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Synthesis and Properties of a Photoaffinity Labeling Reagent for Protoporphyrinogen Oxidases, the Target Enzymes of Diphenyl Ether Herbicides

Norval O'Connor,[1] René Mornet,[1*] Michel Matringe,[2†] Denis Clair,[2] René Scalla,[2‡] Ted T. Fujimoto[3] and Colin Swithenbank[3]

[1]*Laboratoire de Chimie Organique Fondamentale et Appliquée, Faculté des Sciences, 2 Boulevard Lavoisier, 49045 Angers Cedex, France*

[2]*Laboratoire des Herbicides, INRA, BV 1540, 21034 Dijon, France*

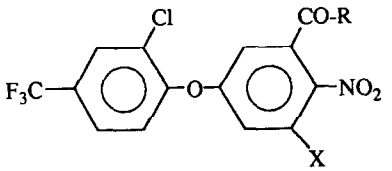
[3]*Rohm and Haas Company, Research Laboratory, 727 Norristown Road, Spring House, PA 19447, U.S.A.*

Abstract—A diazoketone **3** has been synthesized in two steps from acifluorfen **1**, a diphenyl ether herbicide. Like the parent compound **1**, the diazoketone **3** is toxic to plant cells and inhibits protoporphyrinogen oxidase, the molecular target of diphenyl ether herbicides. On photolysis of **3** in methanol, the generated carbene mainly undergoes the Wolff rearrangement to a ketene which further adds methanol, but many other products are observed. A tritiated derivative of **3** has been prepared which is suitable for photoaffinity labeling experiments.

Introduction

It has been recently shown that the target enzymes of diphenyl ether herbicides and related compounds are protoporphyrinogen oxidases.[1] In plants and plant cell cultures, these herbicides induce accumulation of protoporphyrin IX, which is a photosensitizer. Protoporphyrin IX generates singlet oxygen when the tissues or cells are exposed to light, resulting in cell membrane damage through peroxidative degradation.[2] Recent studies[3] using tritium-labeled acifluorfen[4] [^{3}H]-**1** demonstrated that the various inhibitors of protoporphyrinogen oxidases of pea and corn etioplasts share the same binding site on the enzyme and that acifluorfen **1** competes with protoporphyrinogen IX at the catalytic site. Kinetic studies on plant and mouse protoporphyrinogen oxidases also showed that diphenyl ether herbicides are competitive inhibitors with respect to the tetrapyrrole substrate. The yeast enzyme behaves differently however, since the inhibition is mixed competitive.[5]

A photoaffinity labeling reagent would be very useful for further investigations on the inhibition of protoporphyrinogen oxidases by diphenyl ether herbicides. We describe here the synthesis and some properties of a diazoketone derivative **3** of acifluorfen **1** which fulfills all the conditions to be a promising tool in this field.



1 : R = OH, X = H

[^{3}H]-**1** : R = OH, X = ^{3}H

2 : R = OMe, X = H

3 : R = CHN_2, X = H

[^{3}H]-**3** : R = CHN_2, X = ^{3}H

4 : R = Cl, X = H

Results and Discussion

Chemistry

Acifluorfen **1** or acifluorfen methyl **2**, its methyl ester, are both powerful inhibitors of protoporphyrinogen oxidases.[3] That opens the possibility of modifying the molecule at the carboxyl group level, without changing its activity significantly. Thus we chose to replace this carboxyl group by a diazoketone function, which has been successfully used in the design of photoaffinity labeling reagents.[6]

The diazoketone **3** was prepared in two steps from acifluorfen **1** through the acid chloride **4** using classical methods. The products **3** and **4** were obtained easily in good yields. IR, ^{1}H NMR, and mass spectra were consistent with the expected structure of the diazoketone **3**. In particular, the diazo group gave an intense absorption at 2110 cm^{-1} in the IR spectrum.

*Author to whom correspondence should be addressed.
†Present address: Rhone Poulenc Agrochimie, Laboratoire Mixte UMR 41, BP 9163, 12–14 rue Pierre Baizet, 69263 Lyon Cedex, France.
‡Present Address: Laboratoire des Xénobiotiques, INRA, BP3, 31931 Toulouse, France.

A diazoketone [^{3}H]-**3** tritiated on the 3 position of the carboxyl-bearing ring was further prepared through a similar scheme from tritiated acifluorfen[4] [^{3}H]-**1**, with benzoic acid as a carrier. This latter compound was expected to be transformed into benzoyl chloride by the action of oxalyl chloride and finally to diazoacetylbenzene in the last step. It was easily separated from the tritiated diazoketone [^{3}H]-**3** by low pressure reversed phase chromatography.

The photolysis of the diazoketone **3** in methanol gave rise to many products. The multiplicity of the products was not surprising, in view of the complexity of the photochemistry of the diaryl ethers[7] and of the nitro group.[8] We did not attempt to characterize the photoproducts, except the major product **6**. This was identified as the result of the Wolff rearrangement[9] of the generated carbene **5** to the ketene **7**, followed by the addition of a solvent molecule (Scheme I). The compound **8** with the same molar mass as the ester **6**, which was expected to occur from insertion of the carbene in O–H bond of methanol, was not characterized. The compound **6** was distinguished from its isomer **8** with the help of IR (C=O ester absorption at 1740 cm^{-1}) and mass spectrometry. The mass spectrum of **6** exhibited a base peak at m/z 343 (M - NO_2), and peaks corresponding to cleavages of the bonds adjacent to the carbonyl group[10] (M - CH_3O; M - $CH_3OC{=}O$ and $CH_3OC{\equiv}O^+$). As a diphenyl ether[7] and a nitro compound,[8] the compound **6** was partially photodegraded and was recovered in low yield. This photolysis study demonstrated that the diazoketone **3** may react in several ways, some of them probably leading to covalent attachment to neighbouring molecules. Thus, if nucleophilic groups are present in the binding site of protoporphyrinogen oxidases, **3** is expected to bind covalently to these enzymes in photoaffinity labeling experiments, through addition of these nucleophiles to the photogenerated ketene **7**.

Biological activity

The biological activity of the diazoketone **3** was compared to that of acifluorfen methyl **2**, which is a typical diphenyl ether herbicide. The growth of nonchlorophyllous soybean cells was strongly reduced when cultured in light in the presence of **3** (Figure 1), while much less difference was observed when the cells were cultured in the dark, except at the higher concentration of the diazoketone **3**. In the same conditions, very similar effects were induced by acifluorfen methyl **2**. The diazoketone **3** thus induces the same type of light-dependent effects as acifluorfen methyl **2** and other peroxidising herbicides.[2,11]

The diazoketone **3** at a concentration of 10^{-5} M, also induced protoporphyrin IX accumulation in dark-treated soybean cells, like acifluorfen methyl **2**[2,11] (0.74 and 0.87 µg per mg of cells dry weight respectively, after 2 days of treatment in the dark).

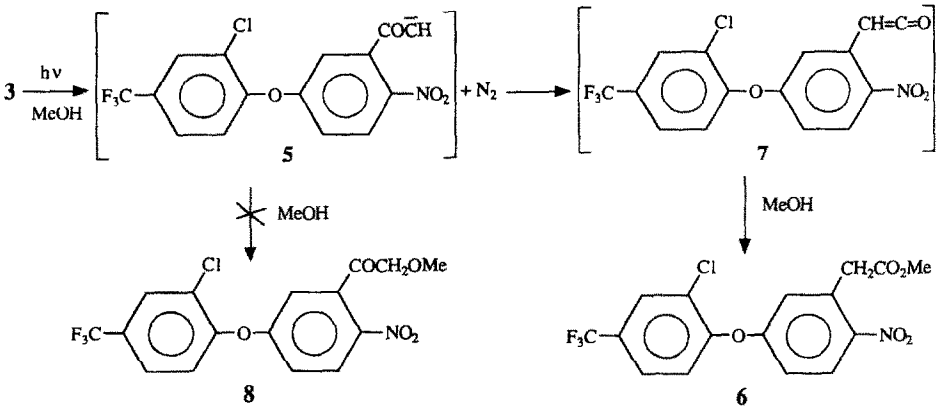
Finally, **3** inhibited protoporphyrinogen oxidase from yeast mitochondrial membranes (IC_{50} = 3 x 10^{-8} M) and from corn etioplasts (IC_{50} = 10^{-8} M) while the corresponding inhibition characteristics of acifluorfen methyl **2** were 7 x 10^{-9} M and 4 x 10^{-9} M respectively.

Thus, all these results clearly showed that the diazoketone **3** behaves in the same way as diphenyl ether herbicides, and consequently appears, in its tritiated form, as a promising reagent for photoaffinity labeling of protoporphyrinogen oxidases, the target enzymes of this class of herbicides.

Experimental

Chemistry

Melting points were taken on a Kofler bank and are uncorrected. ^{1}H NMR spectra were recorded with TMS as internal standard at 270 MHz on a JEOL GSX 270 WB spectrometer. Attribution of ^{1}H NMR spectra were done from Literature data.[12] IR spectra were recorded on a Beckman Acculab 3 spectrometer. EI and FAB MS spectra were recorded on VG spectrometers at the Service Central d'Analyses du CNRS at Vernaison (France). HPLC analyses were run on a Waters apparatus using Merck Lichrospher® RP8 columns, and water–methanol mixtures as eluents. Radioactivity controls on fractions from HPLC were effected with a Beckman LS 1800 scintillation counter, using the Beckman Ready Value™ scintillation cocktail.



Scheme I. Photolysis of the diazoketone **3** in methanol. The major compound **6** is obtained through the Wolff rearrangement of the carbene **5**. The expected insertion product **8** of the carbene in the O–H bond of methanol has not been characterized.

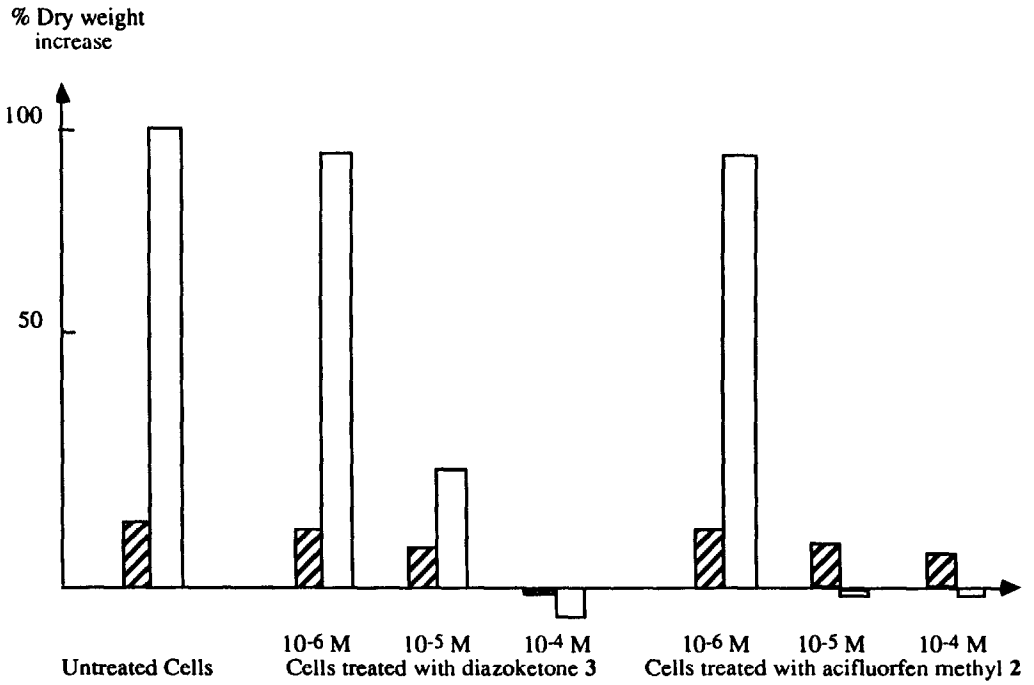


Figure 1. Growth of nonchlorophyllous soybean cells treated with the diazoketone **3** or acifluorfen methyl **2**, referred to that of untreated cells cultured in the dark and then in the light: growth after 48 h in the dark □ or after 48 h in the dark + 72 h in the light ■.

2-Chloro -3'- chlorocarbonyl -4'- nitro -4- trifluoromethyldiphenyl ether 4. 5-(2-Chloro-4-trifluoromethylphenoxy)-2-nitrobenzoic acid **1** (5 g, 13.8 mmol) was dissolved in dichloromethane (50 mL). Pyridine (2 drops) and oxalyl chloride (2.7 g, 21.2 mmol) were added under dry nitrogen atmosphere, and the mixture was heated to reflux overnight. The reaction was followed by TLC on silica gel (hexane:diethyl ether, 1:1). Further pyridine (2 drops) and oxalyl chloride (2.7 g, 21.2 mmol) were added and the solution was heated for an additional 4 h. Dichloromethane (100 mL) was added and the solution was washed with water (100 mL), with a K_2CO_3 saturated solution in water (100 mL), and was then dried over $MgSO_4$. The solvent was removed to yield white crystals which were recrystallized from hexane to give pure **4** (4 g, 76 %) mp 63–66 °C; ^{1}H NMR ($CDCl_3$) 7.14 (IH, d. 8.45 Hz, 6'-H), 7.21 (1H, d, 8.46 Hz, 6-H), 7.24 (1H, d, 2.36 Hz, 2'-H), 7.59 (1H, d, 8.46 Hz, 5-H), 7.78 (1H, d, 1.88 Hz, 3-H), 8.00 (1H, dd, 7.99 and 0.94 Hz, 5'-H); MS m/z 379, 381 (M^+, 1.3 and 0.8 %), 344, 346 (M–Cl, 96 and 32), 166 (100); HRMS Found 343.9940 (M–Cl), $C_{14}H_6{}^{35}ClF_3NO_4$ requires 343.9937.

2-Chloro -3'- diazomethylcarbonyl -4'- nitro -4- trifluoromethyldiphenyl ether 3. 2-Chloro-3'-chlorocarbonyl-4'-nitro-4-trifluoromethyldiphenyl ether **4** (1.72 g, 4.5 mmol) was dissolved in diethyl ether (50 mL) and this solution was added dropwise to a 0.3 M solution (50 mL) of diazomethane in diethyl ether, in a flask carefully protected from light by wrapping with aluminum foil. After the addition was complete, the yellow colour gradually faded, and further diazomethane solution (20 mL) was added. The mixture was then allowed to stand for 1 h. Nitrogen was bubbled through the solution to remove excess diazomethane, and the solvent was evaporated under reduced pressure. The thick yellow liquid residue was chromatographed in the dark trough silica gel with petroleum ether–diethyl ether (2:1) as eluent, giving pure **3** as an oil (1.05 g, 60 %). ^{1}H NMR ($CDCl_3$) 5.53 (1H, s, CH–N=N), 6.95 (1H, d, 2.59 Hz, 2'-H), 7.03 (1H, dd, 8.93 and 2.58 Hz, 6'-H), 7.27 (1H, d, 8.69 Hz, 6-H), 7.60 (1H, d, 8.69 Hz, 5-H), 7.80 (1H, d, 1.65 Hz, 3-H), 8.08 (1H, d, 8.69 Hz, 5'-H); IR 2110, 1625 cm^{-1}; FAB-MS m/z 386, 388 (70 and 27 %, M + H), 358–360 (89 and 32, M + H – N_2); HRMS Found 386.0157 (M + H), $C_{15}H_8{}^{35}ClF_3N_3O_4$ requires 386.0155.

Photolysis study of 2-chloro-3'-diazomethylcarbonyl-4'-nitro-4-trifluoromethyldiphenyl ether 3 in methanol. The diazoketone **3** (200 mg, 0.518 mmol) was dissolved in 400 mL of methanol. The solution was photolyzed for 30 min using a medium pressure mercury vapour lamp located in a quartz immersion apparatus cooled internally by running water. HPLC monitoring of the reaction showed the formation of many products. The amount of the major product initially increased and then decreased. After photolysis, the complex mixture was chromatographed through silica gel with petroleum ether–diethyl ether (1:1) as eluent, giving 2-chloro-3'-methoxycarbonylmethyl-4'-nitro-4-trifluoromethyldiphenyl ether **6** (12 mg, 6 %), which was identical by HPLC to the major compound found during the photolysis; mp 108 °C; IR 1740 cm^{-1}; ^{1}H NMR ($CDCl_3$) 3.72 (3H, s, CH_3O), 4.01 (2H, s, CH_2), 6.89 (IH, d, 2.82 Hz, 2'-H), 6.94 (1H, dd, 8.93 and 2.82 Hz, 6'-H), 7.24 (1H, d, 8.46 Hz. 6-H), 7.60 (1H, dd, 8.46 and 1.88 Hz, 5-H), 7.81 (1H, d, 1.88 Hz, 3-H), 8.19 (1H, d, 9.17 Hz, 5'-H); EI-MS m/z 389–391 (2 and 0.7 %, M^+), 358, 360 (6.5 and 2.3, M - CH_3O), 343, 345 (100 and 34, M - NO_2), 330, 332 (5 and 1.5, M - CH_3OCO), 59 (34, CH_3OCO^+); HRMS Found 389.0270 (M^+),

$C_{16}H_{11}{}^{35}ClF_3NO_5$ requires 389.0278; Found 343.0340 (M $-NO_2$), $C_{16}H_{11}{}^{35}ClF_3O_3$ requires 343.0349.

2-Chloro -3'- diazomethylcarbonyl -4'- nitro -4- trifluoromethyl-5'-tritiodiphenyl ether [^{3}H]-3. 2-Chloro-3'-carboxy-4'-nitro-4-trifluoromethyl-5'-tritiodiphenyl ether[4] [^{3}H]-**1** (4.5 mCi), benzoic acid (42 mg, 0.35 mmol) and two drops of pyridine were added to a 2 M solution of oxalyl chloride in dichloromethane (10 mL). The mixture was heated to reflux for 3 h. The solvent and excess of reagent were evaporated under reduced pressure. The residue was taken up in diethyl ether (3 mL) and transferred into 20 mL of a 0.3 M solution of diazomethane in diethyl ether, in a flask protected from light by aluminum foil. The mixture was stirred at room temperature for 1 h and then evaporated to dryness. The residue was taken up in 3 x 2 mL of a water–methanol (70:30) mixture. The resulting solutions were injected through a Millex® filter in a glass (310 x 25 mm) RP 18 Lichroprep® column (40–63 μm). The column was eluted using a linear gradient (methanol 30 % to 100 % over 1 h—rate 1 mL min^{-1}). The major peak of radioactivity was collected and the solvents evaporated. The residue was taken up in 2 mL of MeOH–H_2O (30:70) and the solution was chromatographed through a Lichrospher® 100 RP 18 (5 μm) column (steel 125 x 4mm) using a linear gradient of water–methanol (30 % to 100 % methanol over 15 min—rate 1 mL min^{-1}). The main peak of radioactivity was collected (1.3 mCi, 29 %). This radioactive material coeluted with the cold diazoketone **3** in HPLC.

Biological studies

The effect of the diazoketone **3** and acifluorfen methyl **2** on nonchlorophyllous soybean cells cultured in the dark or in the light was studied according to a previously described method.[11] Growth of cell cultures was estimated from the increase of cell dry weight after 48 h in the dark, or 48 h in the dark and 72 h in the light (Figure 1).

The accumulation of protoporphyrin IX in soybean cells was measured as previously described,[11] after 48 h of cell culture in the presence of 10^{-5} M diazoketone **3** and acifluorfen methyl **2**.

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Inhibition of protoporphyrinogen oxidase was estimated as previously described.[1a]

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