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Mutagenic Activity of the Amido Derivatives and Their Hydroxamic Acids of Nitrobiphenyl Ethers

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Nitrobiphenyl ethers are used as herbicides all over the world, especially in Japan. 2,4,6-Trichloro 4'-nitrobiphenyl ether (CNP) produced by chlorination of 2,4-dichloro 4'-nitrobiphenyl ether (NIP) is extensively applied to rice fields. However, the wide application of CNP and NIP has resulted in environmental pollution(WATANABE et al. 1983). Moreover, NIP was reported to be carcinogenic(QUEST et al. 1989) and CNP is under suspicion in Japan as a possible inducer of gallbladder cancer(YAMAMOTO et al.1993). NIP and CNP are known to be dechlorinated(AIZAWA 1982) and reduced to the corresponding amines by verious factors (NAKAGAWA et al.1974; NIKI et al.1976; MIYAUCHI et al. 1980). Their amino, acetamido and formamido derivatives have been detected as metabolites in mammals(BURKE et al.1983), fish and shellfish(SUZUKI et al.1983). In chemical carcinogenesis testing, attention must be paid to decomposed products that occur in the environment and to metabolites formed in animals. In view of the fact that most carcinogens are also mutagens(McCANN et al.1975), it is of interest to determine whether or not the dechlorinated forms and metabolites of NIP and CNP are capable of inducing mutations. We reported previously that the amino derivatives of nitrobiphenyl ethers used in this study induced mutations in Salmonella typhimurium TA100 strain only after metabolic activation(MIYAUCHI et al.1983). However, there have been no reports on the mutagenic activity of the amido derivatives of NIP or CNP, although they have been detected as metabolites in animals. The present study was undertaken to determine the mutagenic activity of the acetamido and formamido derivatives of 4 kinds of nitrobiphenyl ethers (4'-nitrobiphenyl ether, 4-chloro, 2,4-dichloro and 2,4,6-trichloro 4'-nitrobiphenyl ethers) in the presence and absence of liver homogenates of rats treated with Kaneclor-500. Additionally, N-acetylated and N-formylated hydroxamic acids of these amides were also tested for mutagenic activities, because N-hydroxylation of aromatic amides is a well-known metabolic route in mammals, and N-aryl-O-acylhydroxamic acids can be further activated metabolically to the ultimate carcinogens(BELAND et al.1990).

MATERIALS AND METHODS

β-NADPH, D-glucose-6-phosphate and D-glucose-6-phosphate dehydrogenase were from Oriental Yeast(Tokyo, Japan). Bacto agar and nutrient broth

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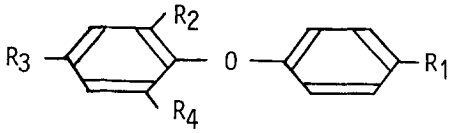
were from Difco. Diisopropyl fluorophosphate was from Aldrich. All other chemicals were reagent grade and purchased from Wako(Osaka, Japan). 4'-Acetylaminobiphenyl ether(4'-NHAc), 4-chloro 4'-acetylaminobiphenyl ether(4-NHAc) and 2,4-dichloro 4'-acetylaminobiphenyl ether(2,4-NHAc) were synthesized by reacting the corresponding amino derivatives, prepared by the method described previously(MIYAUCHI et al. 1983), with acetic anhydride. [4'-NHAc: Yield 89%; m.p. 130-131℃; Anal. Calcd. for $C_{14}H_{13}NO_2$(%)C 73.97, H 5.77, N 6.16, Found C 74.13, H 5.84, N 6.36; MS 227(parent), 4-NHAc: Yield 90%; m.p. 145-146℃; Anal. Calcd. for $C_{14}H_{12}NO_2Cl$(%) C 64.24, H 4.63 N 5.35, Found C 64.10, H 4.63, N 5.40; MS 261(parent), 2,4-NHAc: Yield 90%; m.p. 148-150℃; Anal. Calcd. for $C_{14}H_{11}NO_2Cl_2$(%)C 56.78, H 3.75, N 4.73, Found C 56.72, H 3.71, N 4.77; MS 295(parent)]. 2,4,6-Trichloro 4'-acetylaminobiphenyl ether (2,4,6-NHAc) was prepared according to the method previously reported (SUZUKIet al. 1983). 4'-Formylaminobiphenyl ether(4'-NHCHO) and 4-chloro 4'-formylaminobiphenyl ether(4-NHCHO) were synthesized by reacting the corresponding amino derivatives with formic acid. [4'-NHCHO: Yield 75%; m.p. 85-86℃; Anal. Calcd. for $C_{13}H_{11}NO_2$(%) C 73.21, H 5.20, N 6.56, Found C 72.96, H 5.10, N 6.74; MS 213(parent), 4-NHCHO: Yield 65%; m.p. 91-92℃; Anal. Calcd. for $C_{13}H_{10}NO_2Cl$(%) C 63.03, H 4.07, N 5.65, Found C 63.06, H 4.08, N 5.63; MS 247(parent)]. 2,4-Dichloro 4'-formylaminobiphenyl ether(2,4-NHCHO) (NAKAGAWA et al. 1974) and 2,4,6-trichloro 4'-formylaminobiphenyl ether(2,4,6-NHCHO) (SUZUKI et al. 1983) were prepared accordingto the previously reported procedures.

4'-Hydroxyacetylaminobiphenyl ether(4'-N(OH)Ac)(MIYAUCHI et al. 1984), 4-chloro 4'-hydroxyacetylaminobiphenyl ether(4-N(OH)Ac)(KUMANO et al. 1986), 2,4-dichloro 4'-hydroxyacetylaminobiphenyl ether(2,4-N(OH)Ac) (MIYAUCHI et al. 1984)and 2,4,6-trichloro 4'-hydroxyacetylaminobiphenyl ether(2,4,6-N(OH)Ac)(MIYAUCHI et al. 1984) were synthesized according to the previously described procedures. N-Formylated hydroxamic acids were synthesized as follows; to 1g of the corresponding hydroxylaminobiphenyl ether, prepared by the method reported previously(MIYAUCHI et al. 1984), 60 ml of dry pyridine was added and then acetic-formic anhydride (15 ml of acetic anhydride and 9.3 ml of formic acid) was added dropwise with stirring for 1 hr at 0℃. To the mixture, 20 ml of ethyl ether and 20 ml of water were added, and the aqueous layer was discarded and the organic layer was washed with water. To the organic layer, 30 ml of 1N sodium hydroxide saturated with sodium chloride was added and the mixture was extracted twice with ethyl ether after adjustment pH at 5.0 by 1N hydrochloric acid and then dried over anhydrous sodium sulfate. The ether layer was evaporated in vacuo to give crystals. [4'-N(OH)CHO: Yield 44%(calcd. from corresponding nitro compound); m.p. 86-87℃; Anal. Calcd. for $C_{13}H_{11}NO_3$(%) C 68.10, H 4.84, N 6.11, Found C 68.10, H 4.87, N 6.33; MS 229(parent), 4-N(OH)CHO: Yield 36.8%(calcd. from corresponding nitro compound); m.p. 131-132℃; Anal. Calcd. for $C_{13}H_{10}NO_3Cl$(%) C 59.20, H 3.83, N 5.31, Found C 59.20, H 3.77, N 5.25; MS 263(parent), 2,4-N(OH)CHO: Yield 48%(calcd. from corresponding nitro compond); m.p. 81-82℃; Anal. Calcd. for $C_{13}H_9NO_3Cl_2$ (%) C 52.36, H 3.04, N 4.69, Found C 52.44, H 3.05, N 4.64; MS 297 (parent), 2,4,6-N(OH)CHO: Yield 45%(calcd. from corresponding nitro compound); m.p. 161-162℃; Anal. Calcd. for $C_{13}H_8NO_3Cl_3$(%) C 46.94, H 2.42, N 4.21, Found C 46.99, H 2.40, N 4.16; MS 331(parent)].

Male Wistar rats weighing 200-250g were used. They were maintained on



Compound	R_1	R_2	R_3	R_4
NIP	NO_2	H	Cl	Cl
MO	NO_2	Cl	Cl	Cl
4'-NHAc	$NHCOCH_3$	H	H	H
4-NHAc	$NHCOCH_3$	H	Cl	H
2,4-NHAc	$NHCOCH_3$	H	Cl	Cl
2,4,6-NHAc	$NHCOCH_3$	Cl	Cl	Cl
4'-NHCHO	NHCHO	H	H	H
4-NHCHO	NHCHO	H	Cl	H
2,4-NHCHO	NHCHO	H	Cl	Cl
2,4,6-NHCHO	NHCHO	Cl	Cl	Cl
4'-N(OH)Ac	$N(OH)COCH_3$	H	H	H
4-N(OH)Ac	$N(OH)COCH_3$	H	Cl	H
2,4-N(OH)Ac	$N(OH)COCH_3$	H	Cl	Cl
2,4,6-N(OH)Ac	$N(OH)COCH_3$	Cl	Cl	Cl
4'-N(OH)CHO	N(OH)CHO	H	H	H
4-N(OH)CHO	N(OH)CHO	H	Cl	Cl
2,4-N(OH)CHO	N(OH)CHO	H	Cl	Cl
2,4,6-N(OH)CHO	N(OH)CHO	Cl	Cl	Cl

Figure 1. Structures and abbreviations of nitrobiphenyl ether derivatives and related compounds.

a commercial diet and tap water ad libitum. and were injected i.p.(500 mg/Kg) with Kaneclor-500 dissolved in olive oil for induction of drug-metabolizing enzymes. On the fifth day after injection, rats were killed and S-9(the supernatant of liver homogenate centrifuged for 10 min at 9,000 x g) fractions were prepared according to the previously described procedure(AMES et al.1975) and stored at -80℃. The protein content of the S-9 fractions was measured by the previously described method(LOWRY et al.1951).

Tests for mutagenicity were carried out mainly according to the Ames method as modified by Yahagi(1975) with tester strain TA100, because nitrobiphenyl ethers are primarily base-substitution mutagens(MIYAUCHI et al.1983). Titrations were performed with the concentration of 0-200 nmoles/plate of each compound and with three separate protein concentrations(0.5,2.0 and 5.0 mg/plate). To investigate the effect of deacylase inhibition on the mutagenicity of the hydroxamic acids, diisopropyl fluorophosphate dissolved in 0.05 ml of dimethylsulfoxide (DMSO) at a concentration of 10^{-4}M was added to the test tubes containing 0.1ml of bacterial culture, 0.1ml of DMSO containing the test compound at the concentration of 150 nmoles, and 0.5ml of mixture containing S-9 and other cofactors. It was confirmed that this amount of inhibitor and DMSO had no toxic or mutagenic effect on the tester strain. All mutation assays were carried out in triplicates.

Statistical tests for differences were performed using Student's t-test.

RESULTS AND DISCUSSION

Table 1 shows ratios of the number of revertant colonies with each compound observed on the test plate(at 5 mg protein S-9/plate, and 200 nmol the amido derivative/plate or 150 nmol the hydroxamic acid /plate) to the number of revertant colonies appearing on the corresponding control(S-9 blank) plate, i.e. the ratio of test to background. None of the acetamido or formamido derivatives of nitrobiphenyl ethers induced any mutations in Salmonella typhimurium TA 100 strain without the S-9 fraction. However, in the presence of the S-9 fraction they induced mutations and their dose-response curves were linear in the range of 200 nmoles/plate, indicating that they were metabolized by S-9 to mutagenic products. In our previous report (MIYAUCHI et al.1983), the amino derivatives of nitrobiphenyl ether used in this study were shown to induce mutations in Salmonella typhimurium TA 100 strain only after metabolic activation and their N-hydroxylated metabolites, the N-hydroxyarylamines, were thought to be responsible for inducing mutations because the amino derivatives caused methemoglobin formation with rat liver homogenates(MIYAUCHI et al.1981), and the N-hydroxyarylamines showed mutagenic activity by themselves(MIYAUCHI et al.1984).The mutagenic metabolites of the amido derivatives were thought to be the N-hydroxyarylamines as well as the amino derivatives because it is known that the mutagenic activity of aromatic amides depends on N-hydroxylation forming hydroxamic acids,

Table 1. Mutagenic activity of the acetamido and formamido derivatives and their hydroxamic acids of nitrobiphenyl ethers with and without rat liver homogenates.

Chemicals	-S-9	+S-9	Chemicals	-S-9	+S-9
4'-NHAc	0.8	7.8**	4'-NHCHO	0.7	9.0**
4-NHAc	0.8	9.5**	4-NHCHO	0.8	12.6**
2,4-NHAc	0.9	3.4*	2,4-NHCHO	0.9	3.8*
2,4,6-NHAc	1.0	2.4*	2,4,6-NHCHO	0.9	3.0*
4'-N(OH)Ac[1]	0.8	39.5**	4'-N(OH)CHO[1]	0.7	45.6**
4-N(OH)Ac[1]	0.9	47.6**	4-N(OH)CHO[1]	0.8	55.6**
2,4-N(OH)Ac[1]	0.9	14.2**	2,4-N(OH)CHO[1]	0.8	17.5**
2,4,6-N(OH)Ac[1]	0.9	10.4**	2,4,6-N(OH)CHO[1]	0.9	12.6**

Values in the table are ratio of numbers of revertant colonies observed on the test plate(200 nmol chemical,[1]150 nmol chemical/plate) to the number appearing on the corresponding control(S-9 blank) plate, i.e. the ratio of test to background.
Statistical significant *at $P<0.05$ and **at $P<0.001$.

and following enzymatic deacylation, N-hydroxyarylamines(THORGEIRSSON et al.1983). To validate this point, N-acetylated and N-formylated hydroxamic acids of the amido derivatives of nitrobiphenyl ethers were prepared and tested for their mutagenic activity, and the effect of diisopropyl fluorophosphate, a deacylase inhibitor, on their mutagenic

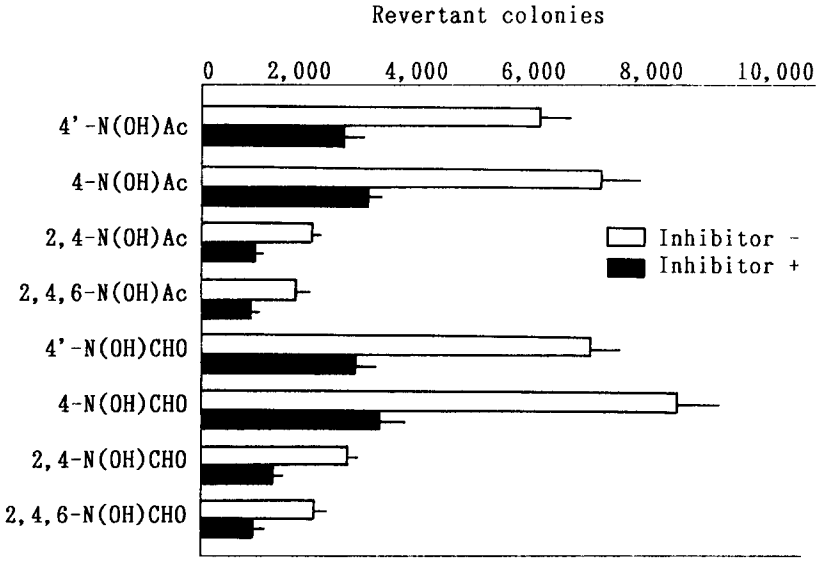


Figure 2. Effects of the deacylase inhibitor(diisopropyl fluorophophate on mutagenicity of the hydroxamic acids of the amido derivatives of nitrobiphenyl ethers in the presence of rat liver homogenates. The inhibitor was added at the concentration of 10^{-4}M per plate. Bars are the mean ± S.E. for three experiments.

activities was examined. The hydroxamic acids of the amido derivatives showed mutagenic activity only after metabolic activation, and their dose-response curves were linear in the range of 150 nmoles/plate. They showed no mutagenic activity by themselves although the hydroxamic acid of 2-acetylaminofluorene shows low mutagenic activity without metabolic activation(AMES et al.1972). N-Hydroxy-2-acetylaminofluorene could be N-deacetylated more easily, or the resultant N-hydroxy-2-aminofluorene could undergo O-acetylation in the bacteria (FU 1990). It seems that the hydroxamic acids of the amido derivatives could not be N-deacetylated easily in the bacteria compared with fluorene derivatives. However, with the S-9 fraction they showed high mutagenic activities compared with their corresponding amides, even though low concentrations. Their high mutagenic activities indicated that they are metabolized more quickly to the products responsible for inducing mutations than their correspoding amides. The metabolite responsible for mutagenic activity were considered to be the deacylated form or the hydroxylamine because rat microsomal deacylase, sensitive to organophosphate, can convert N-hydroxy-2-acetylaminofluorene and N-hydroxy-2-formylaminofluorene to N-hydroxy-2-aminofluorene (KING et al.1983). Figure 2 illustrates the number of revertant colonies with hydroxamic acids of the amido derivatives in the presence of S-9 fraction with and without the deacylase inhibitor diisopropyl fluorophosphate. Addition of the inhibitor blocked the mutagenic activity of N-acetylated and formylated hydroxamic acids by about 60-65%, showing that these hydroxamic acids were mainly converted to the hydroxylamine by microsomal deacylase. The imcomplete disappearance of mutagenicity of these hydroxamic acids by this inhibitor would be due to the actions of cytosolic deacylases,

N-aryl-hydroxamic acid N,O-acyltransferase(ALLABEN et al.1984) and/or N-arylacyl-hydroxamic acid-dependent N-acyltransferase (KUMANO et al. 1986) which are resistant to this inhibitor. To validate the pathway of mutagenic activation of these hydroxamic acids of the amido derivatives, additional experiments using the purified enzyme are needed. As shown in Table 1, the compounds became less effective in inducing mutations with increasing numbers of chlorine substituents. This tendency has also been found in the ability to induce methemoglobin formation(MIYAUCHI et al.1981) and the mutagenic activities of chlorinated nitrobiphenyl ethers and their amino derivatives (MIYAUCHI et al.1983). However, that the monochlorocompound was more effective in inducing mutations than deschlorocompound shows that the mutagenic activity depends not only on thenumber of chlorines but also on their position in the phenoxygroup.

The results reported here clearly demonstrate that the amido derivatives of nitrobiphenyl ethers are mutagens, and suggest that mutagenic activity of the amides is due to N-hydroxylation and subsequent deacylation forming the N-hydroxyarylamine as judged from the high mutagenic activities of their hydroxamic acid drivatives (Table 1)and from the effects of organophosphate(Fig. 2). Formation of N-hydroxyarylamine and hydroxamic acid are the key steps in the induction of carcinogenesis by aromatic amides(BELAND et al.1990). Therefore it is very important to pay attention to the mutagenic activity of the amido derivatives and the hydroxamic acids of the herbicides, NIP and CNP. In addition, the finding that successively dechlorinated compounds produced in the environment(AIZAWA 1982) are more effective in inducing mutations is important from the toxicological point of view. Moreover, the high mutagenic activity of hydroxamic acids of the amido derivatives is significant because there is a possibility that the hydroxamic acid derivatives are formed from aromatic nitroso compounds by thiamine-dependent enzymes such as pyruvate decarboxylase(CORBETT et al.1982).

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