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Oxidative transformation of 2-acetylaminofluorene by a chemical model for cytochrome P450: A water-insoluble porphyrin and *tert*-butyl hydroperoxide

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Abstract—Oxidation of 2-acetylaminofluorene (AAF), a carcinogen, by a chemical model for cytochrome P450 was investigated to identify an active mutagen and elucidate the oxidation pathway. The oxidation system consisted of a water-insoluble tetrakis(pentafluorophenyl)porphyrinatoiron(III) chloride and *tert*-butyl hydroperoxide. The mutagen derived from AAF by the chemical model was 2-nitro-9-fluorenone (NO_2F=O), which was mutagenic in *Salmonella typhimurium* TA1538. AAF was oxidized initially at position 9 of the fluorene carbon by the chemical model forming 2-acetylamino-9-fluorenol (AAF–OH), and then oxidized further to 2-acetylamino-9-fluorenone (AAF=O) as a major product. Initial oxidation of the nitrogen formed 2-nitrofluorene (NO_2F), and further oxidation yielded 2-nitro-9-fluorenol (NO_2F–OH) as a minor product. These products, AAF–OH, AAF=O, NO_2F, and NO_2F–OH, and their presumable common intermediate, *N*-hydroxy-2-acetylaminofluorene, were oxidized by the chemical model, and the formation of NO_2F=O was determined. These results showed that NO_2F=O was the mutagen derived from AAF in the presence of the chemical model and was formed via oxidation of *N*-OH–AAF, NO_2F, and NO_2F–OH. These results may lead to a new metabolic pathway of AAF.

1. Introduction

Environmental carcinogens are usually activated through metabolic enzymes, such as cytochrome P450.[1,2] In metabolic studies of xenobiotics, medicine, or chemical carcinogens, it is troublesome to handle enzymes because of their restrictions in available reaction conditions (temperature, solvent, and pH), and enzymes react with oxidized products or reactive intermediates.[3] Consequently, instead of enzymes, biomimetic chemical models have been helpful in metabolic studies.[4] Okochi et al. have detected mutagenicity of *N*-nitrosodialkylamines in *Salmonella typhimurium* YG7108 and reproduced the enzymatic metabolic pathway of *N*-nitrosodialkylamines by isolating the corresponding alcohols and aldehydes as their products, which suggest that *N*-nitrosodialkylamine underwent oxidation through α-hydroxynitrosamine in tetrakis(pentafluorophenyl)porphyrinatoiron(III) chloride (F_5P) and *tert*-butyl hydroperoxide (*t*-BuOOH).[5] Thus, the chemical model did oxidize and activate carcinogens in the mutation assay as a substitute for the metabolic activation system. The metabolic activation mechanism of 2-acetylaminofluorene (AAF) is well known. The acetyl group of AAF is oxidized to *N*-hydroxy-AAF by cytochrome P450 in the metabolic activation, followed by *O*-acetylation of *N*-hydroxy-2-acetylaminofluorene (*N*-OH–AAF) to *N*-acetoxy-2-aminofluorene.[6,7] The ester contains an excellent leaving group and readily breaks down to produce a nitrenium ion, and then induces mutation by reaction with DNA bases. We have reported that AAF is mutagenic in the presence of chemical models.[8] In this study, the oxidation mechanism of AAF is elucidated by isolation and identification of the mutagen and the oxidation products derived from AAF in the presence of the chemical model.

2. Results

2.1. Mutagenicity of AAF by a chemical model for P450 in a mutation assay

A chemical model system for cytochrome P450, water-insoluble F_5P and *t*-BuOOH was used in the Ames assay as a substitute for the S9 mix (Fig. 1).

Keywords: Chemical model; Cytochrome P450, 2-Acetylaminofluorene; Oxidation.
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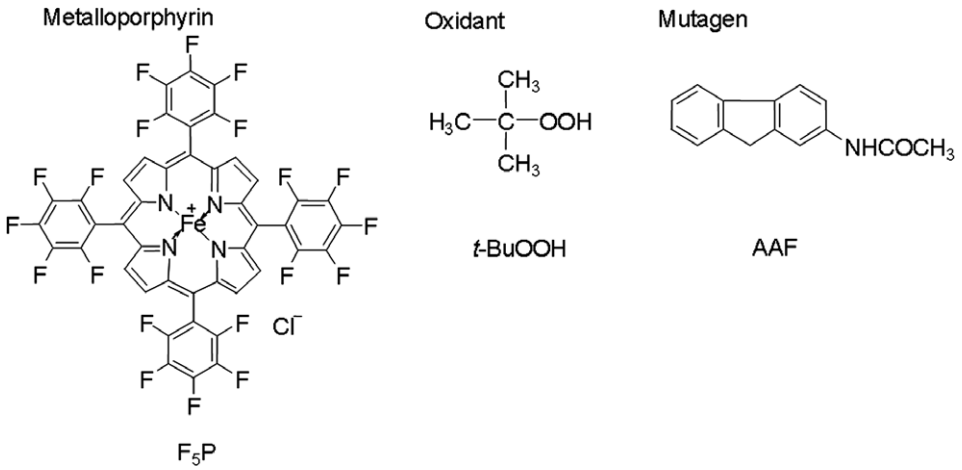


Figure 1. Chemical structure of the metalloporphyrin (F_5P), oxidant (*t*-BuOOH), and mutagen (AAF).

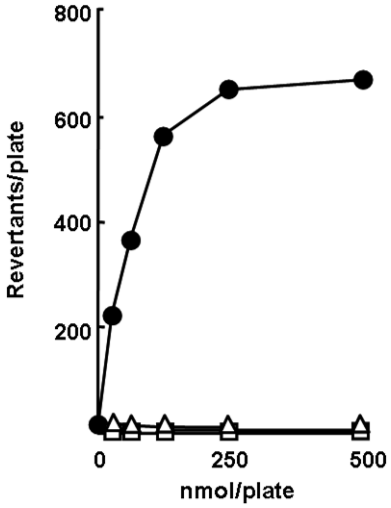


Figure 2. Mutagenicity of AAF in *Salmonella typhimurium* TA1538 in the presence of F_5P/*t*-BuOOH. F_5P (250 nmol/plate) and *t*-BuOOH (750 nmol/plate) in acetonitrile. The complete system contained AAF, F_5P, and *t*-BuOOH (●). Control systems lacked F_5P (□) or *t*-BuOOH (△).

AAF was mutagenic in a dose-dependent manner in the presence of F_5P/*t*-BuOOH in *S. typhimurium* TA1538 (Fig. 2). The activating system itself, F_5P and *t*-BuOOH, was not mutagenic in the absence of mutagens. In addition, the reaction mixture was not mutagenic in the absence of F_5P or *t*-BuOOH.

2.2. Isolation and identification of AAF oxidation products from the reaction mixture

AAF and F_5P were dissolved in acetonitrile, and *t*-BuOOH was then added. The resulting solution was stirred for 2 h at room temperature. The reaction mixture was extracted with ethyl acetate, and was dried over anhydrous sodium sulfate, and the solvent was subsequently evaporated. The residue was fractionated by silica gel column chromatography, and the fractions were tested for mutagenicity. A fraction showing strong mutagenicity contained a single compound, which was recrystallized from CCl_4, yellow needles, mp 221.0–222.0 °C. ^{1}H NMR, IR, and MS analyses showed that oxidization of AAF in the presence of F_5P/*t*-BuOOH produced 2-nitro-9-fluorenone ($NO_2F{=}O$). This finding was confirmed by a comparison of spectra with those of authentic $NO_2F{=}O$. ^{1}H NMR (500 MHz; $CDCl_3$; Me_4Si) δ: 7.43–7.49 (1H, m), 7.58–7.65 (1H, m), 7.68–7.73 (2H, m), 7.76–7.79 (1H, m), 8.41–8.49 (2H, m); ν_{max}/cm^{-1} 1714 (CO), 1520 (NO), 1338 (NO), EI-MS *m*/*z*: 225 (M^+, 100%), 195 (22), 151 (83). The mutagenicity of the product was the same as that of the authentic sample (data not shown).

2.3. Contribution of the direct mutagen derived from AAF and the chemical models to the total mutagenicity of the reaction solution

AAF decreased with an increase of $NO_2F{=}O$ in the presence of the chemical model (Fig. 3A and B).

AAF mutagenicity was assayed using *S. typhimurium* YG7131, which is deficient in nitroreductase. The result was compared to that of parent strain *S. typhimurium* TA1538 (Fig. 4A). The nitro compound was activated by reduction into hydroxylamine by nitroreductase in *S. typhimurim* TA1538, and then the mutagenicity derived from the hydroxylamine was detected, thus the mutagenicity of the nitro compound decreased in nitroreductase-deficient strain. Mutagenicity of the mixture of products derived from AAF oxidation decreased significantly in *S. typhimurium* YG7131, and the mutagenicity of $NO_2F{=}O$ in the same strain also decreased significantly. Furthermore, the amount of $NO_2F{=}O$ formed, calculated by HPLC, accounted for the mutagenicity of the reaction mixture in *S. typhimurium* TA1538 (Fig. 4B).

A possible mechanism of AAF oxidation is shown in Scheme 1. In pathway A of Scheme 1, the formation of the nitro compound $NO_2F{=}O$ was initiated by the oxidation of the methylene carbon of the fluorene ring. While in pathway B of Scheme 1, the formation of $NO_2F{=}O$ was initiated by the oxidation of nitrogen of

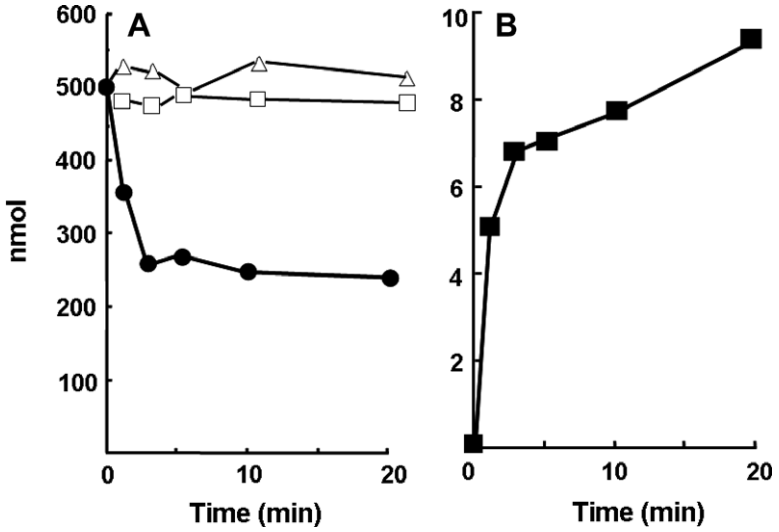


Figure 3. Oxidation of AAF by F_5P/*t*-BuOOH. (A) Decrease of AAF by F_5P/*t*-BuOOH. AAF (500 nmol), F_5P (250 nmol), and *t*-BuOOH (750 nmol) were incubated in 3 mL of acetonitrile at 25 °C. The complete system contained AAF, F_5P, and *t*-BuOOH (●). Control systems lacked F_5P (□) or *t*-BuOOH (△). (B) Formation of NO_2F=O by F_5P/*t*-BuOOH. AAF (500 nmol), F_5P (250 nmol), and *t*-BuOOH (750 nmol) were incubated in 3 mL of acetonitrile at 25 °C.

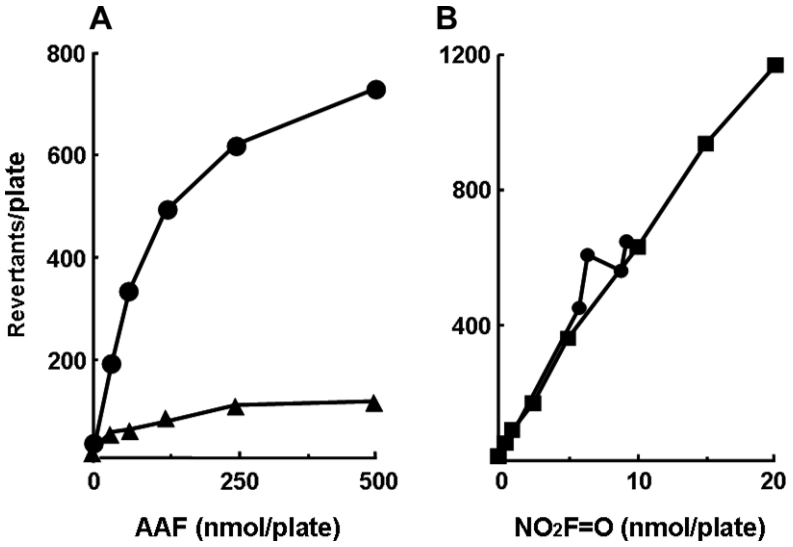


Figure 4. Contribution of NO_2F=O to total mutagenicity in the reaction mixture. (A) Mutagenicity of AAF under F_5P/*t*-BuOOH in *Salmonella typhimurium* TA1538 (●) and *Salmonella typhimurium* YG7131 (▲). (B) Correlation of mutagenicity of AAF by F_5P/*t*-BuOOH with the calculated mutagenicity based on NO_2F=O formed. Mutagenicity of NO_2F=O from AAF by F_5P/*t*-BuOOH (●); mutagenicity of authentic sample (■).

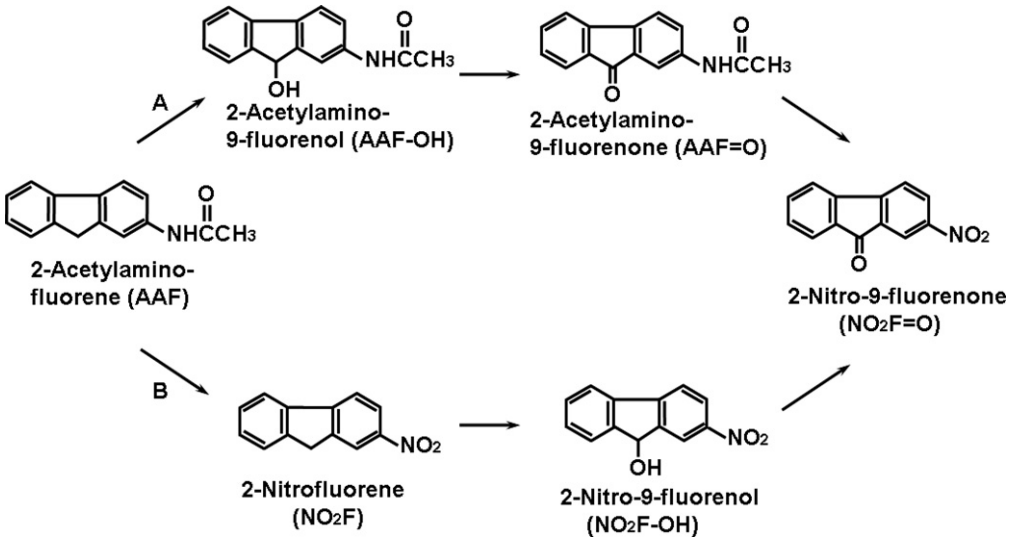
the acetylamino group, followed by the oxidation of the fluorene ring.

2.4. Quantitative analysis of AAF oxidation products

For the purpose of elucidating the pathway in Scheme 1, the oxidation products of AAF were detected using the chemical model. The AAF oxidative products were analyzed by HPLC in the presence of the chemical models. AAF–OH and AAF=O were detected in the reaction mixture (Fig. 5A). Their retention times and UV spectra were identical to those of the authentic samples. NO_2F and NO_2F–OH were detected in the presence of the chemical model using HPLC with a photodiode array detector (Fig. 5B).

To examine the detailed oxidation pathway by the chemical model, each of the four oxidation products, AAF–OH, AAF=O, NO_2F, and NO_2F–OH, was oxidized using the chemical model. AAF–OH was exclusively oxidized to AAF=O by the chemical model (Fig. 6A). AAF=O was barely oxidized by the chemical model and NO_2F=O was not significantly formed (Fig. 6B). On the other hand, NO_2F was oxidized to NO_2F–OH and NO_2F=O (Fig. 7A), and NO_2F–OH was oxidized to NO_2F=O (Fig. 7B).

We proposed that NO_2F=O was formed via *N*-OH–AAF in the presence of the chemical model, as in the S9 mix. *N*-OH–AAF could not be detected in the reaction mixtures because of its high reactivity. *N*-OH–



Scheme 1. Proposed mechanism of $NO_2F{=}O$ formation.

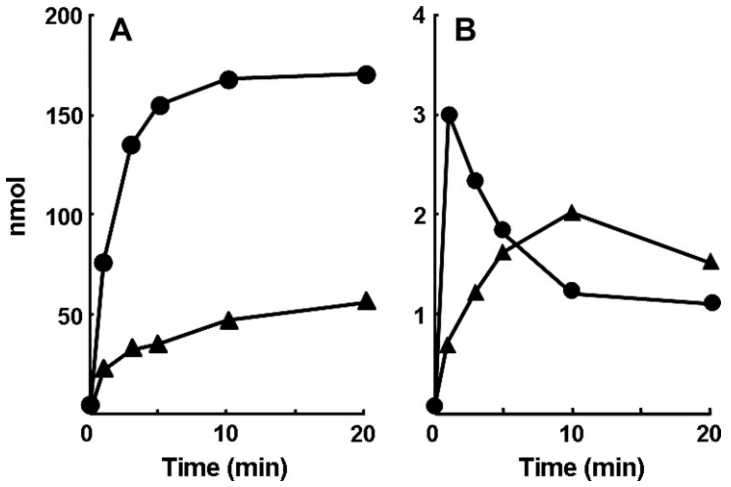


Figure 5. Formation of oxidation products from AAF and by F_5P/t-BuOOH. (A) Formation of AAF–OH (●) and AAF=O (▲). (B) Formation of NO_2F (●) and NO_2F–OH (▲).

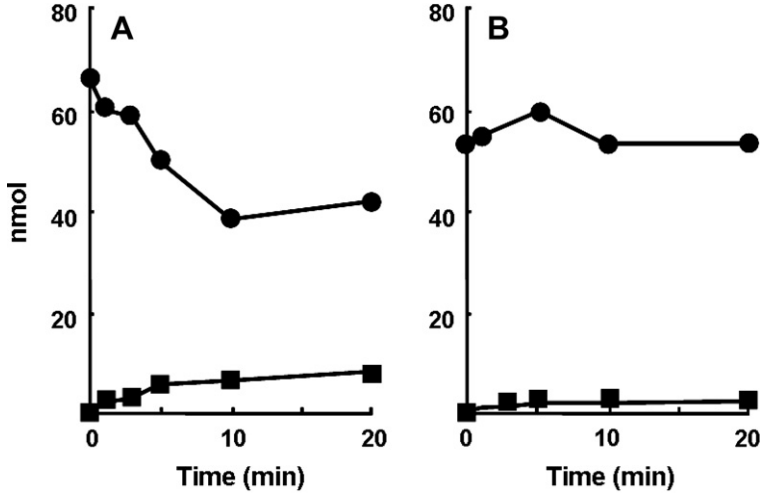


Figure 6. Oxidation of AAF oxidation products by F_5P/t-BuOOH. (A) Oxidation of AAF–OH (●) and formation of AAF=O (■) by F_5P/t-BuOOH. (B) Oxidation of AAF=O (●) and formation of $NO_2F{=}O$ (■) by F_5P/t-BuOOH. AAF–OH or AAF=O (50 nmol), F_5P (250 nmol), and t-BuOOH (750 nmol) were incubated in 3 mL of acetonitrile at 25 °C.

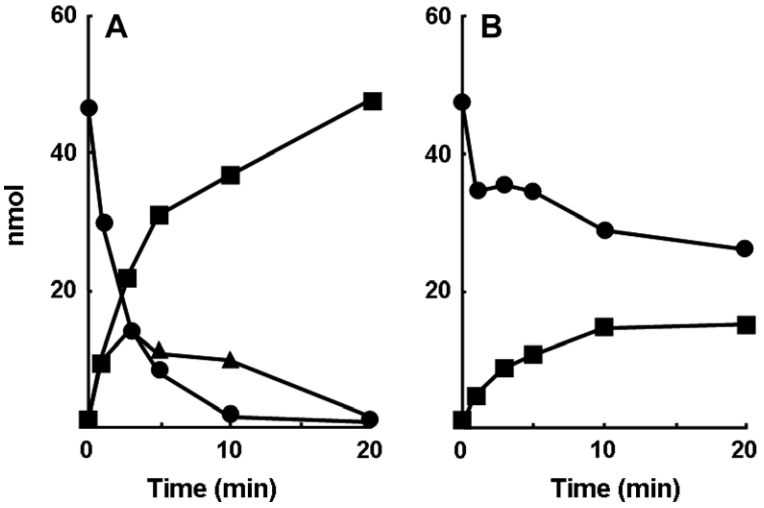


Figure 7. Oxidation of AAF oxidative products by F_5P/t-BuOOH. (A) Oxidation of NO_2F (●) and formation of NO_2F–OH (▲) and NO_2F=O (■) by F_5P/t-BuOOH. (B) Oxidation of NO_2F–OH (●) and formation of NO_2F=O (■) by F_5P/t-BuOOH. NO_2F or NO_2F–OH oxidative product (50 nmol), F_5P (250 nmol), and t-BuOOH (750 nmol) were incubated in 3 mL of acetonitrile at 25 °C.

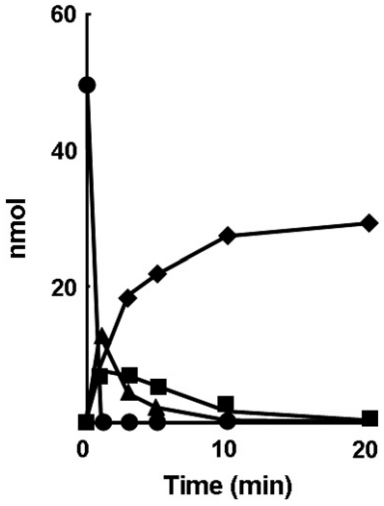


Figure 8. Decrease of N-OH–AAF (●) and formation of NO_2F (▲), NO_2F–OH (■), and NO_2F=O (◆). N-OH–AAF (50 nmol), F_5P (250 nmol), and t-BuOOH (750 nmol) were incubated in 3 mL of acetonitrile at 25 °C.

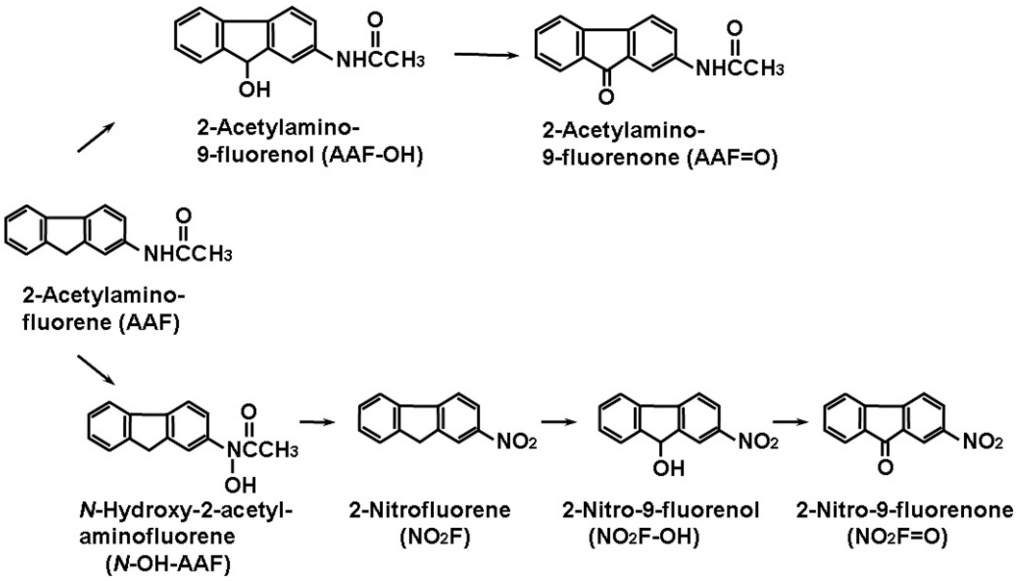
AAF was oxidized in the presence of the chemical model, and NO_2F, NO_2F–OH, and NO_2F=O were detected (Fig. 8).

3. Discussion

A chemical model system for cytochrome P450, a porphyrin and an oxidant, was used in the Ames assay as a biomimetic system of metabolic activation. In this study, we elucidated the activation pathway of AAF in the presence of water-insoluble F_5P/t-BuOOH, which activated AAF in a dose-dependent manner in *S. typhimurium* TA1538 (Fig. 2). We isolated and identified NO_2F=O as a mutagen from a reaction mixture. It has been reported that NO_2F=O exists in the environment,[9] and also that 2-aminofluorene is oxidized to NO_2F by prostaglandin H synthase,[10,11] and NO_2F is metabolized to form NO_2F=O by *Caenorhabditis elegans*.[12,13] We have results that mutagenicity of AAF in the presence of S9 mix was not derived from the nitro compound, since the mutagenicity under S9 mix was similar in *S. typhimurium* TA1538 and YG7131 (data not shown). The result suggested that reductases had high activities in the S9 mix. We are speculating the formation of NO_2F=O in the absence of the reductases, and we are working presently on detecting the nitro compound in the reaction mixture containing AAF and recombinant human CYP isoforms or prostaglandin H synthase.

In order to estimate the contribution of NO_2F=O to the total mutagenicity in the reaction mixture, AAF mutagenicity was assayed using *S. typhimurium* YG7131, which is deficient in nitroreductase, and the result was compared to that in a parent strain, *S. typhimurium* TA1538 (Fig. 4A). The mutagenicity of the mixture of products derived from AAF by the chemical model decreased significantly in *S. typhimurium* YG7131 similar to that of authentic NO_2F=O. The results suggested that the nitro compound formed was responsible for the mutagenicity of AAF in the presence of the chemical model. Furthermore, the amount of NO_2F=O formed was in accord with the mutagenicity of the reaction mixture in *S. typhimurium* TA1538. Those results showed that the mutagen derived from AAF in the model was NO_2F=O alone (Fig. 4B).

As expected on the basis of the proposed oxidation pathway outlined in Scheme 1, AAF was shown to be metabolized to AAF–OH, AAF=O, NO_2F, NO_2F–OH, and NO_2F=O in the presence of the chemical model. The yield of AAF–OH, AAF=O, NO_2F, NO_2F–OH, and NO_2F=O was 68.2%, 22.2%, 0.4%, 0.6%, and 3.7%,



Scheme 2. Pathway of formation of NO_2F=O in the presence of the chemical model.

respectively. All formed oxidative products corresponded to at least 95% of the theoretical based on the observed AAF decomposition. AAF–OH and AAF=O were reported to be formed from AAF by endogenous enzymes.[14,15] Furthermore, to examine the detailed oxidation pathway by the chemical model, each of the four oxidation products, namely, AAF–OH, AAF=O, NO_2F, and NO_2F–OH, was oxidized using the chemical model (Fig. 5). In pathway A in Scheme 1, AAF=O was barely oxidized to form NO_2F=O (Fig. 6). NO_2F=O was formed in the amount of 0.3 nmol (yield 0.6%) after oxidation of AAF=O (50 nmol). On the other hand, in pathway B in Scheme 2, NO_2F=O was apparently formed from NO_2F and NO_2F–OH in the presence of the chemical model (Fig. 7). Thus, position 9 of fluorene was oxidized as a major pathway; however, the pathway to NO_2F=O was not a major one.

We expected that NO_2F=O was formed via *N*-OH–AAF in the presence of the chemical model, as in the S9 mix. Although *N*-OH–AAF could not be detected in the reaction mixtures because of its high reactivity, NO_2F, NO_2F–OH, and NO_2F=O were formed from *N*-OH–AAF in the presence of the chemical model (Fig. 8). It has been reported that the *N*-hydroxyacetylamino group was converted to a nitroso group, which then was easily oxidized to a nitro group.[16,17] Floyd et al. have reported that *N*-OH–AAF reacted with hydroperoxide to form 2-nitrosofluorene via a nitroxyl radical.[17] In addition, Nongkunsarn and Ramsden have reported that a nitroso group was formed from imine via oxidation of the oxaziridine ring.[18,19]

Scheme 2 summarizes the oxidative transformation of AAF in the presence of F_5P and *t*-BuOOH. We showed a pathway in which the *N*-hydroxyacetylamino group was oxidized to a nitro compound in the presence of the chemical model, and that the nitro compound formed was mutagenic in *S. typhimurium* TA1538.

4. Conclusion

The activation mechanism AAF has been studied in detail. AAF was oxidized *in vivo* by cytochrome P450 to *N*-OH–AAF, which is an active form with mutagenicity. In this study, we showed that AAF was oxidized to *N*-OH–AAF, followed by NO_2F and NO_2F–OH, and then finally to NO_2F=O, which was mutagenic in *S. typhimurium* TA1538 in the presence of the chemical model, as shown in Scheme 2. We demonstrated that the use of a chemical model made it easy to identify the oxidative products, and then elucidated the activation mechanisms of AAF in the presence of chemical models. This new pathway of AAF activation may be another pathway in the metabolism of AAF, which has to be further examined for its demonstration. It is worth using a chemical model for cytochrome P450 to predict possible oxidative metabolites including mutagens.

5. Experimental

5.1. Reagents

2-Amino-9-fluorenol (CAS No. 33417-27-5), 2-amino-9-fluorenone (CAS No. 3096-57-9), 2-acetylaminofluorene (AAF, CAS No. 53-96-3), tetrakis- (pentafluorophenyl)porphyrinatoiron(III) chloride (F_5P, CAS No. 36965-71-6) and *tert*-butyl hydroperoxide (*t*-BuOOH, CAS No. 75-91-2) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Hydrazine hydrate (CAS No. 10217-52-4), 5% palladium/carbon, 2-nitrofluorene (NO_2F, CAS No. 607-57-8), and 2-nitro-9-fluorenone (NO_2F=O, CAS No. 3096-52-4) were obtained from Wako Pure Chemical Industries, Ltd. (Okasa, Japan).

5.2. Experimental

Melting points were measured on a Yanagimoto micro-apparatus and were uncorrected. The EI-MS was ob-

Table 1. HPLC analysis conditions

Compound	Conditions
AAF	Acetonitrile:20 mM sodium phosphate buffer (pH 7.4) 1:1, 0.5 mL/min, 7.5 min, 284 nm
NO_2F═O	Acetonitrile:20 mM sodium phosphate buffer (pH 7.4) 1:1, 0.5 mL/min, 13.0 min, 284 nm
NO_2F	Acetonitrile:20 mM sodium phosphate buffer (pH 7.4) 6:5, 0.5 mL/min, 23.4 min, 332 nm
NO_2F–OH	Methanol:20 mM sodium phosphate buffer (pH 7.4) 3:2, 0.3 mL/min, 17.0 min, 332 nm
AAF–OH	Methanol:20 mM sodium phosphate buffer (pH 7.4) 1:1, 0.5 mL/min, 3.6 min, 284 nm
AAF═O	Acetonitrile:methanol:H_2O 7:3:3, 0.1 mL/min, 17.5 min, 284 nm

Eluent, elution rate, retention time, and UV detector wavelength are shown for each compound.

tained with a Hitachi M-4100 mass spectrometer. The NMR experiments were performed with JEOL JNM-GX270, with Me_4Si as an internal standard. HPLC was performed using a Shimadzu LC-6A system [SPD-6AV UV/vis spectrometric detector, Mightysil RP-18 column (5 μm, 250 × 3.0 mm)]. TLC was performed on precoated Kieselgel 60F_{254} (Merck), and spots were visualized under UV light.

5.3. Chemical preparation

2-Acetylamino-9-fluorenol (AAF–OH) was prepared by acetylation of 2-amino-9-fluorenol with acetic anhydride. mp 248.0–249.0 °C (lit., 249–250 °C).[20] 2-Acetylamino-9-fluorenone (AAF═O) was prepared by acetylation of 2-amino-9-fluorenone with acetic anhydride and pyridine. mp 231.0–232.0 °C (lit., 233–236 °C).[20] *N*-Hydroxy-2-acetylaminofluorene (*N*-OH–AAF)[21] and 2-nitro-9-fluorenol (NO_2F–OH)[22] were synthesized by the method reported.

5.4. Bacterial mutation assay using the chemical model

A bacterial mutation assay was based on the Ames test,[23] and the chemical model, an iron(III) porphyrin and an oxidant, was used as a substitute for the metabolic activation system. The bacterial strains were *S. typhimurium* TA1538, provided by Professor B.N. Ames, the University of California, Berkeley, and *S. typhimurium* YG7131, provided by Dr. T. Nohmi, National Institute of Health Science, Japan.[24]

An aliquot of F_5P (250 nmol/20 μL) and AAF (500 nmol/20 μL) in acetonitrile was mixed, and *t*-BuOOH (750 nmol/20 μL) in 0.1 M sodium phosphate buffer (pH 7.4) was added. The mixture was then incubated (25 °C, 120 strokes/min) for 20 min, and a 0.1 M sodium phosphate buffer (pH 7.4, 0.5 mL), a culture of tester strains (0.1 mL), and top agar (2 mL) were added. The mixture was then poured onto a minimal-glucose agar plate. After incubation for 44 h at 37 °C, the colonies were counted. Duplicate plates were used for each dose, and the experiments were repeated at least twice.

5.5. Reaction conditions with the model system

AAF (0.5 mmol) and F_5P (0.06 mmol) were dissolved in acetonitrile (30 mL), and *t*-BuOOH (15 mmol) was added to the solution. The reaction mixture was stirred for 2 h at room temperature, then added to water (70 mL), and extracted three times with ethyl acetate (40 mL). The organic layer was dried over anhydrous sodium sulfate, and the solvent evaporated, leaving a black solid (205 mg). The residue was fractionated using silica gel column chromatography.

5.6. Quantitative analysis of the oxidation product

F_5P (250 nmol), AAF (500 nmol), and *t*-BuOOH (750 nmol) were incubated in acetonitrile (3 mL) at 25 °C for various times (1, 3, 5, 10, and 20 min). The products were analyzed by reverse-phase HPLC. Eluent, elution rate, retention time, and UV detector wavelength for each compound are listed in Table 1.

Acknowledgments

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