123
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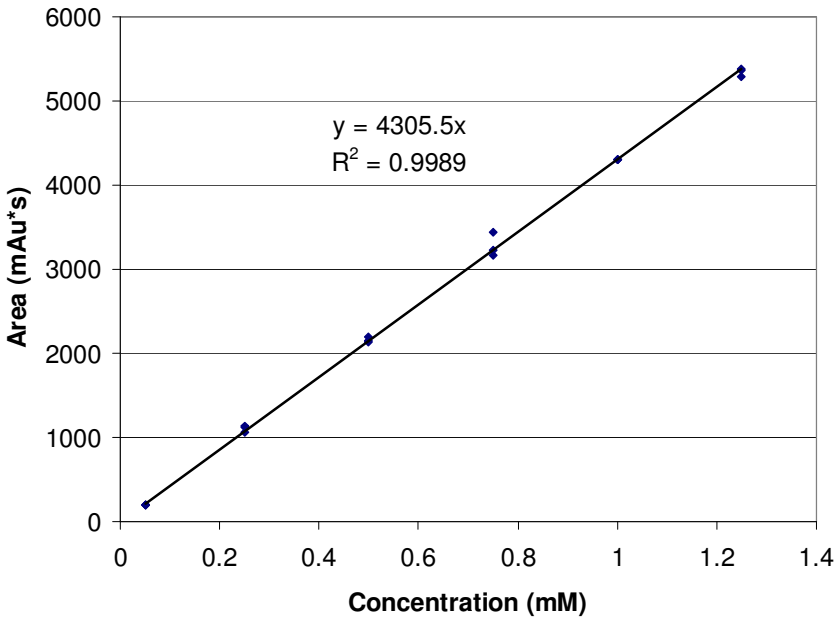
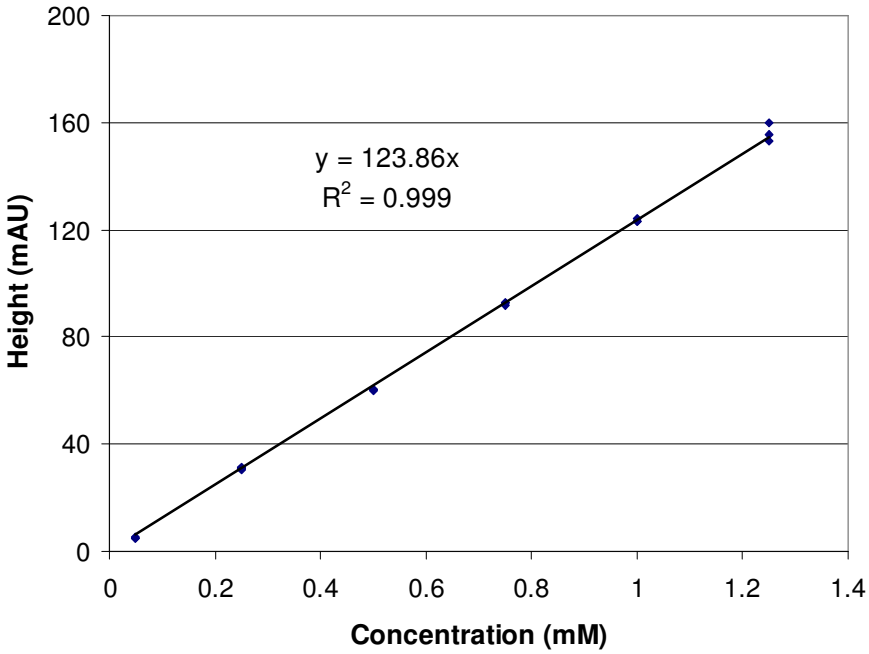


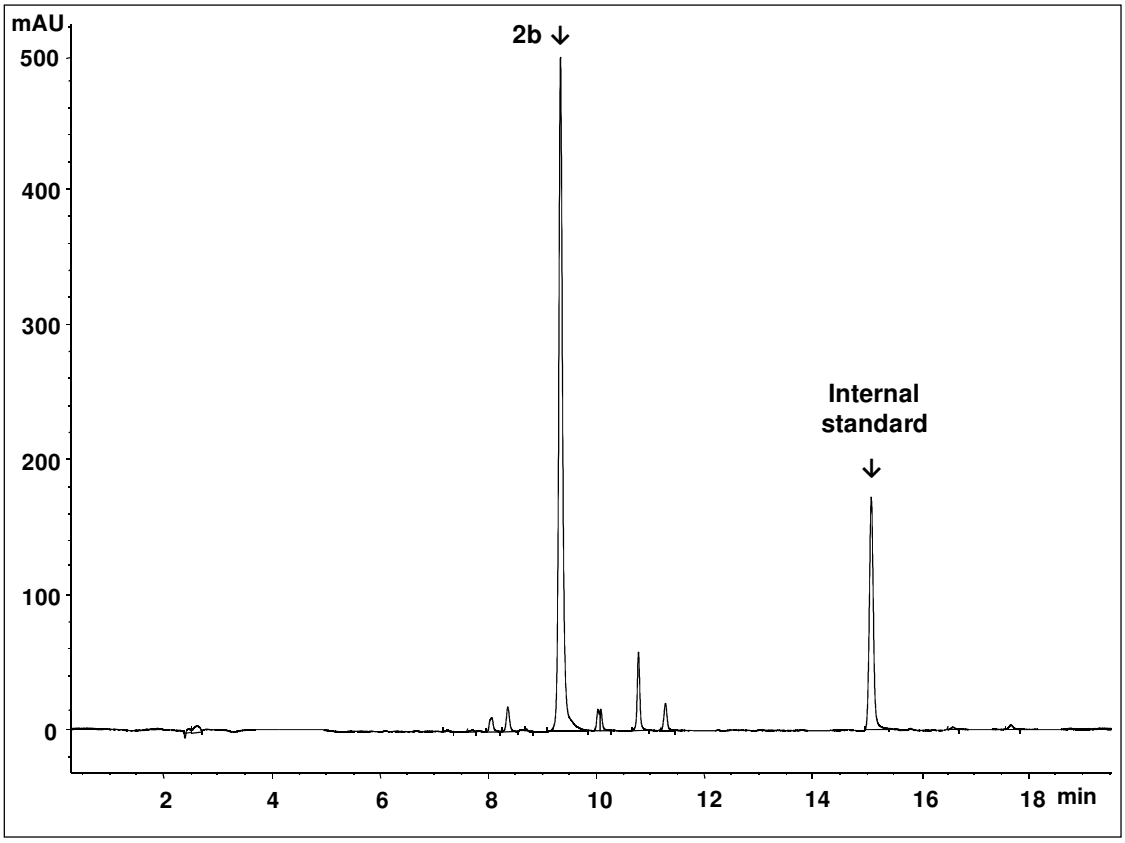
Standard curve for compound 2d at 265.4 nm

concentration (mM)	height (mAU)
0.05	5.28264
0.05	4.61798
0.05	4.65527
0.25	31.33968
0.25	30.96445
0.25	30.24621
0.50	59.82303
0.50	60.15421
0.50	60.26698
0.75	91.69833
0.75	92.95715
0.75	92.8127
1.00	123.13513
1.00	123.97116
1.00	123.13584
1.25	159.75093
1.25	153.27783
1.25	155.68581

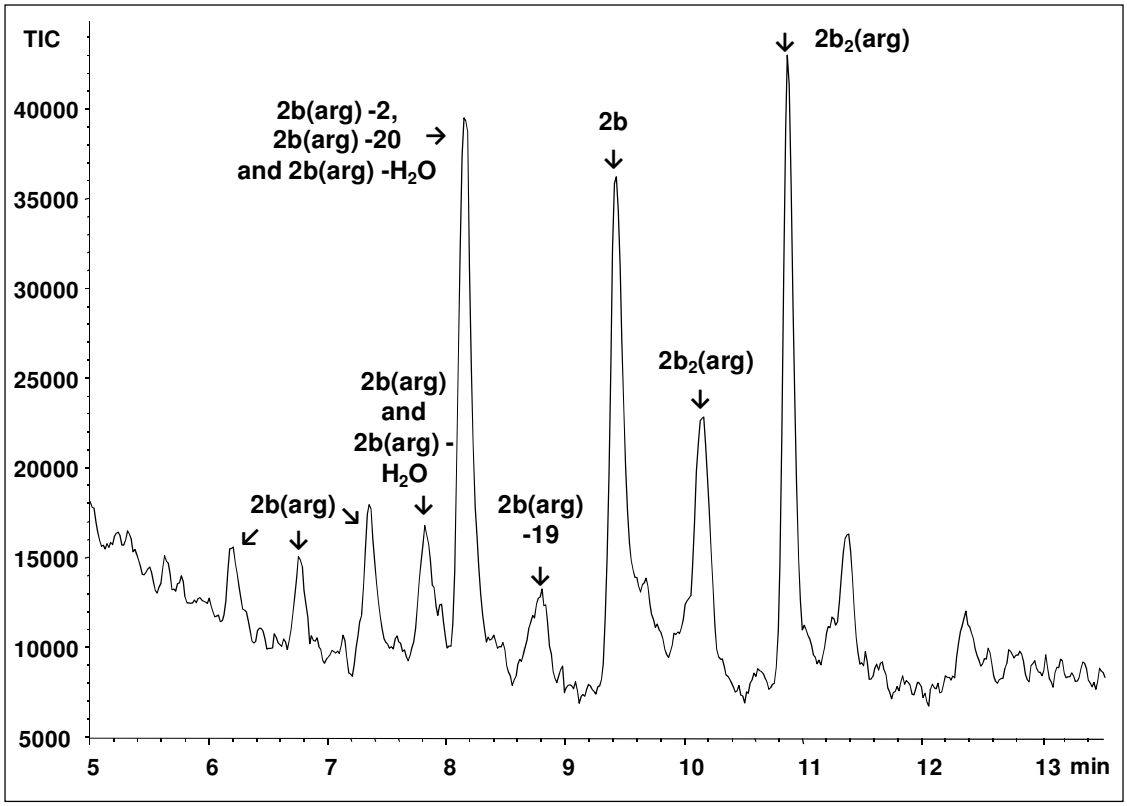


Chromatograms and spectrum from reactivity experiment of compound 2b toward N_α-acetyl-L-Arg

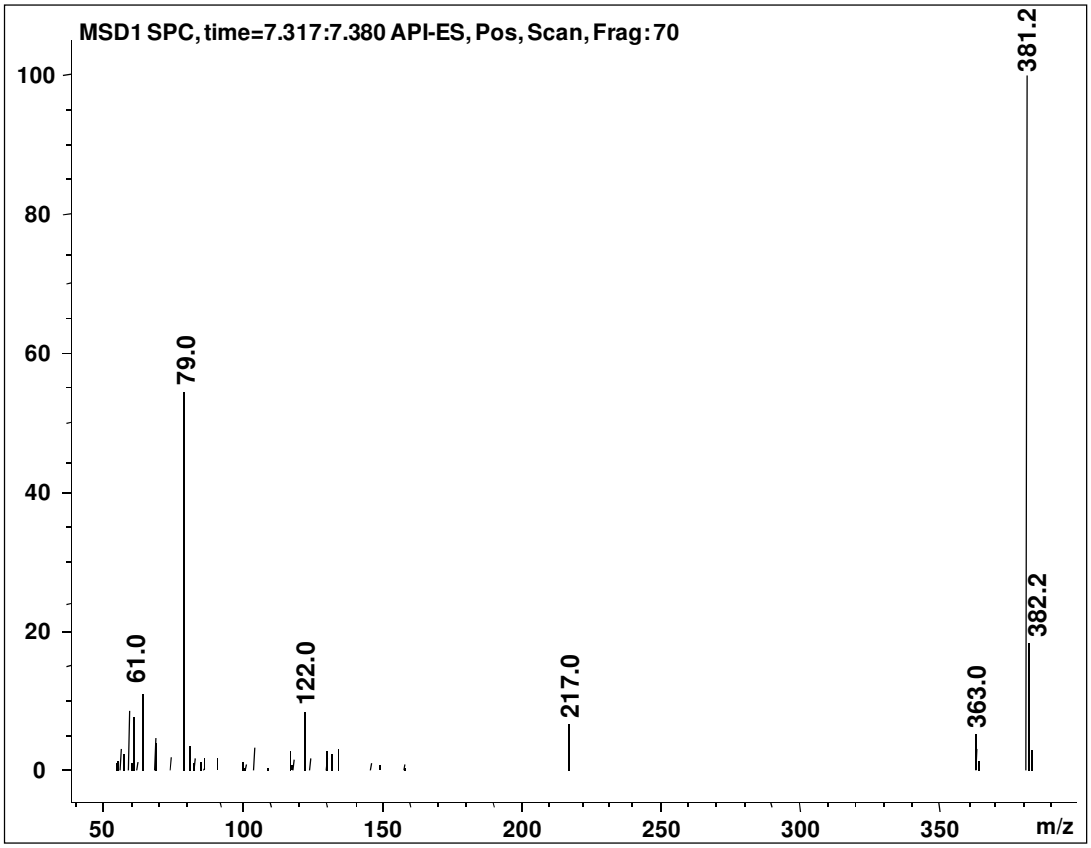
UV chromatogram after 200 minutes at 280.4 nm



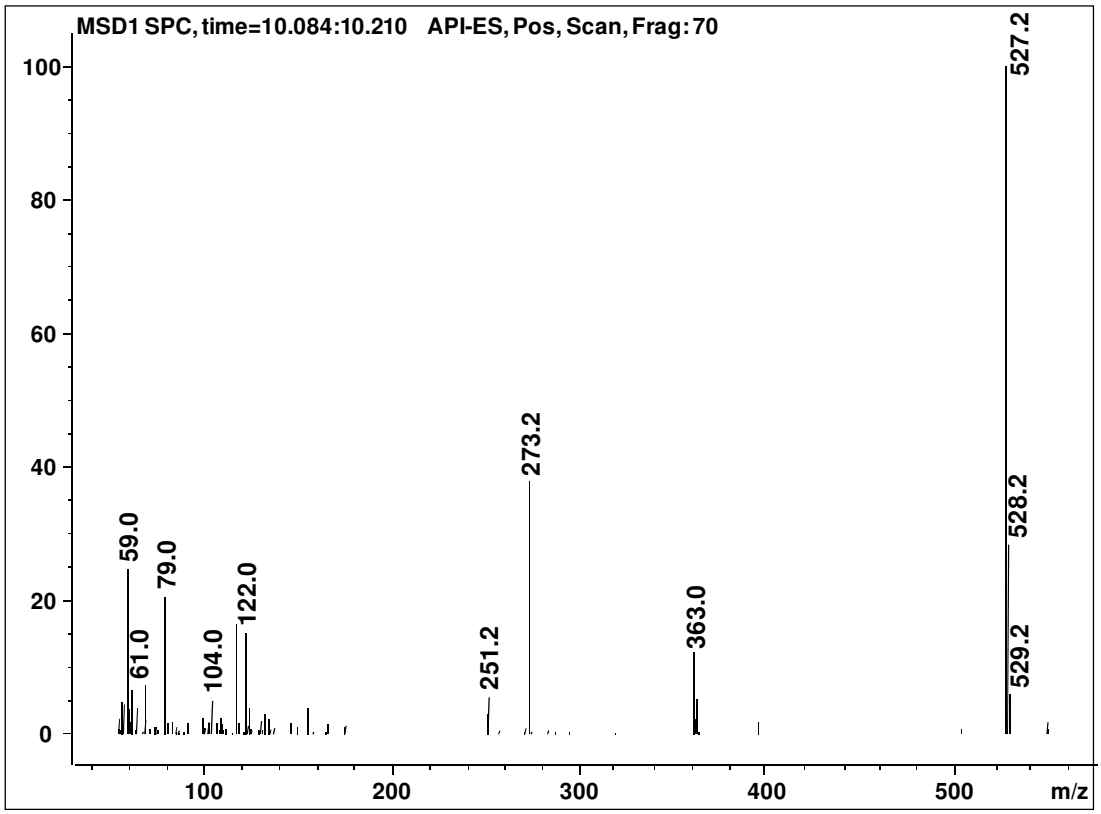
TIC chromatogram after 200 minutes



Mass spectra of a monohaptenated conjugate with m/z 381

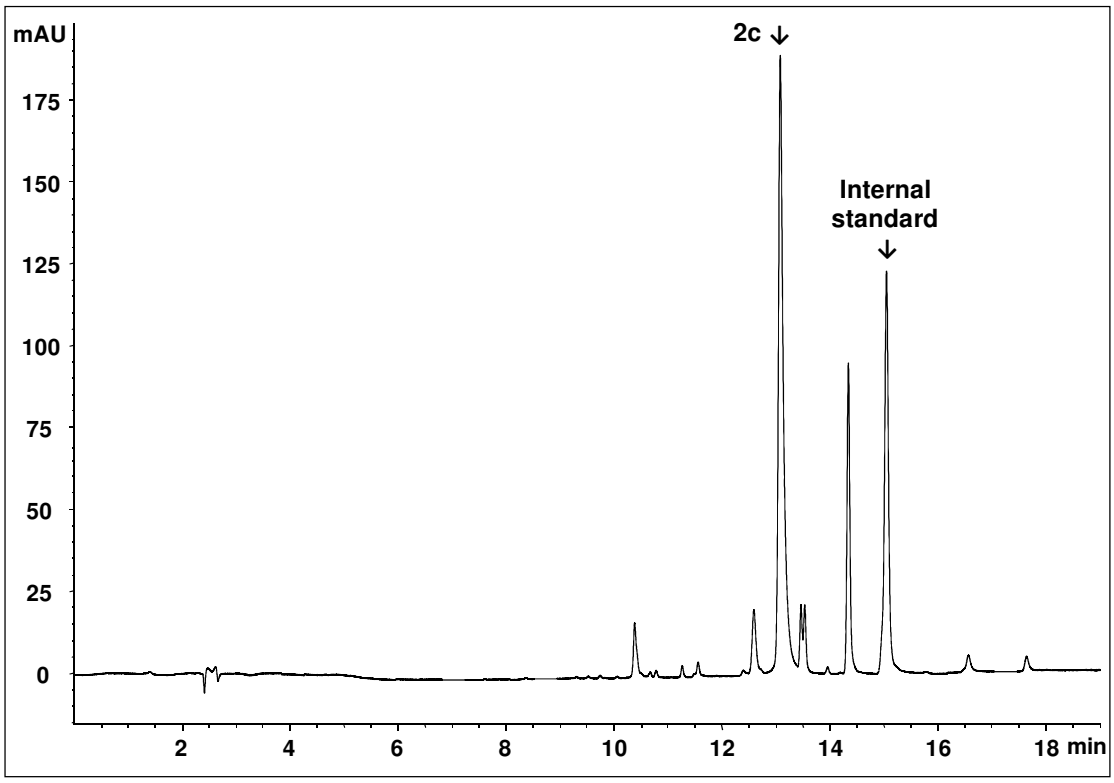


Mass spectra of a dihaptenated conjugate with m/z 527

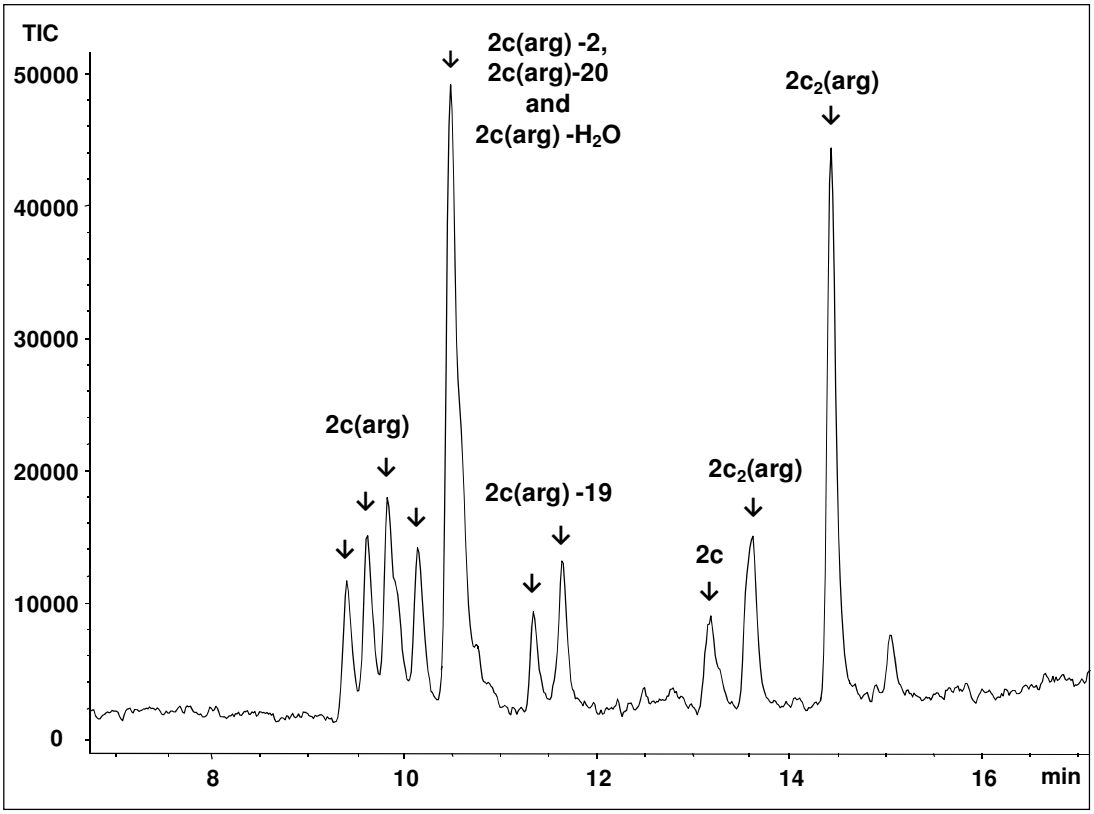


Chromatograms and spectrum from reactivity experiment of compound 2c toward N_α-acetyl-L-Arg

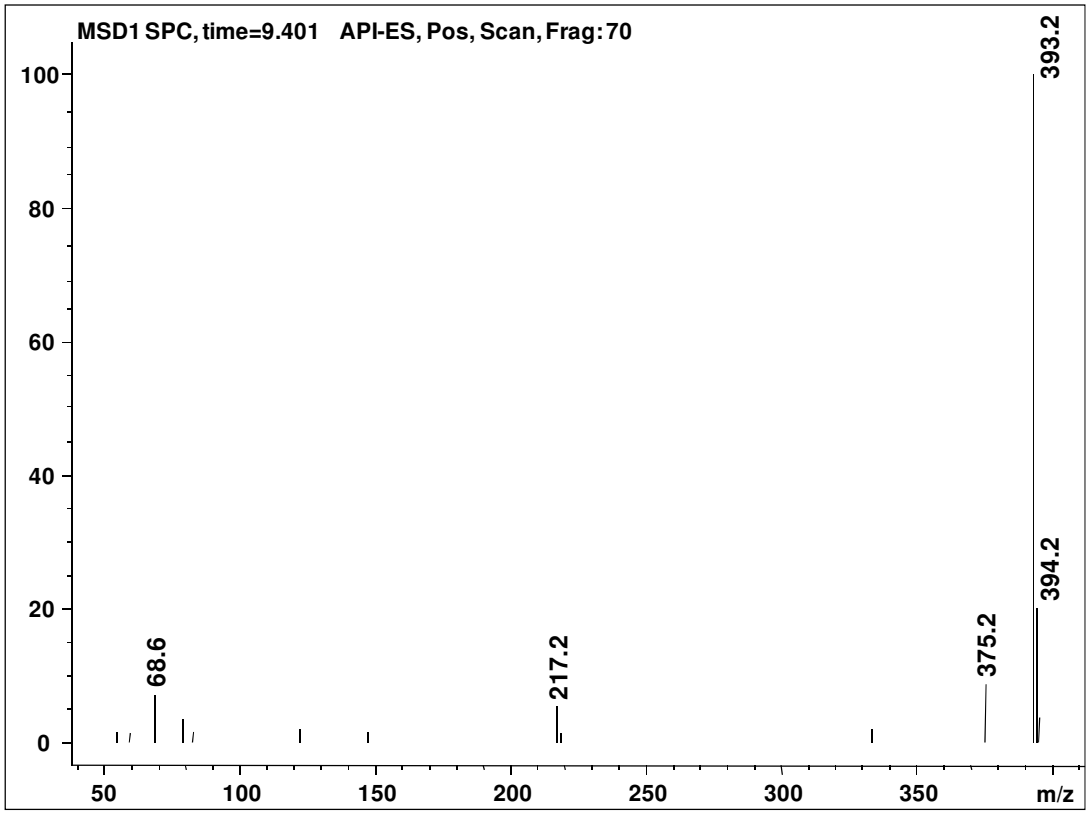
UV chromatogram after 200 minutes at 265.4 nm



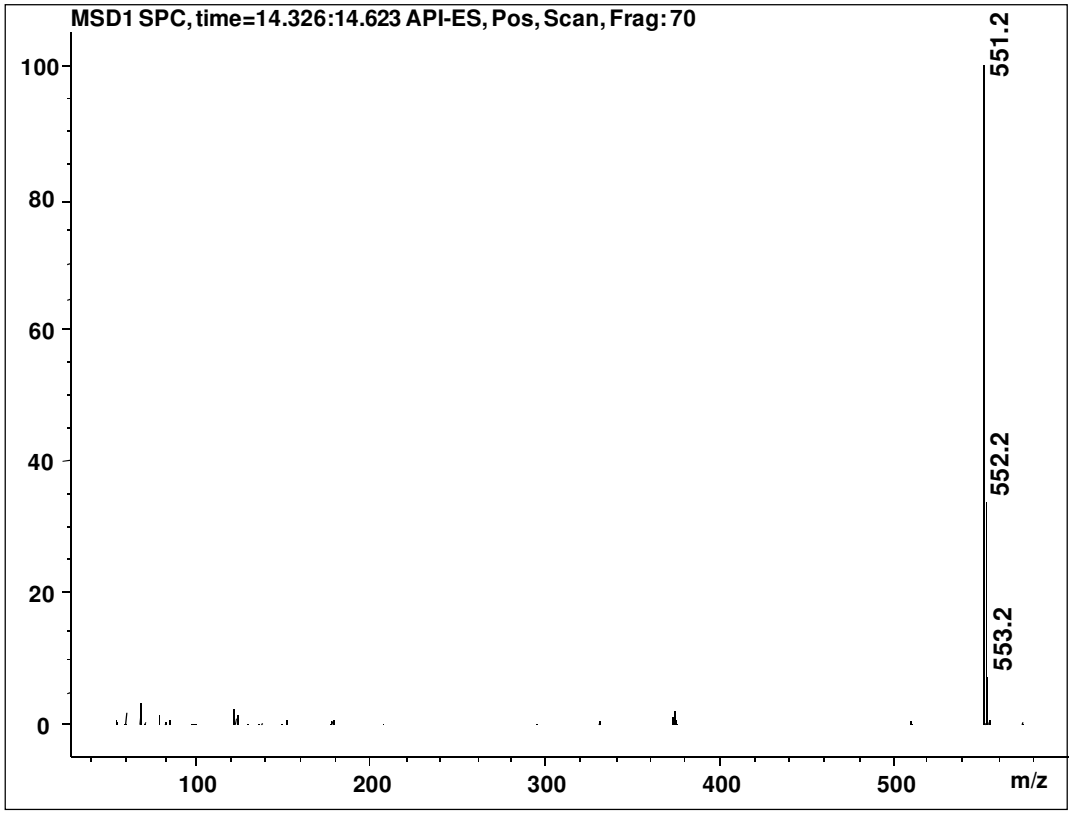
TIC chromatogram after 200 minutes



Mass spectra of a monohaptenated conjugate with m/z 393

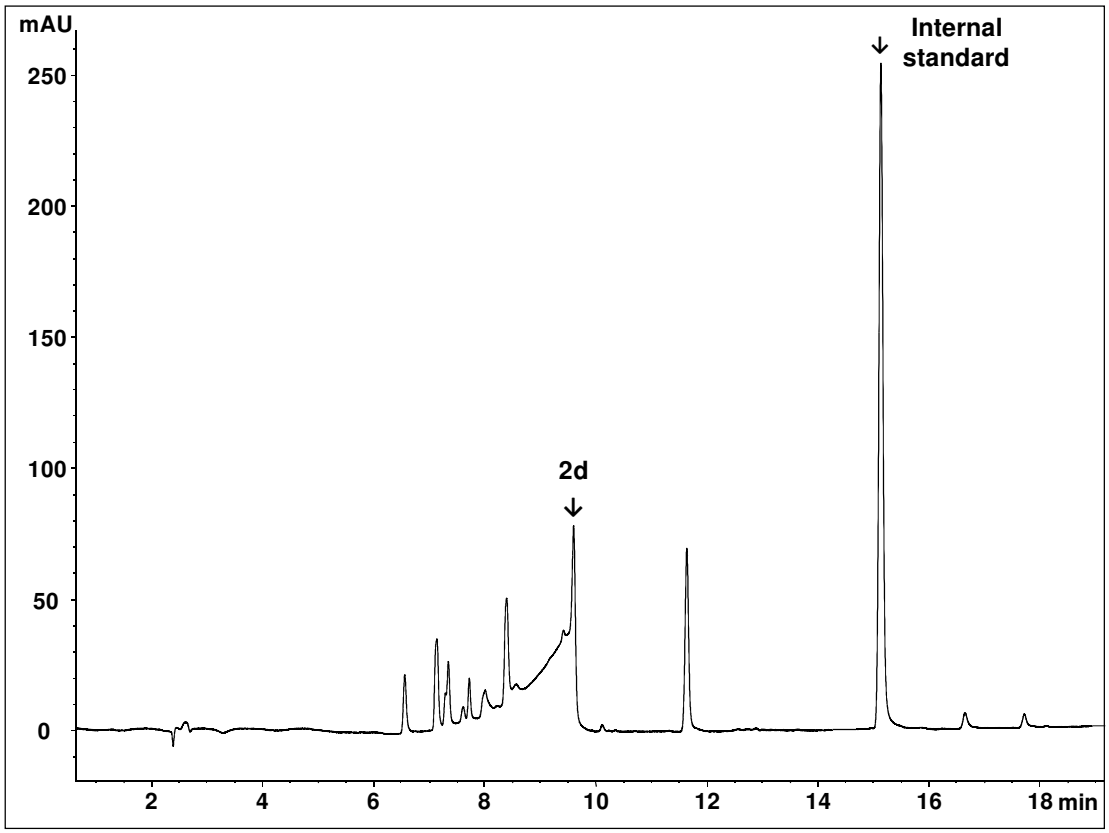


Mass spectra of a dihaptenated conjugate with m/z 551

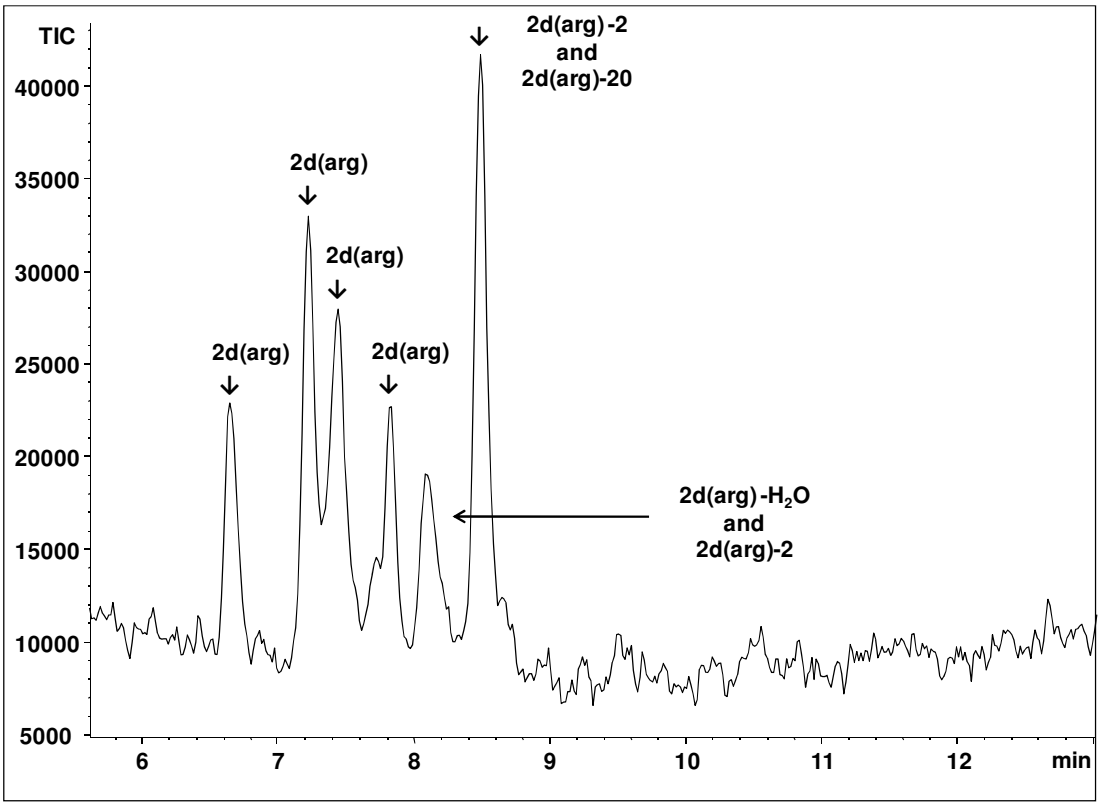
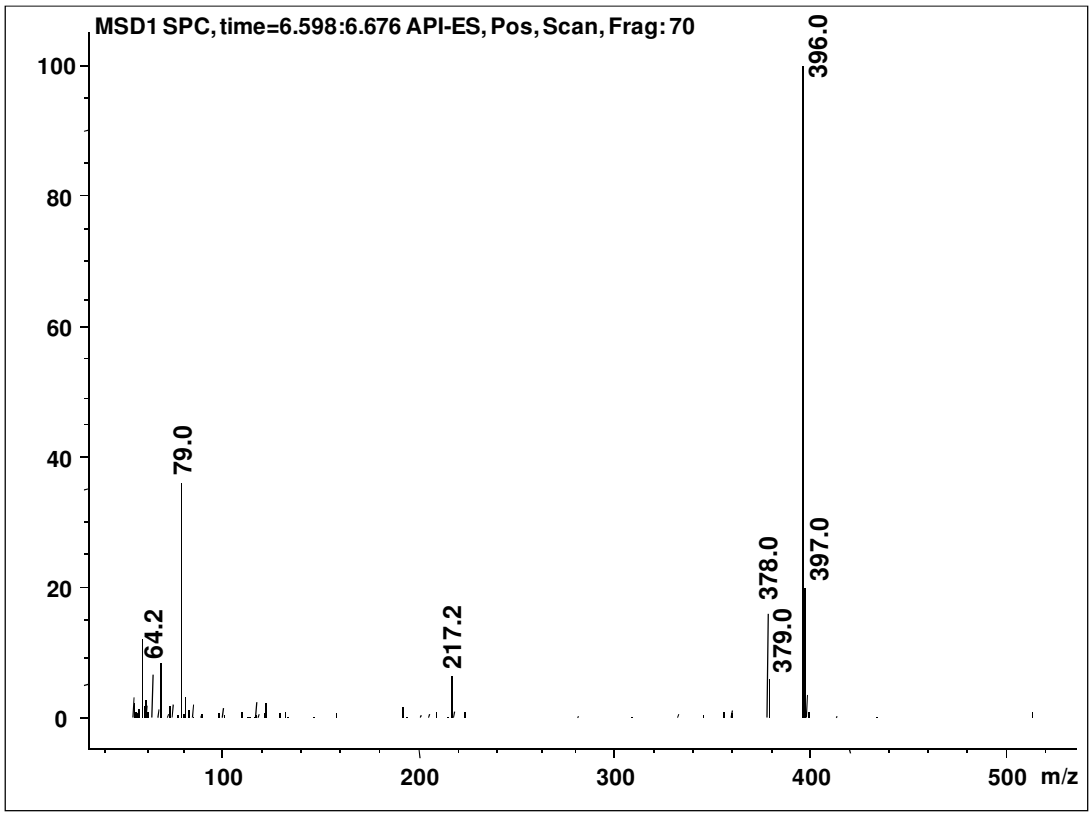


Chromatograms and spectrum from reactivity experiment of compound 2d toward N_α-acetyl-L-Arg

UV chromatogram after 200 minutes at 265.4 nm



TIC chromatogram after 200 minutes



Mass spectra of a monohaptenated conjugate with m/z 396