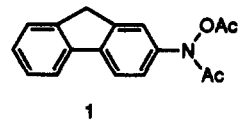
Generation and Identification of the Amidyl Radical Resulting from Homolytic N–O Cleavage in Carcinogenic *N*-Acetyl-*N*-(acyloxy)-2-aminofluorene

Josette Michon, André Jeunet, Nadia Pelloux, Eric Defrancq, Marie-France Lhomme, and Jean Lhomme*

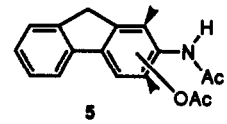
L.E.D.S.S., URA CNRS 332, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

Received May 18, 1993

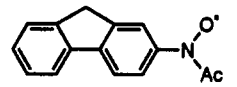
Aromatic amides represent a major class of carcinogenic substances.[1] *In vivo* *N*-hydroxylation followed by esterification[2] produces reactive species which alter cellular macromolecules including DNA.[3] *N*-Acetoxy-*N*-acetyl-2-aminofluorene (1) is representative of this class of carcinogens and is the object of a large number of biological and chemical studies.[2,4] Although the fine details of the reaction are still under active investigation, it is generally accepted that the primary event in carcinogenesis is covalent binding to guanine residues in DNA involving the intermediacy of a nitrenium ion species[5] generated by heterolytic N–O bond cleavage in 1. Homolytic cleavage



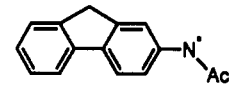
1



5

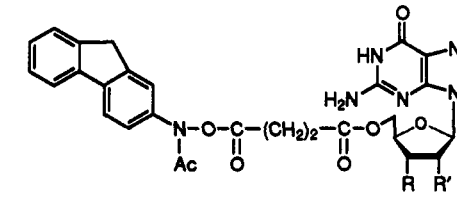


7

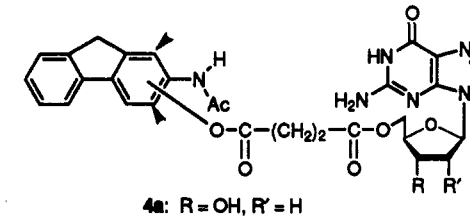


6

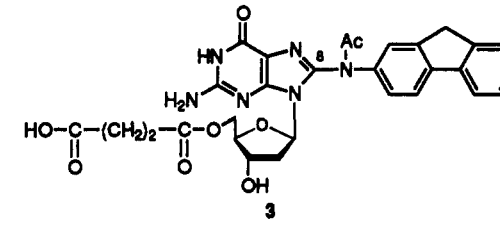
has also been considered leading to radicals[4,6] which may intervene at different levels, including lipids alteration, DNA–protein cross linking, or DNA modification. Formation of radical species derived from model *N*-pivaloxyacetanilides has indeed been reported.[7] In order to mimic the reaction of the carcinogen intercalated in DNA and to obtain an insight into the mechanism which leads to binding of the carcinogen 1 to the guanine ring at position C-8, we have previously prepared the model compounds 2. We have reported that **2a** reacts in water through the



2a: R = OH, R' = H
2b: R = OTBDMS, R' = H
2c: R, R' = acetonide



4a: R = OH, R' = H
4b: R = OTBDMS, R' = H
4c: R, R' = acetonide



3

intermediacy of a nitrenium ion to give essentially the arylamidation product 3 and that all kinetic and products data can be interpreted in terms of a major influence of intramolecular hydrophobic guanine–fluorene ring–ring stacking interactions.[8] In non-nucleophilic solvents, however (dichloromethane, acetonitrile, or benzene), products 2 rearrange to their ortho isomers 4[9] as 1 gives isomers 5.[10] We have now searched for possible homolytic behavior of the metabolite 1 and of the corresponding models 2. We describe here the generation and identification of the amidyl radical 6 by ESR spin trapping experiments.[11]

When 10^{-2}M solutions of *N*-acetoxy-*N*-acetyl-2-aminofluorene (1) in water or in acetonitrile were heated in an ESR cavity at 70 °C, or in dichloromethane at 40 °C, no resonance could be detected. When heating was performed in benzene at 70 °C, ESR signals appeared which remained at a steady state for at least two h at 70 °C. The spectrum (Figure 1A) shows a triplet due to coupling with nitrogen (a_N = 6.7 G ; Lande factor g = 2.0065 ± 0.0002). However when 1 was heated in degassed benzene, the ESR signal was very weak and each line of

(1) Garner, R. C.; Martin, C. N.; Clayson, D. B. *Chemical Carcinogens*; Searle, C. E., Ed. *ACS Monograph* **1984**, *1*, 175–276. Parkes, H. G.; Evans, A. E. J. *Chemical Carcinogens*; Searle, C. E., Ed. *ACS Monograph* **1984**, *1*, 277–301. King, M. C.; Romano, J. L.; Schnetzle, D. *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes*; Elsevier: New York, 1988.

(2) Kriek, E.; Westra, J. G. *Chemical Carcinogens and DNA*; Grover, P. L., Ed.; CRC Press: Boca Raton, FL, 1979; Vol. 2, p 1–28. McManus, M. E.; Burgess, W. M.; Veronese, M.E.; Hugett, A.; Quattrochi, L. C.; Tukey, R. H. *Cancer Res.* **1990**, *50*, 3367–3376. Shen, J. H.; Wegenke, M.; Wolff, T. *Carcinogenesis* **1990**, *11*, 1441–1444. Singer, B.; Grunberger, D. *Molecular Biology of Mutagens and Carcinogens*; Plenum Press: New York, 1983; pp 132–141.

(3) Kriek, E.; Miller, J. A.; Juhl, U.; Miller, E. C. *Biochemistry* **1967**, *6*, 177–182. Westra, J. G.; Kriek, E.; Hittenhausen, H. *Chem. Biol. Interact.* **1976**, *15*, 149–164. Smith, B. A.; Springfield, J. R.; Gutmann, H. R. *Carcinogenesis* **1986**, *7*, 405–411.

(4) Beland, F. A.; Kadlubar, F. F. *Environ. Health Perspect.* **1985**, *62*, 19–30.

(5) Novak, M.; Pelecanou, M.; Pollack, L. *J. Am. Chem. Soc.* **1986**, *108*, 112–130. Novak, M.; Pelecanou, M.; Zemis, J. N. *J. Med. Chem.* **1986**, *29*, 1426–1429. Gassman, P. G.; Granrud, J. E. *J. Am. Chem. Soc* **1984**, *106*, 1498–1499 and 2448–2449. Underwood, G. R.; Kirsch, R. B. *J. Chem. Soc., Chem. Commun.* **1985**, 136–138. Novak, M.; Rangappa, K. S. *J. Org. Chem.* **1992**, *57*, 1285–1290. Griffith-Humphreys, W.; Kadlubar, F. F.; Guengerich, F. P. *Proc. Natl. Acad. Sci. U.S.A* **1992**, *89*, 8278–8282.

(6) Stier, A.; Claus, R.; Bösterling, B.; Reitz, I. *Free Radical and Cancer*; Floyd, R. A., Ed., 1982; pp 63–80.

(7) Novak, M.; Brodeur, B. A. *J. Org. Chem.* **1984**, *49*, 1142–1144.

(8) Defrancq, E.; Pelloux, N.; Leterme, A.; Lhomme, M. F.; Lhomme J. *J. Org. Chem.* **1991**, *56*, 4817–4819.

(9) Defrancq, E.; Leterme, A.; Pelloux, N.; Lhomme, M. F.; Lhomme, J. *Tetrahedron* **1991**, *47*, 5725–5736.

(10) Underwood, G. R.; Kirsch, R. B. *J. Chem. Soc., Chem. Commun.* **1985**, 136–138.

(11) Choice of the solvents and of the model derivatives **2a–c** examined in the ESR experiments was largely determined by the solubility of the products. *N*-acetoxy-*N*-acetyl-2-aminofluorene (1) was studied in a wide range of solvents: water, benzene, acetonitrile, dichloromethane. Model compound **2a** is soluble in water and insoluble in benzene. Models **2b** and **2c** on the other hand could not be studied in water but were soluble in benzene, dichloromethane, and acetonitrile.

0022-3263/93/1958-6143$04.00/0

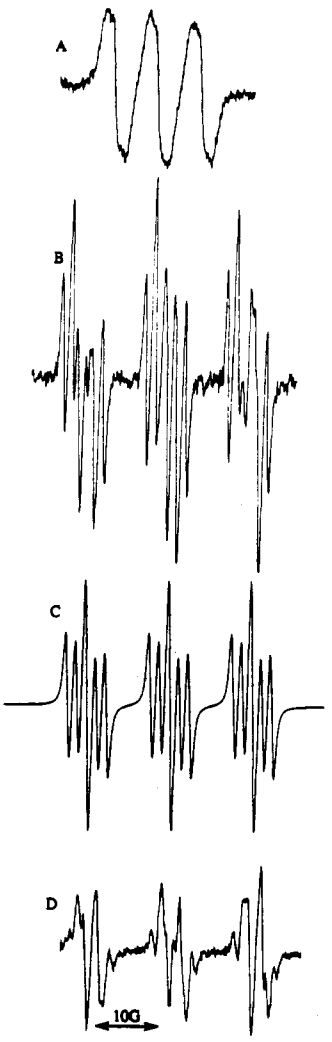
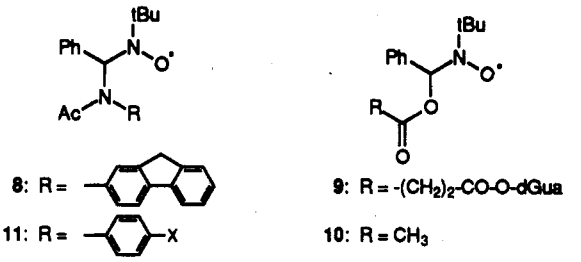


Figure 1. A: Observed ESR spectrum for **1** in benzene. B: Observed ESR spectrum for **2b** with PBN in degassed benzene at 70 °C. C: Simulated spectrum for **8**. D: Observed ESR spectrum for **2b** with PBN in degassed benzene at 20 °C.

the triplet gave also a triplet with an additional splitting $a_H = 1.5\ G \pm 0.1\ G$. Either structure **6** or **7** can be ascribed to this radical. The same spectrum was observed when model compounds **2b** and **2c**[11] were heated in identical conditions in degassed benzene (10^{-2} M, 70 °C), but in this case the intensity of the signals was much higher. Moreover the spectrum is also observed when **2b** and **2c** are heated in dichloromethane solution at 40 °C (triplet with hfcc of 7.0 G which is slightly larger than that observed in benzene as a result of solvent polarity increase). The same weak signals were also detected when **2a** was heated in water and acetonitrile at 70 °C. The nitroxide structure **7** was assigned to this radical formed from **1** and **2** as follows: using the method developed by Forrester,[12] a solution of *N*-acetyl-*N*-hydroxy-2-aminofluorene in benzene was oxidized in the ESR cavity with silver oxide or lead tetraacetate to give the corresponding nitroxide **7**.[13] The ESR spectrum was identical to that generated by thermolysis of **1** and **2**. The calculated Lande *g* factor 2.0065 ± 0.0002 corresponds to a nitroxide radical. A smaller value is expected for an acylamino radical (g = 2.004).[14]

Radical trapping experiments were also performed. When a solution of **2b** (10^{-2}M) and *C*-phenyl-*N*-*tert*-butyl nitrone (PBN) in degassed benzene was heated, ESR signals corresponding to a mixture of the two radicals **8** and **9** were observed (structure assignments are justified below). Due to the difference of stability of those two species, the ESR spectrum of each species could be obtained by varying the temperature.



At 70 °C the signals observed remained constant for several hours, corresponding essentially to the major species **8** (Figure 1B). The 15-line spectrum could be adequately computer simulated (one nitrogen atom with $a_{N_1} = 13.0 \pm 0.1$ G, one proton with $a_H = 3.1 \pm 0.1$ G, and one nitrogen atom with $a_{N_2} = 1.5 \pm 0.1$ G, Figure 1C). In the three groups of lines, the intensities of the second and fourth lines were higher in the experimental spectrum than in the computed spectrum. This was due to the perturbation caused by the minor radical species **9** present in solution. The presence of **9** is also responsible for the nonsymmetrical aspect of the experimental spectrum 1B.

The second species **9** could be observed at 20 °C after rapid heating of the solution of **2b** in benzene and cooling to 20 °C (Figure 1D). The spectrum appeared as a triplet of doublets ($a_N = 13.5 \pm 0.1$ G; $a_H = 2.2 \pm 0.1$ G). A similar behavior was observed for *N*-acetoxy-*N*-acetyl-2-aminofluorene **1** when heated in degassed benzene solutions. The identical 15-line spectrum assigned to radical **8** was observed as previously. A triplet of doublets was equally observed with hfcc $a_N = 13 \pm 0.1$ G, $a_H = 2 \pm 0.1$ G corresponding to species **10**.

These trapping experiment results are consistent with the radical structures as indicated. The same spin adduct **8** is obtained from both compounds **1** and **2b**. The simulated spectrum which correctly fits the experimental data indicates in particular the existence of a coupling with nitrogen a_{N_2}. This is clear evidence that a species of type RN•R′, such as the amidyl **6**, has been trapped. In addition this spectrum is very close to that described by Novak et al.[7] obtained in thermolysis of *N*-pivaloxyacetanilides (structure **11**: $a_{N_1} = 14.4$ G; $a_H = 3.9$ G; $a_{N_2} = 1.5$ G). Structures **9** and **10** corresponding to the trapping of acyloxy radicals are assigned to the second spin adducts that appear as a triplet of doublets. The characteristics of **10** are identical to those reported for this species by Jantzen in the PBN trapping of the acetoxy radical generated in benzene by photolysis of triethyllead acetate and lead tetraacetate.[15]

(12) Forrester, A. R.; Ogilvy, M. M.; Thomson, R. H. *J. Chem. Soc.* **1970**, 1081–1083.
(13) Bartsch, H.; Hecker, E. *Biochem. Biophys. Acta* **1971**, *237*, 556–566 and 567–578.
(14) Danen, W. C.; Gellert, R. W. *J. Am. Chem. Soc.* **1972**, *94*, 6853–6854.
(15) Janzen, E. G.; Blackburn, B. J. *J. Am. Chem. Soc.* **1969**, *91*, 4481–4490.

As a conclusion these results show that thermolysis of *N*-acetoxy-*N*-acetyl-2-aminofluorene (1) and of the corresponding model compound **2** leads to homolytic cleavage of the fragile N–O bond yielding the corresponding two radicals, the amidyl **6** and the acyloxy species which are trapped to give the spin adducts **10** and **9**. However the spectrum of the nitroxide radical **7** is observed when thermolysis is conducted in the absence of the spin trap, while no signal corresponding to the amidyl **6** and acyloxy species can be detected. Two possible explanations for this latter observation can be formulated. (1) Either thermolysis of **1** and **2** leads exclusively to N–O cleavage giving the amidyl **6** and the acyloxy radicals which are not observed due to their short life time. A fraction of the amidyl species **6** is oxidized by the traces of oxygen always present even in carefully degassed solution (see Experimental Section) to give the stable nitroxide radical **7** which is observed. Amido radicals are well known for their propensity to be oxidized to the corresponding nitroxide.[14,16] (2) Or N–O cleavage occurs as a very major pathway leading to the amidyl radical **6**, while a very small fraction undergoes O–C fission. The traces of nitroxide **7** resulting from the latter process are seen in the ESR, while the corresponding PBN adduct cannot be detected.[17] These interpretations are in agreement with the results obtained by Stermitz and al.[16] who observed formation of the amidyl radical accompanied by minor formation of the nitroxide during photolysis of triacylhydroxylamines. The very major if not exclusive homolytic N–O cleavage is good mechanistic support for the observation that thermolysis of *N*-acetoxy-*N*-acetyl-2-aminofluorene (1) and of model compounds **2b** and **2c** in benzene and in dichloromethane yields the corresponding rearranged products.[9]

It should also be noted that an identical behavior is observed for the carcinogen **1** and its models **2**.[18] No intramolecular reaction occurs involving the guanine residue in the case of **2**. In particular no product is formed corresponding to attack at C-8, a position which is well recognized for its radical reactivity.[19] This is in strong contrast to solvolysis of **2** in water where hydrophobic fluorene–guanine interactions control the arylamidation of the guanine ring giving **3**.

Experimental Section

ESR spectra were typically obtained at 10^{-3}–10^{-2} M concentrations. Model **2a** is soluble in H_2O (10^{-3}–10^{-2} M) and in CH_3CN and CH_2Cl_2 (10^{-2} M). Compound **2b** is insoluble in H_2O at 10^{-3} M concentration and soluble in C_6H_6, CH_3CN, or CH_2Cl_2. Product **2c** is insoluble in H_2O and soluble in a mixture of Me_2CO–H_2O (60/40) and in C_6H_6 at 10^{-2} M.

All solvents were of reagent grade quality and were used after distillation and drying on molecular sieves. All the ESR experiments were controlled by HPLC using authentic samples, as previously described.[8,9] Degassed benzene samples were obtained through four freeze-thaw cycles at reduced pressure followed by sealing of the ESR tubes.

Model compounds **2** and their isomers **4** have been described and characterized in previous papers.[8,9] *N*-Acetoxy-*N*-acetyl-2-aminofluorene (1) and the corresponding rearranged products have been reported by M. A. Ioro et al.[20]

Acknowledgment. We thank the "Association pour la Recherche sur le Cancer" (ARC) and the "Ligue Nationale Française Contre le Cancer" for their financial support.

(16) Stermitz, F. R.; Neiswander, D. W. *J. Am. Chem. Soc.* **1973**, *95*, 2630–2634.

(17) In a test experiment we found that the nitroxide **7** generated by oxidation of *N*-acetyl-*N*-hydroxy-2-aminofluorene gave no signal when trapped with PBN. This may be due to decomposition of the spin adduct to give t-butyl benzoyl nitroxide. This latter radical has a nitrogen coupling constant of ≈7 G (cf. Holman, R. J.; Perkins, M. J. *J. Chem. Soc., Chem. Commun.* **1971**, 244–245. Hartgerink, J. W.; Engberts, J. B. F. N.; De Boer, Th. J. *Tetrahedron Lett.* **1971**, *29*, 2709–2712.

(18) The only difference concerns the ESR signals corresponding to the nitroxide, which are observed during thermolysis of the model compounds **2** which include the guanine moiety in their structure, while they cannot be observed or appear with a very weak intensity during thermolysis of *N*-acetoxy-*N*-acetyl-2-aminofluorene (1). In all cases the samples have been carefully degassed through several (four) freeze-thaw cycles.

(19) Zady, M. F.; Wong, J. L. *J. Am. Chem. Soc.* **1977**, *99*, 5096–5101. For an example of intramolecular radical reactivity of purines, see: Barton, D. H. R.; Gero, S. D.; Quiclet-Sire, B.; Samadi, M.; Vincent, C. *Tetrahedron* **1991**, *47*, 9383–9392.

(20) Ioro, M. A.; Mazzeo-Farina, A.; Boniforti, L. *Biomed. Mass Spectrom.* **1985**, *12*, 30–37.