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Anti-mutagenic structural modification by fluorine-substitution in highly mutagenic 4-methylquinoline derivatives

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Abstract

We have previously shown that fluorine-substitution at position 3 of quinoline deprived this molecule of mutagenicity, possibly due to interference with the yield of its metabolically activated form, the 1,4-hydrated 2,3-epoxide (enamine epoxide), which is directly responsible for the mutagenic modification of DNA. To further explore the possibility of a method for anti-mutagenic modification of mutagens by fluorine-substitution, 4-methylquinoline (4-MeQ), the most mutagenic form of all the quinoline derivatives examined so far, was used as a target in the present study. Five mono- and di-fluorinated derivatives of 4-MeQ, 2-fluoro-4-methylquinoline (2-F-4-MeQ), 6-F-4-MeQ, 7-F-4-MeQ, 2,6-difluoro-4-methylquinoline (2,6-diF-4-MeQ), and 2,7-diF-4-MeQ, were subjected to analysis of their structure-mutagenicity relationships. The 2-fluorinated derivatives (2-F-4-MeQ, 2,6-diF-4-MeQ, and 2,7-diF-4-MeQ) were all non-mutagenic in the Ames test. 7-F-4-MeQ was as highly mutagenic as, and 6-F-4-MeQ was less mutagenic than non-fluorinated 4-MeQ. Metabolic studies were also conducted with 4-MeQ, 2-F-4-MeQ, 6-F-4-MeQ, and 7-F-4-MeQ, using a liver microsomal enzyme fraction prepared from the 3-methylcholanthrene-treated rat. The HPLC analytical data showed that, although the metabolic patterns (hydroxylation at 4-methyl group as a main metabolic pathway and 3-hydroxylation as a minor pathway) of these four F-MeQs were similar to one another, only the 3-hydroxy metabolite of 2-F-4-MeQ was not produced under the present experimental conditions employed. These results suggest that fluorine-substitution at position 2 of 4-MeQ inhibited the formation of the enamine epoxide in the pyridine moiety and deprived this molecule of mutagenicity as in the case of quinoline.

Keywords: Anti-mutagenic structural modification; Fluorine-substitution; 4-methylquinoline; Mutagenicity; Metabolism

1. Introduction

Our continued interest in biological effects of a fluorine (F) atom(s) has prompted us to investigate the substituent effect of an F atom(s) on the mutagenicity and metabolism of aza-aromatic compounds. It is well known that, when an aromatic nucleus is fluorinated, enzymatic oxidation is generally inhibited at the site of F-substitution due to its electron-withdrawing nature [1–4]. Therefore, F-substitution at the activation site may decrease the mutagenicity of aromatic compounds. For example, we previously reported that mutagenic [5] and carcinogenic [6,7]

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quinoline was deprived of both in vitro [8,9] and in vivo [10,11] genotoxicity by F-substitution at position 3, whereas 5-fluoroquinoline was as genotoxic as quinoline in the same assay systems. These previous findings have revealed that the genotoxicity of quinoline may be attributable to the formation of an enamine epoxide, 2,3-epoxide of 1,4-hydrated quinoline [8–15].

4-Methylquinoline (4-MeQ) was found to be much more mutagenic in *Salmonella typhimurium* TA100 when compared to quinoline, in the presence of rat liver S9 mix [9,16]. Our previous studies showed that 4-MeQ was metabolized by the liver microsomal preparation from the 3-methylcholanthrene(3-MC)-treated rat mainly to non-mutagenic 4-hydroxymethylquinoline (4-HMeQ) and a small amount of 3-hydroxy-4-methylquinoline (3-OH-4-MeQ), which is possibly derived from the corresponding 2,3-oxide derivative, and that this compound was deprived of its potent mutagenicity by introduction of a chlorine atom to position 3 [16]. From these findings, we proposed that the mutagenic activation of 4-MeQ is achieved through a mechanism common to that of quinoline itself. To confirm this hypothesis, the present study was undertaken to investigate the effect of fluorine-substitution on the mutagenicity and metabolism of 4-MeQ, using three mono- and two di-fluorinated derivatives.

2. Materials and methods

2.1. Materials

4-MeQ (CAS Registry No. 491-35-0), 3-MC (CAS Registry No. 56-49-5), fluorinated anilines, and 2-hydroxy-4-methylquinoline (2-OH-4-MeQ) were purchased from Aldrich. Tetraethylammonium fluoride-5.5 mol eq. of hydrogen fluoride was from Morita Chemical, Osaka, Japan. Melting points were determined with a Yamato MP-500D micro melting point apparatus without correction. Mass spectra were measured with a JEOL JMS-SX102A spectrometer. ^{1}H-NMR spectra were recorded with a JEOL JNM-EX270 or JNM-GSX400 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard. The following compounds were synthesized in this study.

2.1.1. 2-Fluoro4-methylquinoline (2-F-4-MeQ)

2-OH-4-MeQ (499 mg) was dissolved in $POCl_3$ (5 ml), and PCl_5 (804 mg) was added to this solution. After refluxing for 24 h, the reaction mixture was poured into water, neutralized with sodium carbonate, extracted with $CHCl_3$, and evaporated. 2-Chloro-4-methylquinoline (2-Cl-4-MeQ, CAS Registry No. 634-47-9) was obtained as a white solid in 96% yield. MS m/z: 177 (M^+), 179 ($M^+ + 2$). ^{1}H-NMR ($CDCl_3$) δ: 2.69 (s, 3H, CH_3), 7.25 (s, H-3), 7.57 (ddd, H-6), 7.73 (ddd, H-7), 7.96 (dd, H-8), 8.02 (dd, H-5); $J_{5-6} = 7.9$, $J_{6-7} = 6.9$, $J_{7-8} = 8.3$ Hz.

2-Cl-4-MeQ (296 mg) was dissolved in 4 ml of tetraethylammonium fluoride-5.5 mol eq. of hydrogen fluoride. Then the solution was kept stirring for 20 min at 160°C. The reaction mixture was poured into 100 ml of water, neutralized with sodium carbonate, and extracted with $CHCl_3$. Purification of the extract by column chromatography (silica gel, $CHCl_3$) yielded 2-F-4-MeQ as colorless oil in 36% yield. MS m/z: 161 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.69 (s, 3H, CH_3), 6.89 (s, H-3), 7.52 (ddd, H-6), 7.70 (ddd, H-7), 7.93 (m, 2H, H-5 and H-8); $J_{5-6} = 8.3$, $J_{6-7} = 6.8$, $J_{7-8} = 8.3$ Hz. *Anal*. Calcd. for $C_{10}H_8FN$: C, 74.52; H, 5.00; N, 8.69. Found: C, 74.15; H, 4.96; N, 8.64.

2.1.2. 6-Fluoro-4-methylqunoline (6-F-4-MeQ, CAS Registry No. 31598-65-9)

p-Fluoroaniline (2.35 g) was allowed to react with methylvinylketone (1.8 ml, 1.0 eq) in 50 ml of EtOH at 0°C for 1 h. Then the solution was kept stirring at room temperature for 5 h. $ZnCl_2$ (2.98 g), $FeCl_3 \cdot 6H_2O$ (4.47 g) and c-HCl (2.0 ml) were mixed in the reaction mixture. After stirring at 70°C for 13 h, the reaction mixture was poured into water, neutralized with sodium carbonate, and extracted with $CHCl_3$. Purification of the extract by column chromatography (aluminium oxide, hexane:$CHCl_3$ = 1:1 → $CHCl_3$, and silica gel, hexane:$CHCl_3$ = 1:1 → $CHCl_3$) yielded 6-F-4-MeQ as a white solid in 22% yield. mp 180°C (sublimated). MS m/z: 161 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.62 (s, 3H, CH_3), 7.21 (d, H-3), 7.45 (ddd, H-7), 7.54 (dd, H-5), 8.08 (dd, H-8), 8.72 (d, H-2); $J_{2-3} = 4.4$, $J_{5-6F} = 9.9$, $J_{5-7} = 2.8$, $J_{6F-7} = 11.2$, $J_{6F-8} = 5.6$, $J_{7-8} = 8.9$ Hz. *Anal*.

Calcd. for $C_{10}H_8FN \cdot HCl \cdot 3/2\ H_2O$: C, 53.46; H, 5.38; N, 6.23. Found: C, 53.42; H, 5.40; N, 6.29.

2.1.3. 7-Fluoro-4-methylquinoline (7-F-4-MeQ)

m-Fluoroaniline (3.74 g) was allowed to react with methylvinylketone (3.2 ml, 1.0 eq) in 50 ml of EtOH at 0°C for 1 h. Then the solution was kept stirring at room temperature for 18 h. $ZnCl_2$ (4.1 g) and $FeCl_3 \cdot 6H_2O$ (14.2 g) were added to the reaction mixture. After stirring at 70°C for 13 h, the reaction mixture was poured into water, neutralized with sodium carbonate, and extracted with $CHCl_3$. The extract was crudely purified by column chromatography (alumina, hexane:$CHCl_3$ = 1:1 → $CHCl_3$). The crude mixture of 7-F-4-MeQ and 5-F-4-MeQ was purified by recrystallization as a dichromate salt from EtOH. Further purification of the 7-F-4-MeQ dichromate salt by recrystallization as a sulfate salt from EtOH yielded $7F\text{-}4\text{-}MeFQ \cdot H_2SO_4$ as white needles in 21% yield. mp 142–144°C. MS *m/z*: 161 (free form, M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.83 (s, 3H, CH_3), 7.65 (d, H-3), 7.73 (ddd, H-6), 7.84 (dd, H-8), 8.37 (dd, H-5), 8.98 (d, H-2); J_{2-3} = 4.9, J_{5-6} = 9.0, J_{5-7F} = 6.1, J_{6-7F} = 11.5, J_{6-8} = 2.4, J_{7-8} = 9.8 Hz. *Anal*. Calcd for $C_{10}H_8FN \cdot H_2SO_4$: C, 46.33; H, 3.89; N, 5.40. Found: C, 46.14; H, 3.91; N, 5.62.

2.1.4. 2,6-Difluoro-4-methylquinoline (2,6-diF-4-MeQ)

p-Fluoroaniline (5.0 g) was refluxed with diketene (4.2 ml) in THF (100 ml). After 24 h, this solution was evaporated. The resulting residue was kept at 80°C for 30 min in c-H_2SO_4. The reaction mixture was poured into 100 ml of water, neutralized with sodium carbonate and extracted with $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. 6-Fluoro-2-hydroxy-4-methylquinoline (6-F-2-OH-4-MeQ) was obtained in 91% yield. mp 180°C (sublimated). MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.40 (s, 3H, CH_3), 6.45 (s, H-3), 7.32 (dd, H-8), 7.40 (ddd, H-7), 7.51 (dd, H-5), 11.65 (br, –OH); J_{5-6F} = 10.0, J_{5-7} = 2.8, J_{6F-7} = 11.7, J_{6F-8} = 4.9, J_{7-8} = 8.9 Hz. *Anal*. Calcd for $C_{10}H_8FNO$: C, 67.79; H, 4.55; N, 7.91. Found: C, 67.67; H, 4.76; N, 7.61.

6-F-2-OH-4-MeQ (500 mg) was dissolved in $POCl_3$ (5 ml), and PCl_5 (804 mg) was added to this solution. After refluxing for 12 h, the reaction mixture was poured into water, neutralized with sodium carbonate, extracted with $CHCl_3$, and evaporated. 2-Chloro-6-fluoro-4-methylquinoline (2-Cl-6-F-4-MeQ) was obtained as a white solid in 99% yield. mp 112–118°C. MS *m/z*: 195 (M^+), 197 ($M^+ + 2$). ^{1}H-NMR ($CDCl_3$) δ: 2.65 (s, 3H, CH_3), 7.26 (s, H-3), 7.49 (ddd, H-7), 7.55 (dd, H-5), 8.01 (dd, H-8); J_{5-6F} = 9.5, J_{5-7} = 2.7, J_{6F-7} = 11.0, J_{6F-8} = 5.4, J_{7-8} = 9.0 Hz. *Anal*. Calcd. for $C_{10}H_7ClFN$: C, 61.40; H, 3.61; N, 7.16. Found: C, 61.41; H, 3.66; N, 7.14.

2-Cl-6-F-4-MeQ (279 mg) was dissolved in 4 ml of tetraethylammonium fluoride-5.5 mol eq. of hydrogen fluoride. Then the solution was kept stirring for 20 min at 160°C. The reaction mixture was poured into 100 ml of water, neutralized with sodium carbonate, and extracted with $CHCl_3$. Purification of the extract by column chromatography (silica gel, $CHCl_3$) and recrystallization from hexane yielded 2,6-diF-4-MeQ as white needles in 29% yield. mp 97–100°C. MS *m/z*: 179 (M^+). ^{1}H-NMR ($CDCl_3$) δ:2.69 (s, 3H, CH_3), 6.97 (s, H-3), 7.45 (ddd, H-7), 7.58 (dd, H-5), 7.94 (dd, H-8); J_{5-6F} = 9.5, J_{5-7} = 2.8, J_{6F-7} = 11.0, J_{6F-8} = 5.4, J_{7-8} = 9.1 Hz. *Anal*. Calcd for $C_{10}H_7F_2N$: C, 67.04; H, 3.94; N, 7.82. Found: C, 66.91; H, 3.93; N, 7.60.

2.1.5. 2,7-Difluoro-4-methylquinoline (2,7-dif-4-MeQ)

m-Fluoroaniline (5.0 g) was refluxed with diketene (4.2 ml) in THF (100 ml). After 12 h, this solution was evaporated. The resulting residue was kept at 80°C for 30 min in c-H_2SO_4. The reaction mixture was poured into 100 ml of water, and the precipitates were collected by suction. 7-Fluoro-2-hydroxy-4-methylquinoline (7-F-2-OH-4-MeQ) was obtained as a white solid in 66% yield. mp 251–253°C. MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.41 (s, 3H, CH_3), 6.35 (s, H-3), 7.05 (m, 2H, H-6 and H-8), 7.75 (dd, H-5), 11.66 (br, –OH); J_{5-6} = 9.5, J_{5-7F} = 6.1 Hz. *Anal*. Calcd. for $C_{10}H_8FNO$: C, 67.79; H, 4.55; N, 7.91. Found: C, 67.72; H, 4.48; N, 7.67.

7-F-2-OH-4-MeQ (805 mg) was dissolved in $POCl_3$ (10 ml), and PCl_5 (1.27 g) was added to this

solution. After refluxing for 20 h, the reaction mixture was poured into water, neutralized with sodium carbonate, extracted with $CHCl_3$, and evaporated. 2-Chloro-7-fluoro-4-methylquinolin (2-Cl-7-F-4-MeQ) was obtained as a white solid quantitatively. mp 100–106°C. MS *m/z*: 195 (M^+), 197 ($M^+ + 2$). ^{1}H-NMR ($CDCl_3$) δ: 2.69 (s, 3H, CH_3), 7.22 (s, H-3), 7.36 (ddd, H-6), 7.65 (dd, H-8), 7.97 (dd, H-5); $J_{5-6} = 9.1$, $J_{5-7F} = 5.9$, $J_{6-7F} = 11.0$, $J_{6-8} = 2.7$, $J_{7F-8} = 10.0$ Hz. *Anal*. Calcd for $C_{10}H_7ClFN$: C, 61.40; H, 3.61; N, 7.16. Found: C, 61.24; H, 3.60; N, 6.88.

2-Cl-7-F-4-MeQ (105 mg) was dissolved in 2 ml of tetraethylammonium fluoride-5.5 mol eq. of hydrogen fluoride. Then the solution was kept stirring for 20 min at 160°C. The reaction mixture was poured into 100 ml of water, neutralized with sodium carbonate, and extracted with $CHCl_3$. Purification of the extract by column chromatography (silica gel, $CHCl_3$) and recrystallization from hexane yielded 2,7-diF-4-MeQ as white needles in 14% yield. mp 75–76°C. MS *m/z*: 179 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.73 (s, 3H, CH_3), 6.90 (s, H-3), 7.33 (ddd, H-6), 7.58 (dd, H-8), 7.97 (dd, H-5); $J_{5-6} = 9.0$, $J_{5-7F} = 5.9$, $J_{6-7F} = 11.0$, $J_{6-8} = 2.7$, $J_{7F-8} = 10.0$ Hz. *Anal*. Calcd for $C_{10}H_7F_2N$: C, 67.04; H, 3.94; N, 7.82. Found: C, 66.82; H, 4.00; N, 7.81.

2.1.6. 6-Fluoro-4-hydroxymethylquinoline (6-F-4-HMeQ) and 6-fluoro-3-hydroxy-4-methylquinoline (6-F-3-OH-4-MeQ)

6-F-4-MeQ (182 mg) was allowed to react with *m*-chloroperoxybenzoic acid (323 mg) in 30 ml of $CHCl_3$ at room temperature for 40 h. The reaction mixture was washed with 5% sodium carbonate aq. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. 6-F-4-MeQ *N*-oxide was obtained in 79% yield. MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.60 (s, 3H, CH_3), 7.15 (d, H-3), 7.52 (ddd, H-7), 7.57 (dd, H-5), 8.39 (d, H-8), 8.84 (dd, H-2); $J_{2-3} = 6.0$, $J_{5-6F} = 9.3$, $J_{5-7} = 2.7$, $J_{6F-8} = 5.6$, $7_{6-8} = 9.5$ Hz.

6-F-4-MeQ *N*-oxide (150 mg) was refluxed in acetic anhydride (20 ml) for 4 h. After the solvent was evaporated, the residue was dissolved in water, neutralized with sodium carbonate, and extracted with $CHCl_3$. The $CHCl_3$ layer was dried over anhydrous $MgSO_4$ and evaporated. The resulting residue was hydrolyzed in c-NH_3 aq. (10 ml) at 80°C for 2 h. The resulting mixture was extracted with $CHCl_3$. Purification of the extract by column chromatography (silica gel, $CHCl_3$: MeOH = 19:1) yielded 6-F-4-HMeQ and 6-F-3-OH-4-MeQ in 49% and 36% yield, respectively.

2.1.6.1. 6-F-4-HMeQ. mp 146–150°C. MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.41 (s, CH_2OH), 5.16 (s, 2H, CH_2OH), 7.49 (ddd, H-7), 7.57 (m, 2H, H-3 and H-5), 8.14 (dd, H-8), 8.86 (d, H-2); $J_{2-3} = 4.4$, $J_{5-7} = 2.7$, $J_{6F-7} = 12.9$, $J_{6F-8} = 5.6$, $J_{7-8} = 9.5$ Hz. *Anal*. Calcd. for $C_{10}H_8FNO$: C, 67.79; H, 4.55; N, 7.91. Found: C, 67.81; H, 4.76; N, 8.01.

2.1.6.2. 6-F-3-OH-4-MeQ. mp 210°C. MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.41 (s, 3H, CH_3), 7.29 (ddd, H-7), 7.64 (dd, H-5), 7.93 (dd, H-8), 8.55 (s, H-2); $J_{5-6F} = 11.0$, $J_{5-7} = 2.8$, $J_{6F-7} = 11.5$, $J_{6F-8} = 5.9$, $J_{7-8} = 9.3$ Hz. *Anal*. Calcd for $C_{10}H_8FNO$: C, 67.79; H, 4.55; N, 7.91. Found: C, 67.77; H, 4.79; N, 7.76.

2.1.7. 7-Fluoro-4-hydroxymethylquinoline (7-F-4-HMeQ) and 7-fluoro-3-hydroxy-4-methylquinoline (7-F-3-OH-4-MeQ)

7-F-4-MeQ (396 mg) was allowed to react with *m*-chloroperoxybenzoic acid (462 mg) in 30 ml of $CHCl_3$ at room temperature for 24 h. The reaction mixture was washed with 5% sodium carbonate aq. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. 7-F-4-MeQ *N*-oxide was obtained in 73% yield. MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.75 (s, 3H, CH_3), 7.11 (d, H-3), 7.46 (m, H-6), 8.00 (dd, H-5), 8.47 (m, 2H, H-2 and H-8); $J_{2-3} = 6.1$, $J_{5-6} = 9.0$, $J_{5-7F} = 5.4$, $J_{6-8} = 2.7$ Hz.

7-F-4-MeQ *N*-oxide (318 mg) was refluxed in acetic anhydride (20 ml) for 10 h. After the solvent was evaporated, the residue was dissolved in water, neutralized with sodium carbonate, and extracted with $CHCl_3$. The $CHCl_3$ layer was dried over anhydrous $MgSO_4$ and evaporated. The resulting residue was hydrolyzed in c-NH_3 aq. (10 ml) at 60°C for 2 h. The resulting mixture was extracted with $CHCl_3$. Purification of the extract by column chromatography (silica gel, $CHCl_3$: MeOH = 19:1) yielded 7-F-4-HMeQ and 7-F-3-OH-4-MeQ in 38% and 55% yield, respectively.

2.1.7.1. 7-F-4-HMeQ. mp 142–144°C. MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 5.22 (s, 2H, CH_2OH), 7.51 (d, H-3), 7.37 (m, H-6), 7.76 (dd, H-8), 8.00 (dd, H-5), 8.89 (d, H-2); $J_{2-3} = 4.4$, $J_{5-6} = 8.9$, $J_{5-7F} = 5.9$, $J_{6-8} = 2.7$, $J_{7-8} = 10.0$ Hz. *Anal*. Calcd. for $C_{10}H_8FNO$: C, 67.79; H, 4.55; N, 7.91. Found: C, 67.79; H, 4.69; N, 8.01.

2.1.7.2. 7-F-3-OH-4-MeQ. mp 210–215°C. MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.66 (s, 3H, CH_3), 7.37 (m, H-6), 7.76 (dd, H-8), 7.96 (dd, H-5), 8.80 (s, H-2); $J_{5-6} = 9.3$, $J_{5-7F} = 5.9$, $J_{5-8} = 2.7$, $J_{7F-8} = 9.8$ Hz. *Anal*. Calcd for $C_{10}H_8FNO$: C, 67.79; H, 4.55; N, 7.91. Found: C, 68.01; H, 4.75; N, 7.78.

2.2. Isolation of the microsomal fraction

Preparation of the microsomal fraction was performed according to the previous report [14,16] after minor modification with respect to the dose of the inducer. Briefly, male Sprague–Dawley rats (6 weeks old) were intraperitoneally injected with 3-MC dissolved in corn oil at a daily dose of 50 mg/kg body weight for three consecutive days. The rats were fasted for 18 h and sacrificed by decapitation 48 h after the last 3-MC injection. The liver was rapidly excised and homogenized in 0.15 M KCl (3 ml/g liver). The homogenate was centrifuged at $9000 \times g$ for 20 min and the supernatant was used as the S9 fraction. The microsomal fraction was prepared by centrifugation of the S9 fraction at $105{,}000 \times g$ for 90 min. The pellet containing the microsomal fraction obtained from 3 ml of the S9 was suspended in 1 ml of 0.02 M potassium phosphate buffer solution (0.15 M KCl, 20% glycerol, pH 7.5). The microsomal protein content was determined by Lowry's method [17]. The cytochrome *P*-450 content was measured as described by Omura and Sato [18].

2.3. Metabolism with microsomal P-450

2.3.1. HPLC analysis of 4-MeQ metabolites

The incubation mixture (1.0 ml) contained 1 nmol of cytochrome *P*-450 (ca. 1 mg of protein), 0.25 mM 4-methylquinolines, 4 mM NADPH, 4 mM NADH, 5 mM G-6-P, 32.8 mM KCl, 8 mM $MgCl_2$, and 100 mM phosphate buffer (pH 7.4). After incubation at 37°C for an appropriate period, 100 μl of this mixture was combined with a half volume (50 μl) of EtOH and centrifuged at $10{,}000 \times g$ for 15 min. The supernatant was analyzed by HPLC.

2.3.2. Isolation and characterization of 2-fluoro-4-hydroxymethylquinoline (2-F-4-HMeQ)

For structural characterization of the metabolite of 2-F-4-MeQ, the above incubation mixture was scaled up 40-fold and incubated at 37°C for 120 min. The resulting reaction mixture was extracted with 2 volumes of ethyl acetate, and the organic solvent layer was dried over $MgSO_4$ and evaporated. The major metabolite of 2-F-4-MeQ, 2-F-4-HMeQ, was isolated from the ethyl acetate extract by preparative thin-layer chromatography (silica gel, $CHCl_3$:MeOH = 19:1). Its structure was identified by ^{1}H-NMR and high resolution mass spectroscopy: ^{1}H-NMR ($CDCl_3$) δ: 5.07 (s, 2H, CH_2OH), 7.32 (s, H-3), 7.64 (dd, H-6), 7.82 (dd, H-7), 7.90 (d, H-8), 8.04 (d, H-5); $J_{5-6} = 8.3$, $J_{6-7} = 7.2$, $J_{7-8} = 8.3$, $J_{6-8} = 2.7$, $J_{7-8} = 10.0$ Hz; HR-MS *m/z*: 177.059, Calcd. for $C_{10}H_8FNO$: 177.059.

2.4. HPLC analysis

HPLC analysis was performed using a Shimadzu liquid chromatograph equipped with a Model LC-10A solvent delivery system, a Model SPD-M6A UV-VIS photodiode array detector and a Merck LiChrospher 100-18e (ODS) column (4 mm × 250 mm). The solvent system consisted of 30% MeOH in 1/15 M phosphate buffer (pH 6.8) at zero time, followed by a linear gradient of MeOH increasing up to 80% in a 40 min period. The flow rate was 0.8 ml/min. Decreases of the starting materials (4-MeQ, 2-F-4-MeQ, 6-F-4-MeQ, and 7-F-4-MeQ) and increases of hydroxymethyl metabolites (4-HMeQ, 2-F-4-HMeQ, 6-F-4-HMeQ, and 7-F-4-HMeQ) were quantified from the peak areas measured by UV absorption at 290 nm with reference to their respective authentic samples. 3-Hydroxy metabolites of 4-MeQ, 6-F-4-MeQ, and 7-F-4-MeQ were quantified from the peak areas measured by UV absorption at 330 nm with reference to their respective authentic samples, because the λ_{max} values of 3-OH-4-MeQ, 6-F-3-OH-4-MeQ, and 7-F-3-OH-4-MeQ are 331, 326, and 332 nm, respectively. Retention times of the 4-MeQs and their metabolites are given in Table 1, and examples

Table 1
Retention times of 4-MeQs and their metabolites in HPLC

	4-hydroxymethyl (min)	3-OH (min)	Starting material (min)
4-MeQ	15.4	29.0	31.8
2-F-4-MeQ	18.7	n.d.	32.2
6-F-4-MeQ	16.9	30.2	32.8
7-F-4-MeQ	17.0	29.5	31.6

HPLC analysis was performed as described in Section 2.
n.d.; not detected.

of the HPLC profiles of 2-F-4-MeQ and 7-F-4-MeQ metabolites are shown in Fig. 1.

2.5. Mutation assay

Chemicals were tested for mutagenicity as previously reported [19–21], using *S. typhimurium* TA100 in the presence of the S9 fraction (50 μl) and cofactors (Cofactor I™). The S9 fraction and Cofactor I™ were purchased from Oriental Yeast, Tokyo. The S9 fraction was prepared from the male Sprague–Dawley rat liver pretreated with phenobarbital (i.p. injected at a daily dose of 30, 60, 60, and 60 mg/kg for four consecutive days) and 5,6-benzoflavone (i.p. injected at a single dose of 80 mg/kg 2 days after the first injection of phenobarbital). Cofactor I™ consisted of 4 mM NADPH, 4 mM NADH, 5 mM G-6-P, 32.8 mM KCl, 8 mM $MgCl_2$, and 100 mM phosphate buffer (pH 7.4). The TA100 strain was the gift of Dr. Matsushima of the University of Tokyo, and were originally provided by Dr. B.N. Ames of the University of California, Berkeley. Assays were carried out after preincubation of the test chemical with S9 mix at 37°C for 20 min.

3. Results

3.1. Mutagenicity of 4-MeQ and its fluorinated derivatives

The 4-MeQs listed in Scheme 1 were tested for mutagenicity in *S. typhimurium* TA100 in the presence of S9 mix according to the procedure of the Ames test [19–21]. In the present paper, derivatives inducing less than twice the number of revertants in the background were considered non-mutagenic. The dose–response curves for the test compounds are shown in Fig. 2. 7-F-4-MeQ as well as 4-MeQ was highly mutagenic, while 6-F-4-MeQ was less mutagenic than 4-MeQ. None of the 2-F-derivatives, i.e., 2-F-4-MeQ, 2,6-diF-4-MeQ, and 2,7-diF-4-MeQ, showed mutagenicity in the whole dose range examined.

3.2. Metabolism of 4-MeQ and its monofluorinated derivatives

4-MeQ, and its monofluorinated derivatives (2-F-4-MeQ, 6-F-4-MeQ, and 7-F-4-MeQ) were examined for their metabolism profiles using liver microsomes from the 3-MC-treated rat. Liver microsome prepared from rats pretreated with 3-MC was used in the present study for metabolism studies to compare with our previous data [16]. On the other hand, liver S9 fraction pretreated with phenobarbital and 5,6-

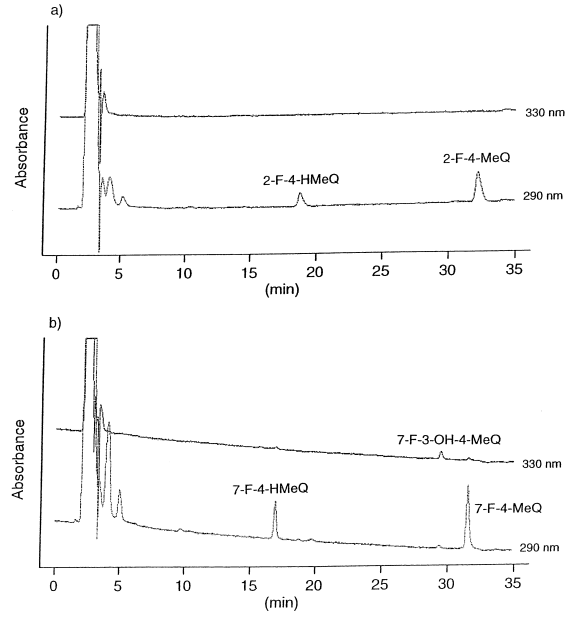
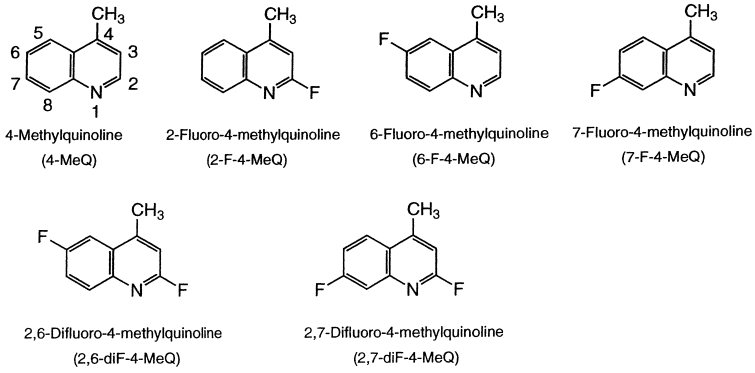


Fig. 1. HPLC profiles of 2-F- and 7-F-4-MeQ metabolites after 30 min incubation in the presence of the microsomal fraction. 2-F- and 7-F-4-MeQ were metabolized for 30 min. The detection wavelengths were 290 and 330 nm. Details are described in Section 2. (a) metabolites of 2-F-4-MeQ. (b) metabolites of 7-F-4-MeQ.



Scheme 1. List of 4-methylquinolines examined.

benzoflavone was used for mutagenicity assay since there is no significant difference in the mutagenicity of the quinoline derivatives between the two exogenous metabolic systems (data not shown). The metabolites were identified and quantified by reverse phase HPLC analysis with reference to their respective authentic samples other than 2-F-4-HMeQ and 2-F-3-hydroxy-4-methylquinoline (2-F-3-OH-4-MeQ), which were difficult to synthesize chemically. 2-F-4-HMeQ was isolated as a metabolite from the large-scale (40-fold) reaction mixture. The retention times of the metabolites (4-hydroxymethyl and 3-hydroxy derivatives) and the starting materials of these 4-MeQs are given in Table 1, and representative charts of HPLC analysis for 2-F-4-MeQ and 7-F-4-MeQ metabolism are also shown in Fig. 1a and b, respectively. The retention times of the fluorinated

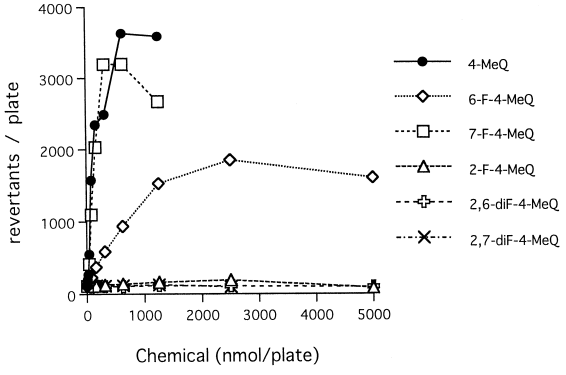


Fig. 2. Mutagenicity of 4-methylquinolines in *S. typhimurium* TA100 in the presence of S9 mix. The symbols shown indicate the means of at least three independent experiments.

derivatives were almost same as (for parent compounds), or slightly longer than (for the two metabolites of each parent compound), those of their respective non-fluorinated analogs (Table 1).

Fig. 3 shows the metabolism of 4-MeQ and its monofluorinated derivatives in the presence of the microsomal fraction. 6-F-4-MeQ was metabolized by the rat liver microsomal fraction to two metabolites: 6-F-4-HMeQ as a major metabolite and 6-F-3-OH-4-MeQ as a minor one. The amount of 6-F-4-HMeQ increased with time up to 120 min after incubation, while that of 6-F-3-OH-4-MeQ reached a maximum in 30 min, followed by a gradual decrease. The metabolism was almost completed in 120 min under the reaction conditions employed. 4-MeQ and 7-F-4-MeQ showed metabolic patterns similar to that observed with 6-F-4-MeQ except for the lesser amounts of the corresponding 4-hydroxymethyl derivatives produced (6-F-4-MeQ: 93%, 4-MeQ: 85%, and 7-F-4-MeQ: 60%).

On the other hand, 2-F-4-MeQ produced only one peak corresponding to 2-F-4-HMeQ other than the starting material on the HPLC chart. Since the retention time of 2-F-3-OH-4-MeQ was predicted to be slightly shorter than that of the starting material, we concluded that 2-F-3-OH-4-MeQ was not produced under the present experimental conditions employed.

4. Discussion

We previously proposed that the genotoxicity of quinoline may be attributable to formation of an

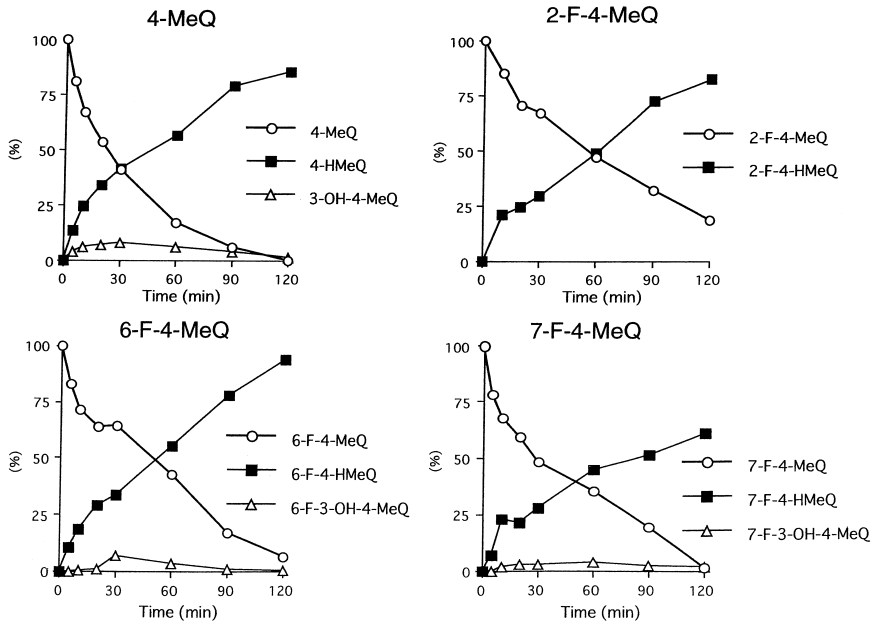


Fig. 3. Metabolism of 4-MeQ and its monofluorinated derivatives in the presence of the microsomal fraction.

enamine epoxide, 2,3-epoxide of 1,4-hydrated quinoline [8,9]. This hypothesis is supported by the fact that its mutagenicity was eliminated by fluorine-substitution at position 3 but not by substitution at position 5, 6, 7, or 8 [8,9,22]. 4-MeQ, a 4-methyl derivative of quinoline, is found to be highly mutagenic in *S. typhimurium* TA100 when compared to quinoline in the presence of rat liver S9 mix [9,16]. Actually, it is the most mutagenic form of all the quinoline derivatives examined so far. As we reported [16], the major metabolite of 4-MeQ in the 3-MC-induced rat liver microsomal enzyme system was identified as non-mutagenic 4-HMeQ and other metabolites identified were 4-MeQ *N*-oxide (4-MeQO), 3-OH-4-MeQ, and 3-hydroxy-4-hydroxymethylquinoline (3-OH-4-HMeQ), while 5,6-epoxi-

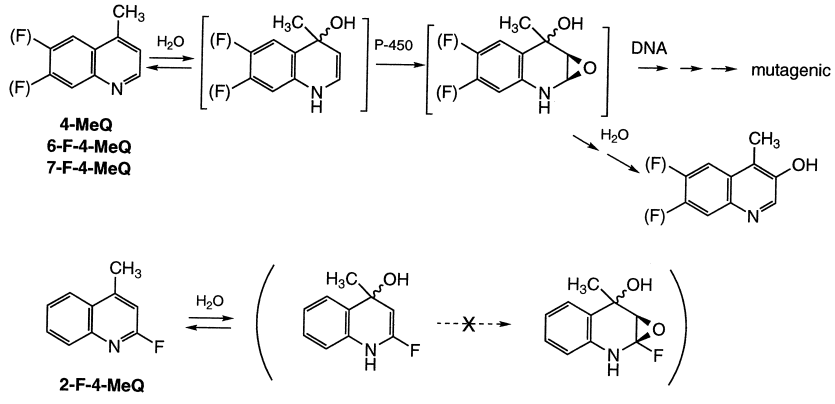


Fig. 4. Proposed mutagenic mechanism of 4-MeQ.

dation, a major detoxification metabolic pathway of quinoline, barely proceeded with 4-MeQ. In addition, our previous studies showed that 4-MeQ was deprived of its potent mutagenicity by introduction of a chlorine atom to position 3 [16]. These findings suggest that the mechanism of mutation induction by 4-MeQ is the same as that applied to mutagenicity of quinoline, which appears to be mediated through the formation of enamine epoxide of 4-MeQ (Fig. 4). The enhancement of mutagenicity of 4-MeQ may be due to its 4-methyl group that interfered with the detoxification process of 5,6-epoxidation at the peri-position and/or due to retarded enzymatic epoxide-ring opening of 1,4-hydrated 2,3-epoxide by epoxide hydrolase or glutathion-*S*-transferase. As a result, the 1,4-hydrated 2,3-epoxide might be stable enough to have greater chance to react with DNA [16].

In the present study, 2-F-4-MeQ, in which an F atom was substituted for H at the position of metabolism in the pyridine moiety, was compared with 6-F- and 7-F-4-MeQ which were fluorinated at the non-metabolic positions, in order to analyze F-substitution effects on the mutagenicity and metabolism of 4-MeQ. The mutagenic activity of 4-MeQ was totally lost by introduction of an F atom to position 2, while 6-F-4-MeQ and 7-F-4-MeQ showed mutagenic potency. In the latter two monofluorinated 4-MeQs, a second F-substitution at position 2 also deprived both compounds of their mutagenicities (Fig. 2). These findings suggest that the 2-fluoro substituent of 2-F-4-MeQs may interfere critically with the enzymatic epoxidation of the 2,3-double bond carrying the fluorine, so that the mutagenicities of these derivatives were completely lost (Fig. 4). This explanation was supported by the observation that 2-F-4-MeQ was not metabolized to its corresponding 3-hydroxy derivative (Fig. 3).

4-MeQ and 7-F-4-MeQ showed metabolic patterns similar to that observed with 6-MeQ except for the lesser amounts of the corresponding 4-hydroxymethyl derivatives produced (6-F-4-MeQ: 93%, 4-MeQ: 85%, and 7-F-4-MeQ: 60%). The amounts of the 3-hydroxy metabolites of above three 4-MeQs increased up to 30 min and decreased thereafter. On the other hand, the amounts of the 4-hydroxymethyl metabolites increased up to 120 min with all the four 4-MeQ derivatives tested (4-MeQ, 6-F-4-MeQ, 7-F-4-MeQ, and 2-F-4-MeQ). These results suggest that the 3-hydroxy metabolites may be further metabolized to 3-hydroxy-4-hydroxymethyl derivatives etc. In fact, we detected the 3-OH-4-HMeQ metabolite in the previous study, where the liver microsomal enzyme fraction was prepared from rats treated with 3-MC for 1 day instead of 3 days as in the present study [16]. The longer treatment of rats with 3-MC may have affected the expression of cytochrome *P*-450 proteins.

In conclusion, the present study affords further evidence to support the enamine epoxide theory as a common activation mechanism of the genotoxicity of the quinoline nucleus and, at the same time, suggests that 2-/3-F-substitution in the pyridine moiety may be responsible for anti-mutagenic structural modification in the quinoline nucleus.

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