Photoproducts of benzydamine and azapropazone: demonstration of their phototoxicity *in vitro*

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Abstract

Red blood cell lysis, photosensitized by the products of the aerobic photolysis of benzydamine (**1**) and azapropazone (**4**), was investigated. Irradiation of a methanol solution of **1** and **4** under oxygen produces the photoproducts 3-hydroxy-benzydamine, (**2**), 2-(3-dimethylaminopropyl)-1-benzylindazolin-3-one (**3**) and 3-dimethylamino-7-methyl-1,2,4-benzotriazine (**5**). The mechanism of the photodegradation of **1** was examined. Photoproducts **3** and **5** produce singlet oxygen as demonstrated by trapping with 2,5-dimethylfuran.

The photohemolysis rate for the photoproducts **3** and **5** was enhanced by deuterium oxide and oxygen. No change was observed in the presence of reduced glutathione. The photohemolysis rate was low under anaerobic conditions.

Keywords: Photodegradation, phototoxicity, photohemolysis, benzydamine, azapropazone.

1. Introduction

Benzydamine (**1**) and azapropazone (**4**) are of therapeutic importance because they exhibit anti-inflammatory and analgesic properties. However, these drugs have been reported to initiate adverse light-induced biological effects. Cases of photodermatitis caused by **1** [1–4] and **4** [5] and cases of photoallergy [6, 7] have been reported.

These drugs are photolabile under aerobic conditions. One of the aims of this paper is the study of the photodegradation of **1**. The photodegradation of **4** has previously been reported by Jones *et al.* [7, 8]. Furthermore, the possibility that the photoproducts derived from these drugs are responsible for the photosensitivity and photoallergic and/or phototoxic reactions is investigated. The structures of the different photoproducts have been determined and the phototoxicity and role of oxygen *in vitro* have been established. The phototoxic effects of the major products of the aerobic photolysis of **1**, namely 2-(3-dimethylaminopropyl)-1-benzylindazolin-3-one (**3**), and of **4**, namely 3-dimethylamino-7-methyl-1,2,4-benzotriazine (**5**), were determined by means of a photohemolysis test [9, 10].

2. Experimental details

Benzydamine (**1**) was extracted from Tantum® (Elmor Laboratory) using a Soxhlet extractor with dichloromethane and recrystallized from the same solvent. The purity was 99.2% as determined by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and UV–visible spectrometry. Azapropazone (**4**) was obtained from the Palenzona Laboratory (99% purity). All solvents were of analytical or spectrograde. Reduced glutathione (GSH) and 2,5-dimethylfuran were obtained from Aldrich.

After irradiation the solvent was evaporated at reduced pressure (14 Torr) at room temperature and the residue was purified by preparative thin layer chromatography (silica gel) using a hexane–dichloromethane (1:3) mixture for the separation of the photoproducts of **1** and petrol ether–dichloromethane (1:4) for the separation of the photoproducts of **4**.

The structures of the isolated products were determined by ^{1}H NMR and ^{13}C NMR (Bruker

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Aspects 3000, 300 and 100 MHz), IR (Nicolet DX V 5.07) and mass spectrometry (MS) and gas chromatography-mass spectrometry (GC-MS) (Carlo Erba/Kratos MS 25 RFA) in connection with a Carlo Erba chromatograph equipped with a capillary column (25 m) of cross-linked 5% phenylmethylsilicone. 3-Hydroxy-benzydamine (**2**), 2-(3-dimethylaminopropyl)-1-benzylindazolin-3-one (**3**) and 3-dimethylamino-7-methyl-1,2,4-benzotriazine (**5**) were identical in all respects with authentic samples synthesized in the laboratory according to the methods of Palazzo *et al.* [11], Schmutz *et al.* [12] and Mixich [13] respectively.

2.1. Photolysis

Photolysis of **1** and **4** was carried out in methanol solution (1.50 mmol in 50 ml) at 20 °C for 6 h. A quartz immersion-well photoreactor (Applied Photophysics, parts 3230 and 3307) and a Rayonet photochemical chamber reactor (model RPR-100) equipped with 16 fluorescent lamps (RPR-300 nm) was used for the irradiation of **1**. For the irradiation of **4** an Osram HQL 250 W medium pressure mercury lamp in a Pyrex immersion-well photoreactor was used. Oxygen was bubbled through the reaction mixture throughout the irradiation process. The photodegradation reaction was followed by UV–visible spectrometry with a Perkin–Elmer 559 spectrophotometer as well as by thin layer chromatography. After irradiation was stopped the solvent was evaporated under reduced pressure (14 Torr) and the residue was chromatographed on a silica gel (230 mesh) column. The elution was carried out using solvent mixtures of hexane and dichloromethane (1:3, v/v).

2.2. Photohemolysis

A red blood cell (RBC) suspension from three different samples of freshly obtained human erythrocytes was prepared by washing four times with a tenfold volume of a phosphate-buffered saline (PBS) solution (pH 7.4; 0.01 M phosphate buffer and 0.135 M NaCl), centrifuging the cells at 2500 *g* for 15 min and carefully removing the supernatant. For the photohemolysis experiments RBCs were diluted in PBS containing the individual compounds **1–5** so that the resultant suspension had an optical density (OD) of 0.4–0.8 at 650 nm. An OD value of 0.5 corresponded to 3.3×10^6 cell ml^{-1}. This was read on a Spectronic 20 spectrophotometer.

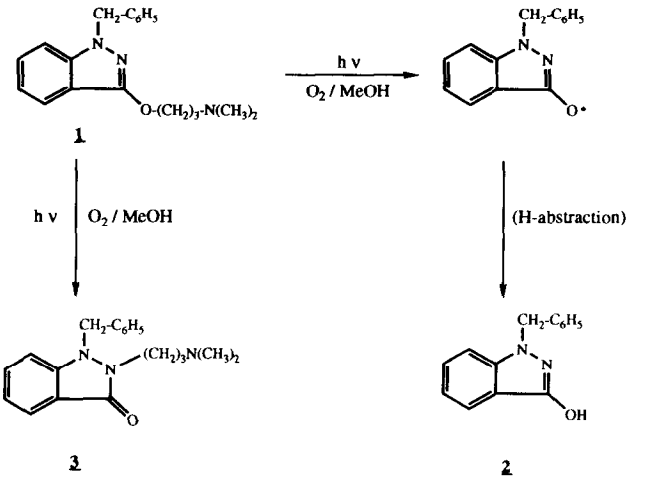
The hemolysis rate was determined by measuring the decreasing OD at 650 nm, since the OD is proportional to the number of intact RBCs [14].

The individual compounds **1–5** were dissolved in the RBC solution and were irradiated at concentrations of 20–80 $\mu g\ ml^{-1}$ in aerobic conditions in a Rayonet photochemical reactor (equipped with 16 phosphor lamps with an emission maximum at 300 and with lamps with an emission maximum at 400 nm) for periods ranging between 10 and 100 min.

3. Results and discussion

The course of the photolysis reaction of **1** is shown in Scheme 1. The major photoproduct of **1** in oxygenated media was **3** (yield, 75%). The formation of photoproduct **2** (yield, 25%) is compatible with an initial excitation of benzydamine after light absorption, followed by cleavage of the O–C bond (O-photodealkylation) to give a radical intermediate (Scheme 1), which via hydrogen abstraction produces compound **2**. The formation of photoproduct **3** is compatible with a rearrangement of **1** after an initial excitation.

Photoproduct **2** (0.375 mmol) (melting point (m.p.) 166–168 °C) showed the following spectroscopic data: IR (KBr): 3500–3200, 2956, 1617, 1530, 1495, 1454, 1363, 1330, 1189, 743, 702, 680 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.34 (m, 1H, aromatic-H), 6.78 (m, 7H, aromatic-H), 6.55 (m, 1H, aromatic-H), 4.74 (s, 2H, N–CH_2–Ph), 4.67 (s, 1H, –OH). ^{13}C NMR ($CDCl_3$): δ 155.06 (s, N=C–OH), 141.43 (s, aromatic-C), 137.11 (s, aromatic-C), 127.85 (d, aromatic-CH), 127.16 (s, aromatic-C), 126.89 (d, aromatic-CH), 126.31 (d, aromatic-CH), 126.20 d, aromatic-CH), 119.62 (d, aromatic-CH), 119.40 (d, aromatic-CH), 118.99 (d, aromatic-CH), 111.40 (d, aromatic-CH),

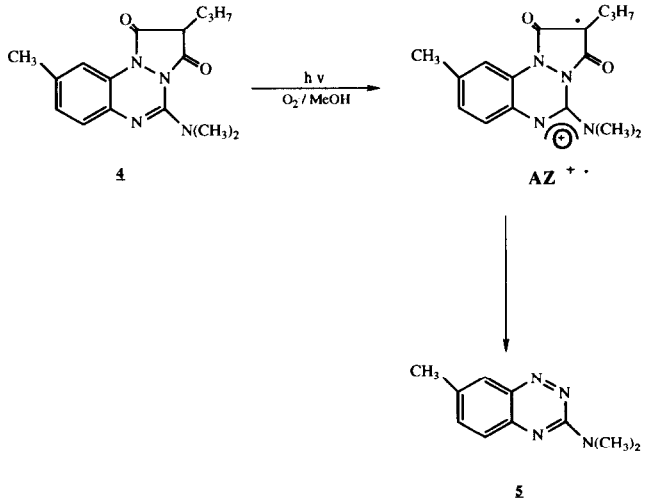


Scheme 1. Proposed mechanism for the photodegradation of **1**.

65.53 (t, N–CH_2–Ph). MS: *m/z* (%) 224 (26, M^+), 207 (18, $M^+ - 17$), 91 (40), 43 (100).

Photoproduct **3** (1.125 mmol) (m.p. 132–134 °C) showed the following spectroscopic data: IR (KBr): 3005, 3000, 2990, 2510, 2436, 2373, 1650, 1490, 1450, 1360, 1200, 1189, 742, 704, 690 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.63 (m, 1H, aromatic-H), 7.19 (m, 7H, aromatic-H), 6.98 (m, 1H, aromatic-H), 5.31 (s, 2H, N–CH_2–Ph), 4.41 (t, $J = 6.0$ Hz, 2H, N–CH_2–), 2.94 (t, $J = 7.7$ Hz, 2H, –$CH_2N(CH_3)_2$), 2.58 (s, 6H, –$N(CH_3)_2$), 2.20 (m, 2H, –CH_2–). ^{13}C NMR ($CDCl_3$): δ 205.89 (s, –C=O), 154.69 (s, aromatic-C), 140.73 (s, aromatic-C), 136.70 (d, aromatic-CH), 127.85 (d, aromatic-CH), 127.64 (d, aromatic-CH), 127.16 (d, aromatic-CH), 126.57 (d, aromatic-CH), 126.27 (d, aromatic-CH), 119.01 (d, aromatic-CH), 118.39 (d, aromatic-CH), 111.96 (d, aromatic-CH), 108.14 (d, aromatic-CH), 65.62 (t, N–CH_2–Ph), 54.62 (t, N–CH_2–), 51.24 (t, –CH_2–$N(CH_3)_2$), 42.79 (q, $N(CH_3)_2$), 24.69 (t, –CH_2–). MS: *m/z* (%) 309 (5, M^+), 264 (8, $M^+ - {}^{\cdot}N(CH_3)_2$), 222 (100, $M^+ - {}^{\cdot}(CH_2)_3N(CH_3)_2$, 91 (60), 85 (90, $CH_2{=}CH{-}CH_2{-}N^{\cdot+}(CH_3)_2$), 86 (65, $^+(CH_2)_3N(CH_3)_2$), 58 (80, $CH_2{=}N^{\cdot+}(CH_3)_2$).

The major photoproduct of the aerobic photolysis of **4** was **5** (yield, 83%) which arises from the cleavage of $\mathbf{4}^{\cdot+}$ (Scheme 2) [7]. The isolated photoproduct **5** (1.24 mmol) was identified spectroscopically by comparison with authentic synthesized material [13] (m.p. 61–62 °C, m.p. (observed) 60–61 °C). The latter showed the following spectroscopic data: IR (KBr): 3374, 2950, 2943, 1737, 1570, 1415, 1093, 820, 705 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.90 (s, 1H, aromatic-H), 7.45 (s, 1H, aromatic-H), 7.43 (s, 1H, aromatic-H), 3.34 (s, 6H, –N–$(CH_3)_2$), 2.42 (s, 3H, –CH_3). ^{13}C NMR ($CDCl_3$): δ 159.14 (s, –C=N–), 141.90 (s, –C–N=N–), 141.00 (s, –C–N–), 137.70 (d, aromatic-CH), 134.47 (s, aromatic-CH), 127.82 (d, aromatic-CH), 125.93 (d, aromatic-CH), 37.05 (q, $-N(CH_3)_2$), 21.17 (q, $-CH_3$). MS: *m/z* (%) 188 (40, M^{+1}), 173 (13, $M^+ - CH_3$), 144 (100, $M^+ - N(CH_3)_2$), 104 (28), 89 (18), 77 (22), 63 (16).



Scheme 2. Proposed mechanism for the photodegradation of **4**.

In order to confirm unambiguously the proposed structures for the photoproducts, independent syntheses of authentic samples were carried out from available reagents [11–13]. The compounds thus obtained exhibited spectra which were indistinguishable from those corresponding to the isolated photoproducts and also from those of the isolated and identified metabolites (compound **2**) from rabbit and rat urine [15, 16].

Information on the photohemolysis mechanism induced by **3** and **5** can be obtained by studying the direct effect of light on these photoproducts. In order to make a direct comparison, most experiments were carried out using the same concentrations of **3** and **5** and the same light sources as in the photolysis experiments.

The possibility that singlet oxygen was produced during the irradiation of **3** and **5** in the RBC solution was examined. In a separate experiment, **3** and **5** were found to be capable of producing singlet oxygen when irradiated in the presence of molecular oxygen in methanol using phosphor lamps ($\lambda_{max} = 300$ nm and $\lambda_{max} = 400$ nm respectively). The production of singlet oxygen was shown when the photolysis of both compounds was carried out in the presence of 2,5-dimethylfuran (DMF) as a singlet oxygen scavenger [10]. Trapping of singlet oxygen in this manner leads to the formation of hexene-2,5-dione, *cis*- and *trans*-3-oxo-1-butenyl acetate and 2-methoxy-5-hydroperoxide-2,5-dimethylfuran, as detected by GC-MS. A control experiment was performed in the presence of DMF in a nitrogen atmosphere and no formation of the corresponding products was detected. The formation of the oxygen trapping products of DMF (derived from the unstable endoperoxide intermediate [17]) was affected by the presence of sodium azide, which acts as a singlet oxygen quencher [9].

In the photohemolysis of RBCs in the presence of **1–5** it was found that only **3**, **4** and **5** caused lysis on irradiation with phosphor lamps ($\lambda_{max} = 300$ nm for **3** and $\lambda_{max} = 400$ nm for **4** and **5**). Similar results were found by Ljunggren [18] for compound **4**, which stimulated UVA-induced hemolysis. The detailed results are given in Figs. 1 and 2. In order to evaluate the photohemolytic potential, the RBC suspensions were irradiated for 20 min in the

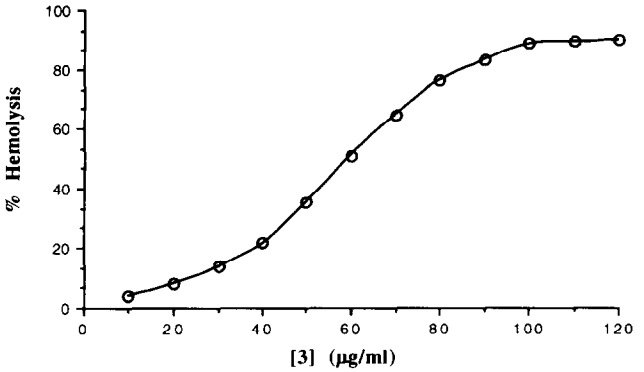


Fig. 1. Photohemolysis of RBCs sensitized by photoproduct **3** under aerobic conditions ([RBC] = 3.3×10^6 cells ml^{-1}; irradiation time, 20 min).

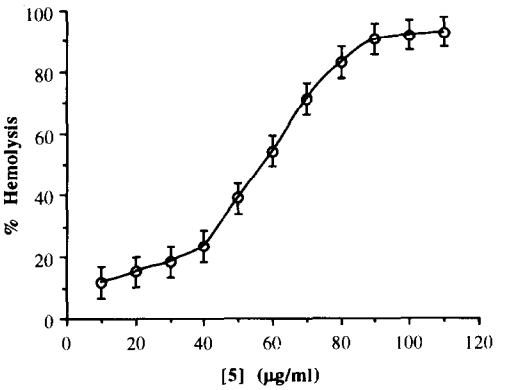


Fig. 2. Photohemolysis of RBCs sensitized by photoproduct **5** under aerobic conditions ([RBC] = 3.3×10^6 cells ml^{-1}; irradiation time, 20 min).

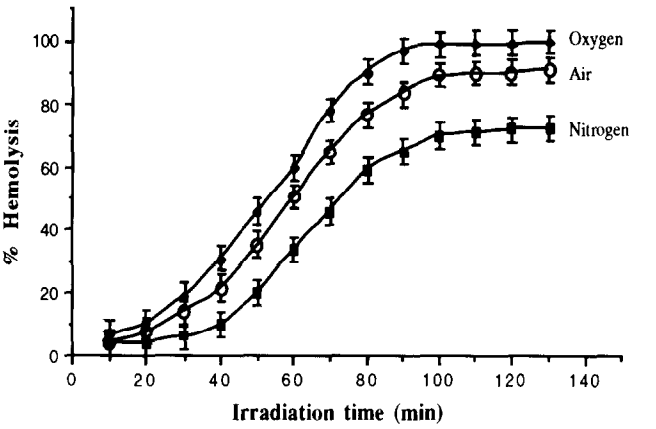


Fig. 3. Photohemolysis of RBCs sensitized by photoproduct **3** in the presence of oxygen, air and nitrogen ([3] = 30 μg ml^{-1}; [RBC] = 3.3×10^6 cells ml^{-1}).

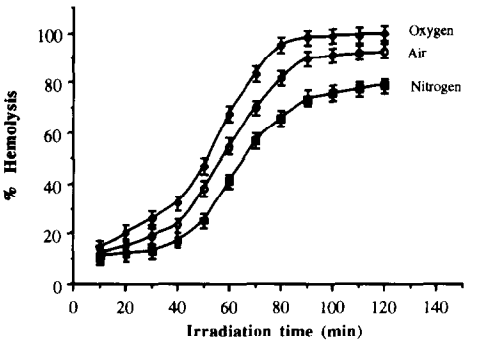


Fig. 4. Photohemolysis of RBCs sensitized by photoproduct **5** in the presence of oxygen, air and nitrogen ([5] = 30 μg ml^{-1}; [RBC] = 3.3×10^6 cells ml^{-1}).

presence of different concentrations of the drugs or its photoproducts (10–120 μg ml^{-1}). No lysis was observed when cells were irradiated for 20 min in the absence of drugs or photoproducts, or when they were incubated for 2 h in the dark at 37 °C with 100 μg ml^{-1} of drug. Furthermore, no lysis was observed when cells were irradiated for 20 min in the presence of **1** and **2** under the above conditions.

It was also found that **3** and **5** are capable of inducing photohemolysis under anaerobic conditions, this effect being markedly enhanced in the presence of oxygen. Typical experiments are illustrated in Figs. 3 and 4.

The oxygen-dependent photohemolysis sensitized by **3** and **5** and the singlet oxygen trapping experiments with DMF prompted us to examine the role of singlet oxygen in the photohemolysis. Therefore the effect of D_2O was investigated. When H_2O is replaced by D_2O in the solvent system, the singlet oxygen lifetime increases [19]. As shown in Table 1, the photohemolysis induced by **3** and **5** is increased proportionally when H_2O is replaced by different proportions of D_2O.

TABLE 1. Effect of D_2O on the photohemolysis induced by the photoproducts **3** and **5**

D_2O (%)	Photohemolysis (%)	
	3	**5**
0	43.2 ± 0.3	52.3 ± 0.2
30	58.4 ± 1.2	68.0 ± 1.5
60	69.2 ± 0.4	80.5 ± 0.8
90	72.1 ± 1.3	84.3 ± 0.9

RBC suspensions in various D_2O–H_2O mixtures plus **3** and **5** (30 μg ml^{-1}) were irradiated for 1 h. Results are the mean ± standard error (SE) ($n = 2$).

In order to provide more information on the photohemolytic mechanism, the studies were repeated in the presence of a radical scavenger GSH. The experiments were carried out with a constant concentration of **3** and **5** (30 μg ml^{-1}) and RBCs (3.3×10^6 cells ml^{-1}) and with a variable concentration of GSH (2.0×10^{-3} to 6.0×10^{-3} M). The

hemolysis rate and the hemolysis percentage were determined 160 min after illumination. No change was observed in comparison with the photohemolysis experiment without GSH. This suggests that free radicals do not play an important role in the membrane damage caused by the photoproducts **3** and **5**.

4. Conclusions

The photoproducts of benzydamine (**1**) and azapropazone (**4**), two photosensitizing drugs, were isolated and characterized. Under the experimental conditions used in this study, the photoproducts **3** and **5** exhibited photohemolytic properties. The experiments with DMF demonstrated that these photoproducts are capable of producing singlet oxygen, and the participation of singlet oxygen in the photohemolysis of RBCs was confirmed using D_2O in the photohemolysis test.

In the light of these results, the phototoxic mechanism of **1** and **4** must involve reactions of stable photoproducts or singlet oxygen with cell membranes following *in vivo* photoactivation.

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