Modification of Mutagenicity by Fluorine-Substitution on Diazachrysene

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Fluorinated derivatives of 4,10-diazachrysene (DAC) were tested for their mutagenicity in *Salmonella typhimurium* TA100 in the presence of rat liver S9 to investigate the metabolic activation pathway of DAC. We have previously reported that metabolism at the pyridine moiety of quinoline was related to its mutagenicity and that quinoline was deprived of its genotoxicity by fluorine-substitution at the position 3 in the pyridine moiety. DAC, an aza-analog of chrysene consisting of two quinoline moieties, has two pyridine and two benzene rings as metabolically susceptible sites in the molecule. The mutagenicity of DAC was only decreased by fluorine-substitution on both pyridine moieties. On the other hand, the mutagenicity of DAC was neither decreased by fluorine-substitution on just one pyridine moiety nor by fluorine-substitution on both benzene moieties. These results suggest that metabolic activation occurs on both pyridine moieties in DAC like in quinoline.

Key words —— aza-polycyclic aromatic hydrocarbon, fluorine-substitution, mutagenicity, metabolic activation, Ames test

INTRODUCTION

We have been investigating the substituent effect of a fluorine atom(s) on the mutagenicity and metabolism of aza-aromatic compounds. It is well known that, when a hydrogen atom on an aromatic nucleus is substituted with a fluorine atom, enzymatic oxidation is generally inhibited at the site of fluorine-substitution due to its electron-withdrawing nature.[1–4] Therefore, fluorine-substitution at the metabolic activation site may decrease the mutagenicity of aromatic compounds. We previously reported that quinoline was deprived of both *in vitro* and *in vivo* mutagenicity by fluorine-substitution at the position 3 in the pyridine moiety[5–7] and that metabolic activation of quinoline might take place in the pyridine moiety to form the ultimate genotoxic form, an enamine epoxide (1,4-hydrated 2,3-epoxide).[8,9]

4,10-Diazachrysene (DAC) is an aza-analog of chrysene (Fig. 1), consisting of two quinoline moieties. We have previously reported that DAC showed mutagenicity in the Ames test in the presence of rat liver S9 or human liver microsomes,[10] although formation of the bay-region diol epoxide, the ultimate mutagenic from of chrysene,[11] from DAC seemed impossible because of the nitrogen atoms in both pyridine rings. Moreover, DAC showed a higher mutagenicity than chrysene in the *in vivo* mutagenesis assay system using the *lac*Z transgenic mouse.[12]

In the present study, we attempted to investigate fluorine-substitution effects on the mutagenicity of DAC with special attention to the metabolic activation mechanism. For this purpose, four fluorinated DACs, 2-fluoro-DAC (2-F-DAC), 6-fluoro-DAC (6-F-DAC), 2,8-difluoro-DAC (2,8-diF-DAC), and 6,12-difluoro-DAC (6,12-diF-DAC), were newly synthesized (Fig. 1).

MATERIALS AND METHODS

Materials —— DAC and 6-amino-DAC (Registry Nos. 218-34-8 and 24628-78-2, respectively) were synthesized according to the reported methods.[10,13] 3-Fluorobenzo[*h*]quinoline was synthesized as in our previous report.[14] Tetraethylammonium fluoride-5.5 mol eq. of hydrogen fluoride was purchased from Morita Chemical Industries Co., Ltd, Osaka, Japan. Melting points were determined with a Yamato MP-500D micro melting point apparatus without correction. Mass spectra (electron impact ionization) were measured with a JEOL AX505HA spectrometer. ^{1}H-NMR spectra were recorded with a JNM-GSX 400 spectrometer in $CDCl_3$ or dimethyl sulfoxide (DMSO)-d_6 using

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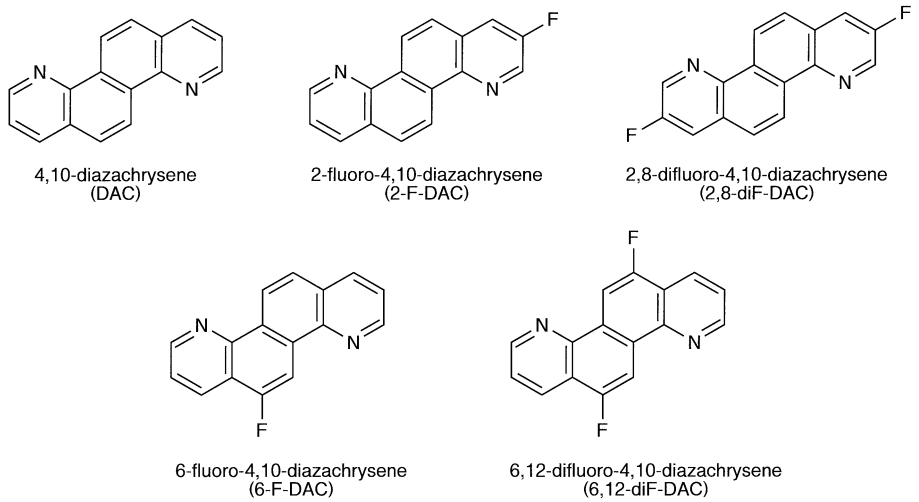

Fig. 1. Chemical Structures of DAC and Its Fluorinated Derivatives

tetramethylsilane as an internal standard. The following compounds were synthesized for the present study.

6-F-DAC —— 6-Amino-DAC (100 mg) was suspended in 5 ml of tetraethylammonium fluoride-5.5 mol eq. of hydrogen fluoride. Sodium nitrite (59.2 mg) was added to the solution, and the mixture was kept stirring at 0°C for 15 min. Then the solution was warmed at 95°C and kept stirring for 1 hr. 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. Purification of the extract by recrystallization with acetone yielded 6-F-DAC in 44% yield. mp 232–234°C (sublimated). ^{1}H-NMR ($CDCl_3$) δ: 7.62 (dd, H-2), 7.68 (dd, H-8), 7.98 (d, H-12), 8.33 (dd, H-1), 8.62 (dd, H-7), 9.06 (d, H-5), 9.08 (dd, H-3), 9.15 (dd, H-9); $J_{1-2} = J_{7-8} = 8.3$, $J_{2-3} = J_{8-9} = 4.4$, $J_{1-3} = 2.0$, $J_{7-9} = 1.5$, $J_{11-12} = 8.8$, $J_{5-F} = 11.7$ Hz. HR-MS m/z: 248.0751, Calcd for $C_{16}H_9FN_2$: 248.0750.

6,12-diF-DAC —— 6-F-DAC (300 mg) was dissolved in 20 ml of c-H_2SO_4 and c-HNO_3 (156 µl in 2 ml of c-H_2SO_4) was added to the solution at 0°C. The resulting solution was kept stirring at 0°C for 15 min. Then the solution was kept stirring at r.t. for 15 hr. The reaction mixture was poured into water, neutralized with aqueous NH_3, and extracted with $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$. The solvent was removed by evaporation to yield 6-fluoro-12-nitro-DAC (6-F-12-NO_2-DAC) in 76% yield. mp 273–275°C. MS m/z: 293 (M^+), 247 (M^+-NO_2). ^{1}H-NMR ($CDCl_3$) δ: 7.76 (dd, H-8), 7.80 (m, H-2), 8.65 (dd, H-7), 9.08 (d, H-5), 9.15 (d, H-1), 9.18 (m, H-3), 9.21 (dd, H-9), 10.20 (s, H-11); $J_{5-F} = 8.3$, $J_{7-8} = 8.3$, $J_{7-9} = 1.7$, $J_{8-9} = 4.3$ Hz.

6-F-12-NO_2-DAC (100 mg) was hydrogenated with 5% Pd-C in methanol for 48 hr. The suspended Pd-C was filtered off and washed with methanol. The solvent was removed by evaporation to yield 6-amino-12-fluoro-DAC (6-NH_2-12-F-DAC) in 62% yield. mp 185–187°C. MS m/z: 263 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 3.49 (br, 2H NH_2), 7.58 (dd, H-8), 7.62 (dd, H-2), 8.31 (dd, H-7), 8.33 (s, H-5), 8.54 (dd, H-1), 8.90 (d, H-11), 9.06 (dd, H-9), 9.08 (dd, H-3); $J_{1-2} = 8.1$, $J_{1-3} = 1.5$, $J_{2-3} = 4.0$, $J_{7-8} = 8.4$, $J_{7-9} = 1.8$, $J_{8-9} = 4.4$, $J_{11-F} = 11.2$ Hz.

6-NH_2-12-F-DAC (37.7 mg) was dissolved in 4 ml of pyridine-HF. Sodium nitrite (9.6 mg) was added to the solution, and the solution was kept stirring at 0°C for 15 min. Then the solution was warmed at 95°C and kept stirring for 1 hr. 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. Purification of the extract by column chromatography (silica gel, $CHCl_3$) yielded 6,12-diF-DAC in 48% yield. mp 250°C (sublimated). ^{1}H-NMR ($CDCl_3$) δ: 7.69 (dd, H-2 and H-8), 8.61 (dd, H-1 and H-7), 8.98 (d, H-5 and H-11), 9.13 (dd, H-3 and H-9); $J_{1-2} = J_{7-8} = 8.2$, $J_{1-3} = J_{7-9} = 1.7$, $J_{2-3} = J_{8-9} = 4.3$, $J_{5-6F} = J_{11-12F} = 11.6$ Hz. HR-MS m/z: 266.0657, Calcd for $C_{16}H_8F_2N_2$: 266.0656.

2-F-DAC —— 3-Fluorobenzo[*h*]quinoline (1.0 g) was dissolved in 10 ml of c-H_2SO_4. 60% HNO_3

(383 μl in 2 ml of c-H_2SO_4) was added to the solution at 0°C and the solution was kept stirring at 0°C for 30 min. The reaction mixture was poured into water, neutralized with aqueous NH_3, and extracted with $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. Purification of the extract by recrystallization from $CHCl_3$ yielded 3-fluoro-7-nitrobenzo[*h*]quinoline in 23% yield. mp 177–178°C. ^{1}H-NMR ($CDCl_3$) δ: 7.81 (dd, H-9), 7.88 (d, H-5), 7.89 (dd, H-4), 8.34 (d, H-6), 8.53 (d, H-8), 8.93 (d, H-2), 9.62 (d, H-10); J_{2-4} = 2.6, J_{5-6} = 7.7, J_{8-9} = 8.1, J_{9-10} = 8.4, J_{4-F} = 9.2 Hz. *Anal*. Calcd for $C_{13}H_7FN_2O_2$: C, 64.47; H, 2.91; N, 11.57. Found: C, 64.46; H, 3.04; N, 11.78.

3-Fluoro-7-nitrobenzo[*h*]quinoline (227 mg) was hydrogenated with 5% Pd-C in benzene for 24 hr. The suspended Pd-C was filtered off and washed with methanol. Purification of the filtrate by recrystallization from benzene yielded 7-amino-3-fluorobenzo[*h*]quinoline in 93% yield. mp 259–261°C. MS *m*/*z*: 212 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 7.02 (dd, H-8), 7.54 (dd, H-9), 7.61 (d, H-6), 7.79 (d, H-5), 7.88 (d, H-4), 8.69 (dd, H-10), 8.85 (d, H-2); J_{2-4} = 3.0, J_{4-F} = 8.8, J_{5-6} = 9.3, J_{8-9} = 7.8, J_{9-10} = 8.3, J_{8-10} = 1.0 Hz. *Anal*. Calcd for $C_{13}H_9FN_2$: C, 73.57; H, 4.27; N, 13.20. Found: C, 73.49; H, 4.41; N, 13.26.

7-Amino-3-fluorobenzo[*h*]quinoline (70 mg), glycerol (73 μl) and sodium *m*-nitrobenzenesulfonate (73.5 mg) were dissolved in 80% H_2SO_4 (2 ml), and the mixture was stirred at 140°C for 4 hr. The reaction mixture was poured into ice water. The filtrate was neutralized with aqueous NH_3 and extracted with $CHCl_3$. Purification of the extract by recrystallization from benzene yielded 2-F-DAC in 34% yield. mp 265–267°C. MS *m*/*z*: 248 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 7.78 (dd, H-8), 8.21 (d, H-6), 8.22 (d, H-12), 8.49 (dd, H-1), 8.58 (dd, H-7), 9.14 (m, H-3 and H-9), 9.27 (d, H-5), 9.40 (d, H-11); J_{1-F} = 9.6, J_{1-3} = 3.0, J_{5-6} = 9.1, J_{7-8} = 8.3, J_{8-9} = 4.6, J_{7-9} = 7.9, J_{11-12} = 9.2 Hz. *Anal*. Calcd for $C_{16}H_9FN_2$: C, 77.41; H, 3.65; N, 11.19. Found: C, 77.09; H, 3.64; N, 11.19.

2,8-diF-DAC ——7-Amino-3-fluorobenzo[*h*]quinoline (205.4 mg) was dissolved in 10 ml of 2% HCl. Sodium nitromalonaldehyde monohydrate (168.3 mg in 1.5 ml of H_2O) was added to this solution at 50°C. The precipitation thus obtained was collected by suction. Purification of the precipitates by recrystallization from benzene yielded *N*-(2-formyl-2-nitroethylidene)-7-amino-3-fluorobenzo[*h*]quinoline in 87% yield. mp 260–263°C. MS *m*/*z*: 292 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 7.73 (d, H-8), 7.85 (dd, H-9), 7.89 (d, H-5), 7.91 (dd, H-4), 8.05 (d, H-6), 8.95 (d, H-2), 9.12 (dd, $CHNO_2$), 9.32 (d, H-10), 10.38 (d, N=CH), 13.07 (br, CHO); J_{2-4} = 2.9, J_{4-F} = 8.8, J_{5-6} = 9.3, J_{8-9} = 8.3, J_{9-10} = 7.8, $J_{CH\text{-}CHNO2}$ = 3.9, $J_{CHNO2\text{-}CHO}$ = 13.7 Hz. This product (200 mg) and $ZnCl_2$ (107 mg) were dissolved in 2 ml of dimethylacetamide, and 6 ml of nitrobenzene was added to this solution. The solution was kept stirring at 210°C for 3 hr. Purification of the reaction mixture by column chromatography (silica gel, $CHCl_3$) yielded 2-fluoro-8-nitro-DAC in 68% yield. mp >300°C. MS *m*/*z*: 293 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 8.00 (dd, H-1), 8.08 (d, H-12), 8.19 (d, H-6), 9.01 (d, H-7), 9.16 (d, H-3), 9.47 (d, H-11), 9.54 (d, H-5), 9.83 (d, H-9); J_{1-F} = 8.8, J_{1-3} = 2.4, J_{5-6} = J_{11-12} = 9.3, J_{7-9} = 2.9 Hz.

2-Fluoro-8-nitro-DAC (87 mg) was hydrogenated with 5% Pd-C in methanol for 3 hr. The suspended Pd-C was filtered off and washed with methanol. Evaporation of the filtrate yielded 2-amino-8-fluoro-DAC in 85 % yield. mp 259–261°C. MS *m*/*z*: 263 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 4.05 (br, NH_2), 7.42 (d, H-1), 7.83 (d, H-6), 7.89 (dd, H-7), 7.91 (d, H-12), 8.64 (d, H-3), 8.92 (d, H-9), 9.22 (d, H-11), 9.30 (d, H-5); J_{1-3} = J_{7-9} = 2.9, J_{5-6} = J_{11-12} = J_{7-F} = 8.8 Hz.

2-Amino-8-fluoro-DAC (54 mg) was dissolved in 2 ml of HF-pyridine. Sodium nitrite (28.5 mg) was added to the solution at 0°C and this solution was kept stirring for 15 min at 0°C. The solution was further kept stirring for 15 min at 30°C, and then for 1 hr at 100°C. The reaction mixture was poured into 30 ml of water, neutralized with 25% aqueous NH_3, and extracted with $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. Purification of the extract by recrystallization with benzene yielded 2,8-difluoro-DAC in 90% yield. mp 218–220°C (sublimated). ^{1}H-NMR ($CDCl_3$) δ: 7.95 (dd, H-1 and H-7), 8.00 (d, H-6 and H-12), 8.97 (d, H-3 and H-9), 9.39 (d, H-5 and H-11); J_{1-F} = J_{7-F} = 8.4, J_{1-3} = J_{7-9} = 2.9, J_{5-6} = J_{11-12} = 8.8 Hz. *Anal*. Calcd for $C_{16}H_8F_2N_2$: C, 72.22; H, 3.03; N, 10.53. Found: C, 71.98; H, 3.33; N, 10.73.

Mutation Assays (Ames Test) —— The DACs were tested for mutagenicity using *Salmonella typhimurium* (*S. typhimurium*) TA100 (kindly provided by Dr. B. N. Ames of the University of California, Berkeley, CA, U.S.A.) in the presence of

rat liver S9 obtained from the phenobarbital- and 5,6-benzoflavone-pretreated male Sprague-Dawley rat liver (Oriental Yeast Co., Tokyo) with co-factors (Cofactor I^{TM}) according to the method by Ames *et al.*[15] with slight modification as previously reported.[16] Cofactor I^{TM} consisted of 4 mM NADPH, 4 mM NADH, 5 mM glucose-6-phosphate, 32.8 mM KCl, 8 mM $MgCl_2$, and 100 mM phosphate buffer (pH 7.4). Assays were carried out by preincubation of the test chemical with the microsome mix at 37°C for 20 min. At least three independent experiments were performed.

RESULTS AND DISCUSSION

The DACs listed in Fig. 1 were tested for mutagenicity in *S. typhimurium* TA100 in the presence of rat liver S9 (1 mg protein/plate) according to the procedure of the Ames test.[15, 16] In general, compounds inducing less than twice the number of revertants in the background were considered non-mutagenic in the Ames test. The dose-response curves for the test compounds are shown in Fig. 2. DAC was highly mutagenic as in our previous report.[10] The mutagenicity of DAC was remarkably decreased to a very weakly positive level by fluorine-substitution in both pyridine moieties, as revealed with 2,8-diF-DAC. The other fluorinated derivatives of DAC showed high mutagenicity. 2-F-DAC, in which only one pyridine moiety is fluorine-substituted, was as mutagenic as, or more mutagenic than, DAC. It seems that fluorine-substitution at only one pyridine moiety was not enough to reduce mutagenicity, probably because the other pyridine moiety site might become more susceptible to metabolic activation. Similar results were obtained in the investigation of the mutagenicity of 1,7-phenanthroline, tricyclic aza-aromatics with the quinoline moiety as a partial structure, and its fluorinated derivatives.[17] Mutagenic 1,7-phenanthroline has three metabolically susceptible sites: two pyridine (position 2–4 and 8–10) and one benzene (position 5 and 6) moieties. Our previous results showed that 9-F-1,7-phenanthroline, in which one pyridine moiety was fluorine-substituted, showed the same mutagenicity as that of 1,7-phenanthroline, and that 3,9-diF-1,7-phenanthroline, in which both pyridine moieties were fluorine-substituted, showed no mutagenicity.[17] Moreover, greater mutagenicity was induced by 6-fluorine-substitution on 1,7-phenanthroline.[17] This suggested that the benzene moiety of 1,7-phenanthroline may not be a metabolic activation site. Like 1,7-phenanthroline, the mutagenicity of DAC was not decreased by fluorine-substitution in the benzene moieties. 6,12-diF-DAC, in which both benzene moieties are fluorine-substituted, and 6-F-DAC were both as mutagenic as DAC. Therefore, the metabolism in the benzene moieties of DAC might be regarded as detoxication rather than mutagenic activation.

In conclusion, the present study affords further evidence to support the enamine epoxide theory as the common activation mechanism of genotoxicity of the quinoline-fused aromatics. In DAC, the metabolic pathway going via the enamine epoxide would be the main activation pathway. Furthermore, substitution with a fluorine atom at a specific position of a mutagenic molecule may be a useful tool to modify its mutagenic potency and to better understand the mechanism of mutation.

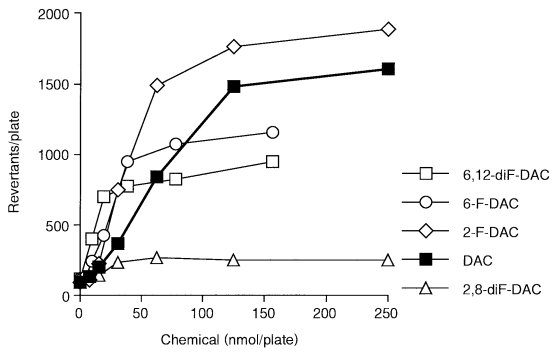

Fig. 2. Mutagenicity of DACs in *S. typhimurium* TA100 in the Presence of S9 Mix

The symbols shown indicate the means of at least three independent experiments.

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