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Photochemistry of flunitrazepam: a product and model study

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Key words: flunitrazepam (Reg no. 1622-62-4) – 5-(2′-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-(2H)-1,4-benzodiazepin-2-one – N-methyl *p*-nitroacetanilide – photoreduction – photoaffinity label – nitrazepam – GABA receptor – benzodiazepines

Summary

Irradiation of 5-(2′-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-(2H)-1,4-benzodiazepin-2-one (flunitrazepam) in methanol with 300 nm light gave the 7-amino reduction product with a photochemical efficiency of 0.002 M/Einstein. A model study was performed with N-methyl-*p*-nitroacetanilide. It was shown that the nitroaniline photochemistry paralleled that of flunitrazepam and demonstrated that these reactions proceed through the series *p*-nitroso, *p*-hydroxylamino, and finally *p*-aminoaniline products with an overall quantum efficiency of 0.01 M/Einstein. The partial reduction product may serve as the electrophilic affinity-labeling intermediate.

Introduction

The photochemistry of the benzodiazepines has been of interest due to the established phototoxicity of certain members of this class of potent tranquilizing drugs (Cornelissen, 1978, 1979a and b; Magnus, 1976). Recently, the 7-nitro derivatives have been shown to act as photoaffinity labels of the benzodiazepine neuroreceptor (Johnson, 1979); one derivative, 5-(2′-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-(2H)-1,4-benzodiazepin-2-one (flunitrazepam, FNP, I), has received considerable attention in this regard (Battersby, 1979a and b; Sieghart, 1980; Sherman-Gold, 1981; Thomas, 1981, 1983; Gee, 1982; Asano, 1983) because of its specificity and high efficiency as a label for the GABA receptor. It is especially noteworthy that these diazepams lack the usual functionalities employed as photoactivators for affinity labeling. Despite this interest, only one study on the photochemical reactions of flunitrazepam has appeared (Cornelissen, 1981).

In order to obtain a quantitative understanding of the importance of the role of partial reduction intermediates on the photochemistry of flunitrazepam, a simplified model derivative, N-methyl-*p*-nitroacetanilide (IV) was also synthesized and the photoreactions detailed. Comparisons of FNP and IV were made which were helpful in delineating the photoreaction sequence and the photochemical efficiency for the reduction.

Methods and Materials

General

Melting points are uncorrected. High-performance liquid chromatography (HPLC) separations were performed on an Alltech 0.5 × 25 cm C-18 ODS 10 μm column using gradient elution with

acetonitrile/water, and a Perkin Elmer Series III pump module with a Rheodyne injector and Perkin Elmer LC-55B UV-vis detector. Peak area determinations were made with a Varian CDS-111 electronic integrator. Spectra were obtained on the following instruments: ^{1}H-NMR spectra, Varian EM-360; Mass Spectra, Varian MAT CH-5; Infrared Spectra, Beckman Acculab 3 or IBM IR32 FT-IR; Ultraviolet Spectra, Perkin Elmer UV-555. Irradiations were performed using a Rayonet Photochemical Reactor fitted with a merry-go-round apparatus with RPR-3000 A lamps in Pyrex reaction vessels or using a Hanovia 450 W lamp with a Pyrex filter. All photolysis solvents were degassed with nitrogen. Flunitrazepam was provided by Hoffmann-La Roche and was used without further purification.

Quantum yield determinations. Light output was determined using ferrioxalate actinometry (Hatchard, 1956). Photolysis progress was monitored using HPLC (detector wavelength = 245 nm) with biphenyl as an internal standard. Solutions were prepared so that 99% of the incident light was absorbed by the substrate.

Photolysis of I. Irradiations were performed in spectrograde methanol with concentrations varying from ca. 1–7 mg/ml of I. Both a Hanovia fitted with a Pyrex filter (preparative runs) and the merry-go-round apparatus fitted with 16 RPR-3000 A lamps were used as light sources. All reactions were followed by HPLC and were terminated at low conversion in order to avoid secondary photoreactions of products. The assignment of the structure of the photoproduct II isolated from a high conversion run was made from its NMR, IR and mass spectra: NMR: ($CHCl_3$) δ 3.5 (s, 3H), 3.9 (m, 1H), 4.9 (m, 1H), 6.8–7.9 (m, 5H), 7.9–8.5 (m, 2H); IR: ($CHCl_3$) 3300, 2990, 1670, 1610, 1570, 1490, 1450, 1415, 1340, 1280, 1210, 1130, 1105, 1075, 1020, 990, 920, 835 cm^{-1}; mass spec. m/e (rel. int. %) 225 (10.5), 241 (5.4), 254 (20.3), 255 (17.9), 256 (8.5), 264 (8.3), 265 (7.8), 267 (31.2), 268 (15.6), 282 (21), 283 (35.9), 284 (25). The reduction product was shown not to be the fully reduced FNZ (see below) by co-injection on HPLC with hydrogenated FNZ and by comparison of the infrared spectra of the two derivatives.

Reduction of I by catalytic hydrogenation. Using a micro-Brown apparatus as a hydrogen source and 10% platinum on carbon as a catalyst in ethanol, 235 mg of I yielded ca. 85% III as determined by HPLC. NMR ($CDCl_3$): δ 8.0–5.5 (7H, m), 4.0 (3H, s), 4.6 (4H, m), 2.0 (1H, s); IR ($CHCl_3$): 3000, 1650 s, 1500, 1450, 1375, 1210, 815 cm^{-1}; UV (methanol): 244 nm (log ϵ 4.2), 350 nm (log ϵ 2.0). Using a Parr shaker, a 30 mg sample of I in 50 ml of methanol was quantitatively reduced with H_2 and 5% Pt/C.

*Synthesis of N-methyl-*p*-nitroacetanilide(IV).* *p*-Nitroacetanilide was alkylated with methyl iodide in methanol by the method of Pachter (1952) employed without modification. Yield, 3.57 g (65% m.p., 150–152°C, recrystallized from water–ethanol). NMR ($CDCl_3$): δ 2.05 (s, 3H), 3.36 (s, 3H), 7.36, 7.51, 8.23, 8.38 ppm (AA′BB′ q, 4H). IR (KBr): 3050, 1660, 1587, 1515, 1495, 1423, 1375, 1340, 1310, 1140, 1110, 1075, 980, 870, 855, 705 cm^{-1}; MS m/e (rel. int. %): 77 (26), 106 (15.7), 122 (49.7), 152 (100), 194 (25.2 $M\cdot^+$). UV (methanol) λ 285 nm (ϵ 7767).

Photolysis of IV. A solution of 173 mg of IV in 100 ml of methanol was degassed with oxygen-free nitrogen and irradiated with a 450 W Hanovia lamp (Pyrex filter). Reaction progress was monitored by HPLC (10–90% acetonitrile/water, t = 15 min, flow = 2 ml/min, detector wavelength = 254 nm) and after ca. 50% conversion the product was isolated by removing the solvent under reduced pressure, dissolving the residue in chloroform, and extracting the chloroform layer with aqueous hydrochloric acid (pH < 2). The aqueous layer was neutralized with aqueous potassium hydroxide, extracted with chloroform, and the extract was passed through a silica gel column (SilicAR CC-7, Merck, 2.5 × 20 cm) using ether/hexane (0–100% gradient). Three major fractions were collected: an early-eluting, highly colored material which was not identified, starting material, and N-methyl-4-aminoacetanilide (V). The product structure was confirmed by comparison of the IR and NMR spectra with those obtained from an authentic sample of V prepared by catalytic hydrogenation of IV.

Catalytic hydrogenation of IV. An ethanol solution of IV and 10% Pt/C was placed in the

micro-Brown apparatus described above. The reaction proceeded very rapidly to give V in essentially quantitative yield. NMR ($CDCl_3$): δ 1.85 (s, 3H), 3.21 (s, 3H), 3.87 (br s, 2H), 6.56, 6.71, 6.88, 7.01 (AA'BB' q, 4H); IR ($CDCl_3$): 3312 br, 1610 s, 1505, 1417, 1375, 1285, 1168, 1137, 1080, 973, 830 cm^{-1}; UV (methanol) λ (ϵ) 292 (1380), 248 (10,900), 234 nm (5030).

Synthesis of N-methyl-4-nitrosoacetanilide (VI). Using a procedure adapted from Hall (1978), in a 250 ml three-necked flask was placed 6.15 g (0.032 M) of N-methyl-4-nitroacetanilide in 105 ml of 2-methoxyethanol. The solution was warmed to 33°C and 2.55 g (0.048 M) of ammonium chloride in 25 ml of water was added with stirring. Approximately 6 g (0.083 M) of zinc dust was added slowly over an hour taking care to maintain the temperature below 40°C. After an additional 30 min, the resulting grey-green suspension was filtered through celite under an inert atmosphere to yield a clear yellow liquid. This was added dropwise over a 40 min period to a mechanically stirred solution of 21 g (0.078 M) of ferric chloride in 135 ml of water and 36 ml of ethanol while the temperature was maintained at 5°C. After 1 h, the dark green mixture was poured into 300 ml of ice water and the remaining solid $FeCl_3$ was removed by filtration. The filtrate was extracted with $CHCl_3$, the $CHCl_3$ layer dried and evaporated to yield 1.02 g (0.005 M, 18%) of a shiny green solid (VI) recrystallized from ether–hexane (m.p. 88–88.5°C). NMR ($CDCl_3$) δ 2.00 (s, 3H), 3.30 (s, 3H), 7.13, 7.40, 7.75, 7.93 (AA'BB' q, 4H); IR (KBr) 3035 br, 1665 s, 1590 s, 1460, 1420, 1380, 1355, 1315, 1290, 1190, 1125, 1085, 990, 865, 835 cm^{-1}; MS m/e (rel intensity %) 56 (35), 77 (35), 79 (31), 106 (52), 136 (36), 178 (21); ^{13}CMR ($CDCl_3$) δ 169 s, 163 s, 150 s, 126 d, 121 d, 36 d, 22 d; UV (CH_3OH) K 324 nm (ϵ 10,200).

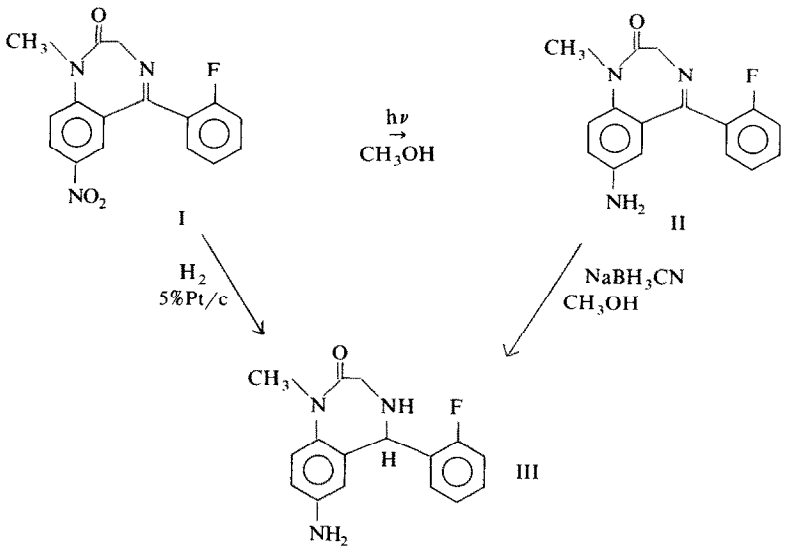
Photolysis of N-methyl-4-nitrosoacetanilide (VI). A solution of ca. 2 mg of N-methyl-4-nitroacetanilide in 30 ml (3.75×10^{-4} M) of methanol was degassed with argon and irradiated at 300 nm in Pyrex. Reaction progress was followed by HPLC on a C-18 reverse-phase column as above. The major photolysis product was the 4-aminoacetanilide; the 4-hydroxylamino acetanilide, VII, was shown to be an intermediate in this reaction. Products were confirmed by coinjection with authentic samples on HPLC.

Synthesis of N-methyl-4-hydroxyaminoacetanilide (VII). Using a procedure adapted from Smissman (1972), a dried, three-necked 250 ml flask was charged with a solution of 3.08 g (0.016 M) of N-methyl-4-nitroacetanilide in 53 ml of 2-methoxyethanol under a nitrogen atmosphere followed by a solution of 1.30 g (0.024 M) of ammonium chloride in 10 ml of water. The solution was warmed to 35°C and 3 g of zinc dust was added with vigorous stirring over a 30 min period. After an additional hour, the resulting grey-green solution was cooled to room temperature and filtered through celite. The filtrate was poured into 30 ml of ice-water and the water layer extracted with CH_2Cl_2, the CH_2Cl_2 layer dried and the solvent evaporated to give a mixture of the nitro and hydroxylamino acetanilides. These were separated with the spinning tlc method. NMR ($CDCl_3$) δ 2.00 (s, 3H), 3.35 (s, 3H), 7.30, 7.35, 7.40, 8.15, 8.30, 8.40 (AA'BB' multiplet, 4H); IR ($CHCl_3$) 3005, 1655, 1600, 1500, 1470, 1420, 1385, 1355, 1310, 1290, 1145, 1015, 980, 860, 700, 640 cm^{-1}

Results and Discussion

In agreement with the studies of Cornelissen et al. (1981), the photoreduction of the nitro group of I in methanol gave the phenylene diamine product II. A modest overall quantum efficiency of 0.002 M/Einstein was determined for this transformation. Comparison of the infrared and mass spectra and the HPLC retention times of the phenylene diamine photoproduct with the product of catalytic reduction of flunitrazepam indicated that both products had lost the characteristic NO_2 bands at 1140 and 1530 cm^{-1} and displayed the characteristic NH absorptions at 3300–3400 cm^{-1}. However, absence of the imine band at 1610 cm^{-1} in the catalytic reduction product and the difference in the HPLC retention times clearly indicated that the two reduction products were not identical. Furthermore, treatment of the photoproduct II with sodium cyanoborohydride gave the fully reduced product III as demonstrated by identical HPLC retention times and by coinjection on

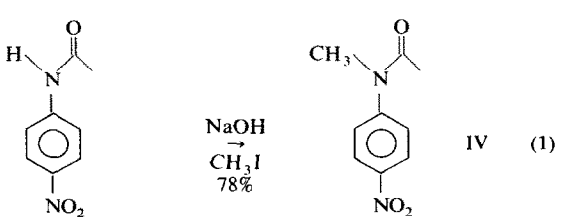
HPLC. These results are summarized in Scheme I.

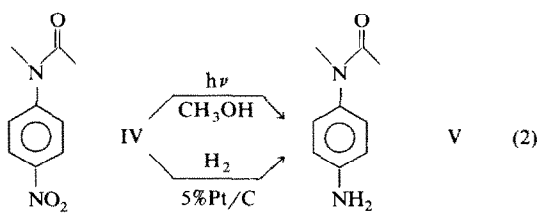


Scheme I. Photochemical and chemical reductions of flunitrazepam.

In order to further examine the nature of the photoreduction sequence for the nitro group, a model derivative, N-methyl *p*-nitro acetamide (IV) was synthesized (Eqn. 1). As shown in Eqn. 2, the photochemistry of IV paralleled that of flunitrazepam, yielding as the major product N-methyl N-acetyl phenylenediamine (V) with a quantum efficiency of 0.01. For the photoreduction of IV to V, two additional intermediate reduction products were detected and were independently identified by comparison with authentic samples synthesized by the routes shown in Scheme II.

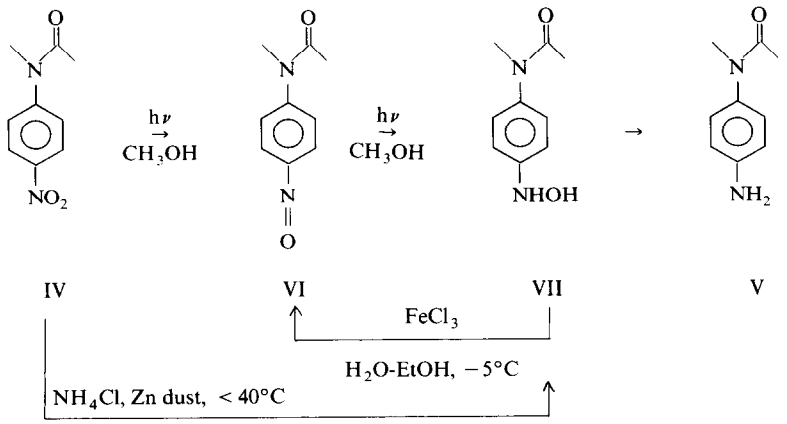
Intermediate reduction products were also detected in the photoconversion of flunitrazepam but proved to be too unstable in methanol to permit isolation. Nevertheless, the course of the flunitrazepam photoreduction appeared to parallel that of N-methyl *p*-acetanilide (IV).





Several features of these two photoreactions bear on the question of how flunitrazepam acts as a photoaffinity label. The first is the requirement that a hydrogen atom source be present in order to effect a reduction. In non-hydrogen atom donating media such as benzene these two nitro-aromatics are unreactive whereas in reducing solvents such as methanol or isopropanol, the disappearance of the nitro group and formation of the intermediate reduction products proceeds very readily.

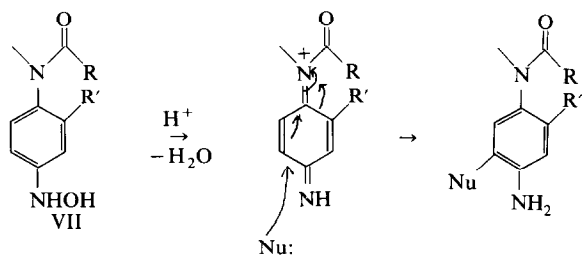
A second, more important feature that emerges from this study is the observation of *sequential photochemical* steps leading to complete reduction. Many of the previous studies on the photochemical reductions of aryl nitro derivatives have indicated that only the initial step is photochemically activated, implying that the subsequent processes are thermal, ground state reactions. We have shown



Scheme II. Chemical and photochemical routes for the photoreduction intermediates.

that each intermediate is converted to the next lower reduction stage photochemically which results in a combined overall low efficiency for the complete reduction. A further consequence of this sequential photochemical process is that the nitroso and hydroxyl amino intermediates will survive for sufficiently long periods of time at these low concentrations and under the low intensity radiation normally available for laboratory photochemical reactions. This permits bimolecular reactions at nucleophilic sites on a substrate (e.g. amino or thiol groups on proteins) to couple with the electrophilic centers on the partially reduced reactive intermediates. Since condensation and nucleophilic substitution reactions of nitroso and hydroxy amino aromatics are well known (March, 1985), these reactions provide a series of potential routes to the covalently linked flunitrazepam.

While little is known at this time about the exact nature of the photolabeling sequence, reactions of amino groups situated in the GABA receptor with the nitroso function generated in situ by the photoreduction of flunitrazepam provide just such a possibility for the irreversible attachment of the partially reduced nitrazepam. An engagingly attractive mechanism for covalent labeling by conjugate addition to the quinodiiminium ion generated from the hydroxyl amine intermediate is shown in Scheme III. This route is particularly intriguing due to the unusually reactive intermediate formed and the parallel conjugate addition chemistry that has been so successfully exploited for the parent quinone derivatives in the trapping of nucleophiles and in suicide inhibitor and affinity labeling studies.



Scheme III. Conjugate addition route to covalent adducts.

While neither of these routes has been established in in vivo studies at this time, they do present possible alternatives to the generally accepted mechanisms for photoaffinity labeling reactions and provide a reasonable pathway for photodegradation of the nitrazepams in general. Furthermore, if these proposed labeling mechanisms do prove to be correct, these studies will provide another approach to the design and implantation of a new class of photoaffinity labels.

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