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# Mutagenicity of Heterocyclic Amines by Biomimetic Chemical Models for Cytochrome P450 in Ames Assay

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**Heterocyclic amines (HCAs) are a family of mutagenic and carcinogenic compounds produced during cooking or other burning processes, and exist in the environment. HCAs are metabolically activated by cytochrome P450, conjugated by phase II enzymes, to react with guanine bases. The aim of this study is to establish a chemical model for cytochrome P450 as an alternative to S-9 mix for detecting HCA mutagenicity in Salmonella strain. A chemical model was developed by comparing the mutagenicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) in the presence of an iron porphyrin and an oxidant. The iron porphyrin derivatives, water-soluble 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrinatoiron (III) chloride (4-MPy) or water-insoluble 5,10,15,20-tetrakis (pentafluorophenyl)porphyrinatoiron (III) chloride ($F_5$P), and the oxidant *tert*-butyl hydroperoxide (*t*-BuOOH), magnesium monoperoxyphthalate or iodosylbenzene were used. 4-MPy or $F_5$P with *t*-BuOOH activated Trp-P-2, and the activity was similar with either porphyrin. Water-soluble model has a better chance to detect unstable compound, since the tester strain was exposed in the whole incubation period in the mutation procedure with 4-MPy. The effectiveness of 4-MPy/*t*-BuOOH was evaluated with other HCAs; IQ, MeIQ, MeIQx, Glu-P-1, Glu-P-2, PhIP, Trp-P-1, MeA$\alpha$C and A$\alpha$C. All HCAs except for MeA$\alpha$C and A$\alpha$C were mutagenic in *Salmonella typhimurium* TA1538. MeA$\alpha$C and A$\alpha$C were not mutagenic in TA1538, but they were mutagenic in *S. typhimurium* TA1538/pYG219, which overexpresses *O*-acetyltranferase on the TA1538 genetic background. Although the HCAs mutagenicity with the chemical model was weaker than that with S-9 mix, the chemical models activated HCAs without S-9 mix in the Ames assay.**

**Key words: iron porphyrin, chemical model, cytochrome P450, metabolic activation, heterocyclic amine**

## Introduction

Heterocyclic amines (HCAs) are found in broiled, fried or barbecued meats and other proteinaceous foods, as well as in tobacco smoke (1–6). The consumption or intake of HCAs is suspected to be correlated with the induction of cancers (7–13). HCAs are a family of mutagenic and carcinogenic compounds produced during the pyrolysis of creatine, amino acids and glucose. HCAs are classified into two groups based on structure: 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-type and non-IQ type. IQ-type comprises HCAs that are characterized by a 2-aminoimidazole structure, while non-IQ type is typically characterized by a 2-aminopyridine structure (14–16) (Fig. 1).

The HCAs are oxidized to *N*-hydroxyamines by cytochrome P450 (17–20), and then conjugated by acetylation, sulfation and proline conjugation, and finally activated to react with guanines (21–25). The HCAs are known to be activated also by prostaglandin H synthase, however, the activation mechanism is not known (26).

Rat S-9 mix is useful in obtaining reliable data when used for activation in the Ames assay, since the S-9 mix includes many kinds of cytochrome P450 containing iron (Fe) porphyrin active sites capable of metabolizing a large number of chemicals to reactive forms (27,28). However the S-9 mix has difficulty maintaining constant enzymatic activity (29). In order to overcome the difficulty, an alternate *in vitro* model for cytochrome P450, consisting of a Fe porphyrin and an oxidant was applied in the Ames mutation assay. This model may be used as an alternative to animals in primary screening. The chemical model has been used previously for detecting the mutagenicity of polyaromatic hydrocarbons (30,31), aromatic amines and amides in the Ames assay (31,32). In this study, a chemical model which combined a Fe porphyrin with an oxidant was developed to detect the mutagenicity of HCAs. The Fe porphyrins, water-soluble 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)

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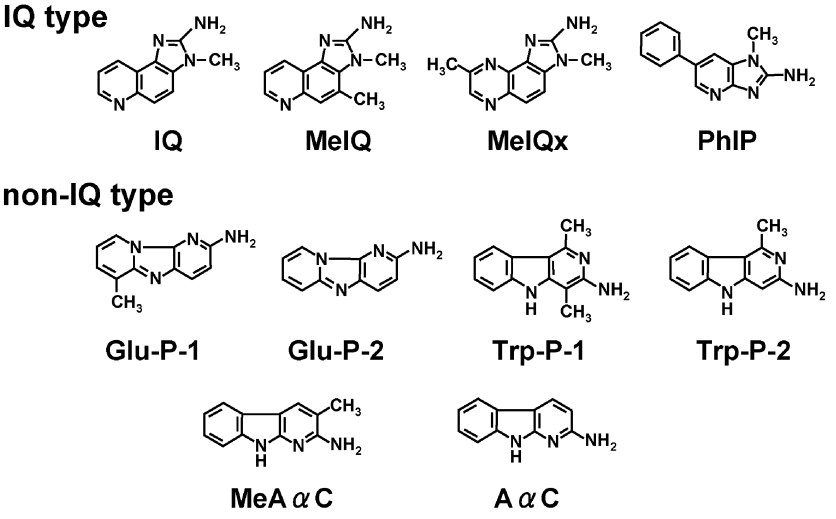


**Fig. 1.** Structure of HCAs.

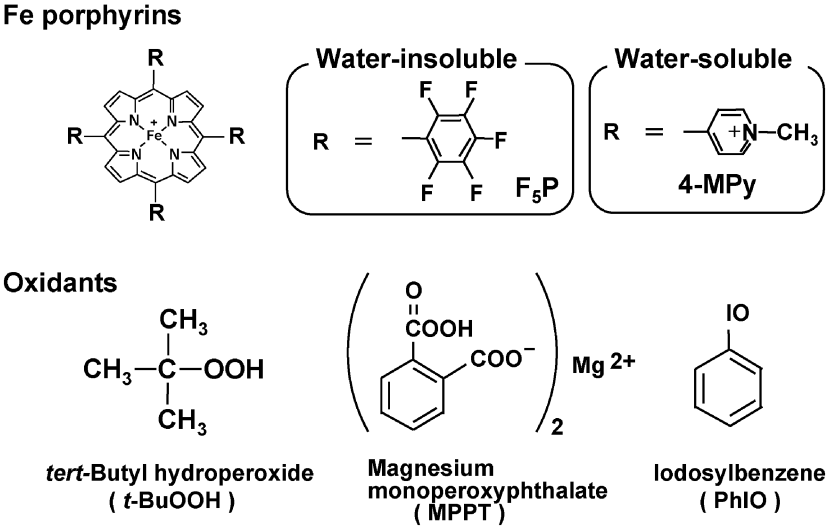


**Fig. 2.** Structure of chemical models Fe porphyrins and peroxides used.

porphyrinatoiron(III) chloride (4-MPy) or water-insoluble 5,10,15,20-tetrakis(pentafluorophenyl)porphyrinatoiron(III) chloride ($F_5P$) was used, and as oxidants, *tert*-butyl hydroperoxide (*t*-BuOOH), magnesium monoperoxyphthalate (MPPT) or iodosylbenzene (PhIO) was used. The reaction conditions were optimized by comparing the mutagenic activity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) in *S. typhimurium* TA1538, and the effectiveness of 4-MPy/*t*-BuOOH with other HCAs using the Ames assay was then evaluated.

## Materials and Methods

**Chemicals:** *t*-BuOOH, $F_5P$ and MPPT were purchased from Sigma-Aldrich Corp. (St. Louis, USA). Sodium ammonium hydrogenphosphate tetrahydrate was purchased from Merck (Darmstadt, Germany). Tetrakis (1-methylpyridinium-4-yl)porphine *p*-toluenesulfonate and PhIO were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Bacto agar and bacto nutrient broth were obtained from Becton Dickinson Microbiology Systems (Sparks, USA). Other reagents used were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-MPy was synthesized as described (33). The purity of 4-MPy was determined by ultraviolet spectroscopy. 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) acetate and Trp-P-2 acetate were recrystallized from ethanol and ethyl acetate before use. The structure of the chemical models used is shown in Fig. 2.

**Mutation assay using the chemical model:** The mutation assay was based on the Ames test (34,27),

utilizing a chemical model consisting of Fe(III) porphyrin and an oxidant as a substitute for the metabolic activation system. Professor B. N. Ames (University of California, Berkeley, USA) provided the *S. typhimurium* TA1538, and Dr. T. Nohmi (National Institute of Health Sciences, Tokyo, Japan) provided the *S. typhimurium* TA1538/pYG219. 4-MPy (50 nmol/20 $\mu$L), *t*-BuOOH (150 nmol/20 $\mu$L) and MPPT (50 nmol/20 $\mu$L) were dissolved in 0.1 M sodium phosphate buffer (pH 7.4), and PhIO (250 nmol/20 $\mu$L) was dissolved in methanol. $F_5P$ (50 nmol/20 $\mu$L), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) hydrochloride, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeA$\alpha$C) and 2-amino-9*H*-pyrido[2,3-*b*]indole (A$\alpha$C) were dissolved in acetonitrile, and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), Trp-P-1 acetate, 2-amino-6-methyldipyrido[1,2-$\alpha$:3′,2′-*d*]imidazole (Glu-P-1) hydrochloride and 2-aminodipyrido[1,2-$\alpha$:3′,2′-*d*]imidazole (Glu-P-2) hydrochloride were dissolved in $H_2O$. Trp-P-2 acetate was dissolved in *N*,*N*-dimethylformamide when water-insoluble $F_5P$ was used, whereas it was dissolved in $H_2O$ when water-soluble 4-MPy was used. All mutagens showed no mutagenicity in the absence of the chemical model system in Salmonella strains.

**Preincubation assay using water-soluble chemical activation system** (35): An aliquot of the water-soluble 4-MPy (20 $\mu$L) and mutagen (20 $\mu$L), both in solutions, were mixed, and oxidant (20 $\mu$L), 0.1 M sodium phosphate buffer (pH 7.4, 0.5 mL), and a culture of the tester strains (0.1 mL) were added. The mixture was then incubated for 10 sec, 1, 3, 5, 10, or 20 min at 25°C with shaking (120 strokes/min), and top agar (2 mL) was added. The mixture was then poured onto a minimal-glucose agar plate. After incubation for 44 h at 37°C, colonies were counted using a colony analyzer (System Science Co. Ltd., Tokyo, Japan). Mutagenic activity was tested at whole incubation periods, however, the highest mutagenic activity was observed at a different incubation period for each chemical model (31). In this study, the optimal incubation period for each of the assay conditions was selected for data presentation. The results were considered positive if the assay produced reproducible, dose-related increases in the number of revertants.

**Preincubation for water-insoluble chemical activation and direct mutagenicity assay** (35): An aliquot of the water-insoluble $F_5P$ (20 $\mu$L) and mutagen (20 $\mu$L) in solution were mixed, and oxidant (20 $\mu$L) in a solution was added. The mixture was then incubated for 1, 3, 5, 10 or 20 min at 25°C with shaking (120 strokes/min), and 0.1 M sodium phosphate buffer (pH 7.4, 0.5 mL), a culture of tester strain (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, colonies were counted. The data are presented as described previously.

## Results

**Optimization of model for detecting HCAs mutagenicity in the Ames assay:** The most efficient chemical model for detecting the HCAs mutagenicity was developed by comparing Trp-P-2 mutagenicity, consisting of a Fe porphyrin, water-soluble 4-MPy or water-insoluble $F_5P$ plus *t*-BuOOH. The assay protocols using the chemical models were already reported (35). The chemical model containing a water-soluble porphyrin was suitable for addition of a tester strain during incubation, whereas the $F_5P$ chemical model was suitable for adding a tester strain after incubation.

Trp-P-2 in the presence of each chemical model showed the highest mutagenicity at the 20 min reaction time using each procedure (Fig. 3). The mutagenicity of Trp-P-2 was similar with either 4-MPy or $F_5P$, in the presence of *t*-BuOOH (Fig. 4). Water-soluble 4-MPy has been shown to detect unstable intermediates formed in the reaction mixture therefore we used preincubation method where the tester strain was added over incubation periods (32).

The models were composed of water-soluble 4-MPy, and an oxidant-*t*-BuOOH, MPPT or PhIO, and were used as alternatives for S-9 mix. One dose of the porphyrin (50 nmol/plate) was used since the mutagenicity of polyaromatic hydrocarbons, aromatic amines and amides have been successfully detected in the presence of 50 nmol/plate of the porphyrin (30,32). The concentration of the oxidants was determined as those not

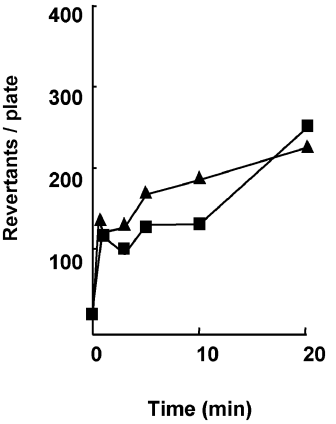


**Fig. 3.** Effect of incubation period on Trp-P-2 mutagenicity in *S. typhimurium* TA1538. Preincubation assay involved exposing a tester strain with reaction mixture using water-soluble 4-MPy (▲) or adding a tester strain into a reaction mixture after the incubation period using water-insoluble $F_5P$ (■). The concentration of Trp-P-2 was 25 nmol/plate.

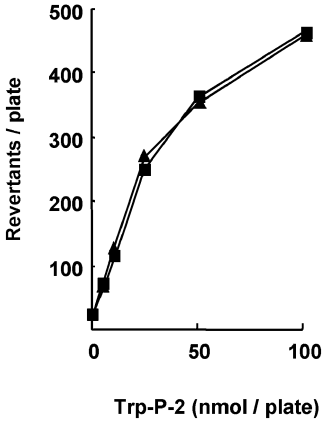


**Fig. 4.** Effect of Fe porphyrin on Trp-P-2 activation in *S. typhimurium* TA1538 in the presence of a Fe porphyrin plus *t*-BuOOH. Preincubation assay involves exposing a tester strain with reaction mixture using water-soluble 4-MPy (▲) or adding a tester strain into a reaction mixture after the incubation period using water-insoluble $F_5P$ (■).

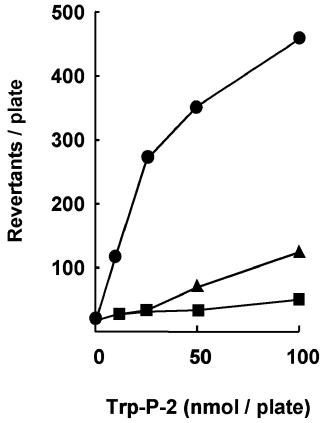


**Fig. 5.** Effect of oxidants on Trp-P-2 activation in *S. typhimurium* TA1538 in the presence of 4-MPy. *t*-BuOOH (●), MPPT (■) or PhIO (▲) preincubated with 4-MPy in 0.1 M phosphate buffer for 20 min.

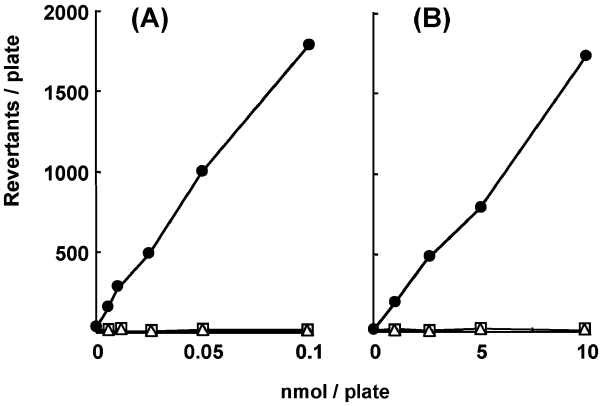


**Fig. 6.** Mutagenicity of IQ (A) and Glu-P-1 (B) in *S. typhimurium* TA1538 in the presence of 4-MPy/*t*-BuOOH. Complete system contained mutagen in water, 4-MPy and *t*-BuOOH in sodium phosphate buffer (pH 7.4) and incubated for 20 min (●). The control system comprised without 4-MPy (□), without *t*-BuOOH (△), and mutagen alone (○), which all overlapped.

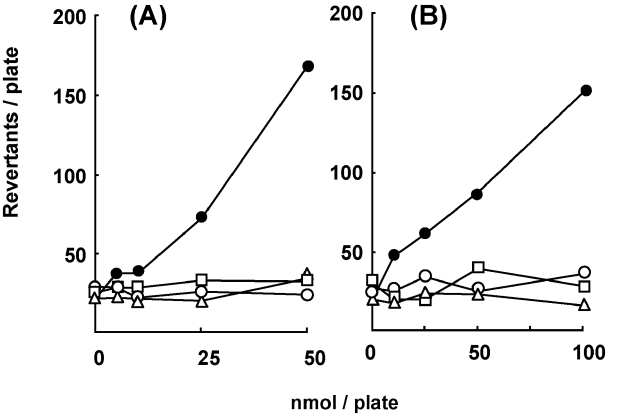


**Fig. 7.** Mutagenicity of MeAαC (A) and AαC (B) in *S. typhimurium* TA1538/pYG219 in the presence of 4-MPy/*t*-BuOOH. Complete system contained a mutagen in acetonitrile, 4-MPy and *t*-BuOOH in sodium phosphate buffer (pH 7.4) preincubated for 10 sec (●). The control system comprised without 4-MPy (□), without *t*-BuOOH (△), and mutagen alone (○).

showing cytotoxicity, and then the ratio of the porphyrin and the oxidant is different among each oxidant. Trp-P-2 mutagenicity in the presence of the various chemicals was compared (Fig. 5).

The activation capacity was in the order: *t*-BuOOH > MPPT > PhIO. The results showed that the 4-MPy/*t*-BuOOH was the most effective chemical model to detect HCAs mutagenicity.

**Detection of HCAs mutagenicity in the presence of 4-MPy/*t*-BuOOH:** Activation of ten HCAs; IQ, MeIQ, MeIQx, Glu-P-1, Glu-P-2, PhIP, Trp-P-1, Trp-P-2, MeAαC, AαC, in the presence of 4-MPy/*t*-BuOOH was evaluated in the Salmonella strains. Figure 6 shows the metabolic activation of IQ and Glu-P-1, which are representative of an IQ-type and a non-IQ type HCA, respectively. All IQ type HCAs (IQ, MeIQ, MeIQx, PhIP) and non-IQ type HCAs (Glu-P-1, Glu-P-2, Trp-P-1) were mutagenic in *S. typhimurium* TA1538 in the presence of 4MPy/*t*-BuOOH with 20 min preincubation. MeAαC and AαC were not mutagenic in the *S. typhimurium* TA1538 strain. When *S. typhimurium* TA1538/pYG219 (36), overexpressing *O*-acetyltransferase on the genetic background of the *S. typhimurium* TA1538, was used for detecting mutagenicity of MeAαC and AαC (Fig. 7), both were mutagenic in a dose-dependent manner in the presence of 4-MPy/*t*-BuOOH.

The mutagenic activity was determined from the slopes of dose-revertants profile in the initial linear part of the profile by the least squares method (Table 1). The

**Table 1.** Relative mutagenicity of HCAs in the presence of 4-MPy/*t*-BuOOH in *S. typhimurium* TA1538

| HCAs | Revertants/nmol |
|---|---|
| IQ | 17444 ± 43.4 |
| MeIQ | 5284 ± 41.0 |
| MeIQx | 4510 ± 16.1 |
| Glu-P-1 | 169 ± 51.2 |
| Glu-P-2 | 29 ± 14.3 |
| PhIP | 48 ± 47.2 |
| Trp-P-2 | 10 ± 2.7 |
| Trp-P-1 | 7 ± 10.4 |
| MeA$\alpha$C* | 3 ± 11.7 |
| A$\alpha$C* | 1 ± 3.1 |

**S. typhimurium* TA1538/pYG219.

study with S-9 mix used *S. typhimurium* TA98 carrying plasmid pKM101, which has higher sensitivity for DNA damage due to SOS repair response (37,38). On the contrary, *S. typhimurium* TA1538 and TA1538/pYG219 were used for detecting the HCAs mutagenicity in the chemical model since, specifically, frame-shift type mutation was detected. Since mutagenicity of some HCAs (IQ, MeIQ, MeIQx, PhIP) with S-9 mix has been reported to be the same in between *S. typhimurium* TA 1538 and TA98, the mutagenic activity using the chemical model was compared with the activity using S-9 mix (39).

The activity with the chemical model was smaller by two orders of magnitude than that with S-9 mix. However, IQ-type HCAs mutagenicity was higher than that of non-IQ type HCAs in both activating systems. Thus, the HCAs mutagenicity with 4-MPy/*t*-BuOOH was similar in tendency with that of S-9 mix.

## Discussion

The chemical model as an alternative for S-9 mix has been studied for application in the Ames assay. The $F_5P$/MPPT and 4-MPy/*t*-BuOOH activate promutagenic polyaromatic hydrocarbons (30,31), aromatic amines and amides (31,32), respectively, to be mutagenic in Salmonella strains. In this study, we developed the test conditions for detecting mutagenicity of Trp-P-2 in the Ames assay and then ten HCAs were evaluated their mutagenicity in *S. typhimurium*.

Application of the chemical model in the Ames assay has been reported by Salmeen *et al.* and Rueff *et al* (40,41). In their procedures, the extract of the reaction mixture was used for detecting the mutagenicity. On the contrary, we added a bacteria strain to the reaction mixture directly (35). The procedure we developed has the advantage of simplifying the handling. In the mutation procedure with water-soluble 4-MPy/*t*-BuOOH where the bacterial strain coexisted during an incubation period, the mutagenicity of 2-acetylaminofluorene was caused by *N*-hydroxyacetylamino compounds (32). Whereas in the procedure with water-insoluble $F_5P$/*t*-BuOOH where the bacterial culture was added to the reaction mixture after an incubation period, and the mutagenicity of 2-acetylaminofluorene was due to 2-nitro-9-fluorenone (42). The results showed that the preincubation method using the 4-MPy/*t*-BuOOH has a better chance to detect unstable intermediates.

As regards an apparent superiority of the oxidants for activation of mutagens, Rueff *et al.* reported that aromatic amines (2-aminofluorene, 2-acetylaminofluorene) and HCAs (IQ) became more mutagenic with alkylhydroperoxide than that with peroxy acid or PhIO (41). In this study, *t*-BuOOH plus Fe porphyrin activated HCAs. Polyaromatic hydrocarbons in the presence of Fe porphyrin are mutagenic with peroxy acid (30,31), while aromatic amines are mutagenic with *t*-BuOOH (31,32). The oxidant specificity for the activation of mutagens is the same as that reported by Rueff *et al* (41). It has been reported that a different active species was formed by alkylhydroperoxide and peroxy acid in the presence of Fe porphyrin (43). In general, peroxy acid or PhIO formed an oxoiron (IV) porphyrin $\pi$ cation radical, whereas alkylhydroperoxide generated alkoxyl radical by a Fenton type reaction. The specificity of the oxidant in a chemical model for activating mutagens might be due to the different active species in the high valent Fe-O complex formed.

The mutagenicity of HCAs activated with the chemical model was lower than that with the S-9 mix (38), probably because of a lack of phase II enzymes in the chemical models. MeA$\alpha$C and A$\alpha$C in the presence of 4-MPy/*t*-BuOOH were not mutagenic in *S. typhimurium* TA1538, however, they were mutagenic in *S. typhimurium* TA1538/pYG219, which overexpresses phase II enzyme *O*-acetyltransferase (36). These results suggest that the phase II enzyme is a key factor in the activation of HCAs. Since HCAs have almost the same mutagenic activity in *S. typhimurium* TA1538 and in *S. typhimurium* TA98 (39), *S. typhimurium* TA1538 was used in this study. The mutagenic potential with 4-MPy/*t*-BuOOH in *S. typhimurium* TA1538 was smaller by two orders of magnitude than that with S-9 mix in *S. typhimurium* TA98, however, the mutagenic strength on the basis of mutagen structure was similar in order in both the S-9 mix and the chemical model. 4-MPy/*t*-BuOOH apparently activated HCAs to mutagenic species in the present investigation.

In conclusion, the metalloporphyrin/oxidant system for cytochrome P450 as a biomimetic system for S-9 mix could be used for detecting promutagenic heterocyclic amines without using an enzymatic activating system in the primary screening assay for DNA damaging agents.

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