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Note

Isolation of Mutagenic β-Carboline Derivatives after Nitrite Treatment of Maillard Reaction Mixtures and Analysis of These Compounds from Foodstuffs and Human Urine

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Mixtures of carbohydrate decomposition products and L-tryptophan were incubated at pH 7.0 and 37 °C for 4 weeks. These mixtures exhibited mutagenic activity toward *S. typhimurium* TA 100 without metabolic activation after a nitrite treatment at pH 4.0. Four β-carboline derivatives were isolated as premutagens from mixtures of methylglyoxal and furfural. These premutagens were also found to be contained in daily foodstuffs and human urine samples.

Key words: β-carboline; Maillard reaction; nitrite; mutagenic activity

Several reaction mixtures of carbohydrates and amino acids have shown mutagenic activities toward certain bacteria and/or mammalian cells.[1,2] The browning reaction mixture consisting of L-ascorbic acid and L-tryptophan showed DNA-damaging and mutagenic effects on *S. typhimurium* TA 100 without metabolic activation. We have identified β-carboline derivatives from the reaction mixtures.[3] We have also reported that β-carboline derivatives, which were formed in the mixtures of 2-furaldehyde and L-tryptophan, showed mutagenic activity after a nitrite treatment.[4] Such other carbohydrate decomposition products as 3-deoxy-D-glucosone, furfural, methylglyoxal, and glyoxal are formed in the Maillard reaction. However, the formation of β-carboline derivatives in the reaction mixtures of these carbohydrate decomposition products with L-tryptophan has not been reported. The extent of human exposure to β-carboline derivatives remains undetermined.

We report in this paper the isolation of β-carboline derivatives as premutagens from the reaction mixtures of some carbohydrate decomposition products and L-tryptophan. Furthermore, we describe the detection of these premutagens in daily foodstuffs and urine samples.

A mixture of 0.1 mol of a carbohydrate (3-deoxy-D-glucosone, furfural, methylglyoxal or glyoxal) and 0.05 mol of L-tryptophan was dissolved in 500 ml of a 0.1 M phosphate buffer solution at pH 7.0 and kept at 37 °C for 4 weeks. This solution (100 ml) was then mixed with 200 ml of a 0.2 M acetate buffer solution at pH 4.0 and 100 ml of a 0.3 M sodium nitrite solution. The solution was kept at 37 °C for 1 hr, before 100 ml of a 0.3 M ammonium sulfate solution was added to stop the reaction. The resulting solution was lyophilized. The lyophilized concentrate was used as sample for submission to a mutagenic assay essentially as reported by Ames *et al.*[5] The numbers of revertant colonies given in figures are averages for three plates. 4NQO was used as a positive control (0.3 μg/plate).

Figure 1 shows the mutagenic activity (Net His^+ revertants/200 μg of a sample (the lyophilized Maillard reaction mixture after a nitrite treatment)/plate) toward *S. typhimurium* TA 100 without metabolic activation. Each sample of the carbohydrate decomposition products and L-tryptophan showed no mutagenic activity before the nitrite treatment. However, the mixtures of furfural, methylglyoxal and glyoxal after the nitrite treatment each showed strong mutagenic activity. In particular, the reaction mixture containing furfural exhibited the strongest mutagenic activity after the nitrite treatment.

We then determined the products after the reaction of methylglyoxal or furfural with L-tryptophan. The lyophilized samples were extracted with methanol, and the resulting methanol extracts were analyzed by HPLC, using an SPD-6AV UV detector (Shimadzu Co., Ltd., Kyoto, Japan), an L-7100 intelligent pump (Hitachi Ltd., Tokyo, Japan), and a L-7300 column oven (Hitachi Ltd., Tokyo Japan). The UV wavelength was 254 nm. Each sample was chromatographed in a YMC-Pack A-314 column (6.0 mm i.d. and 300 mm in length; YMC Co., Ltd., Kyoto, Japan) at 40 °C, the flow rate being 0.8 ml/min. The mobile phase was a mixture of methanol and

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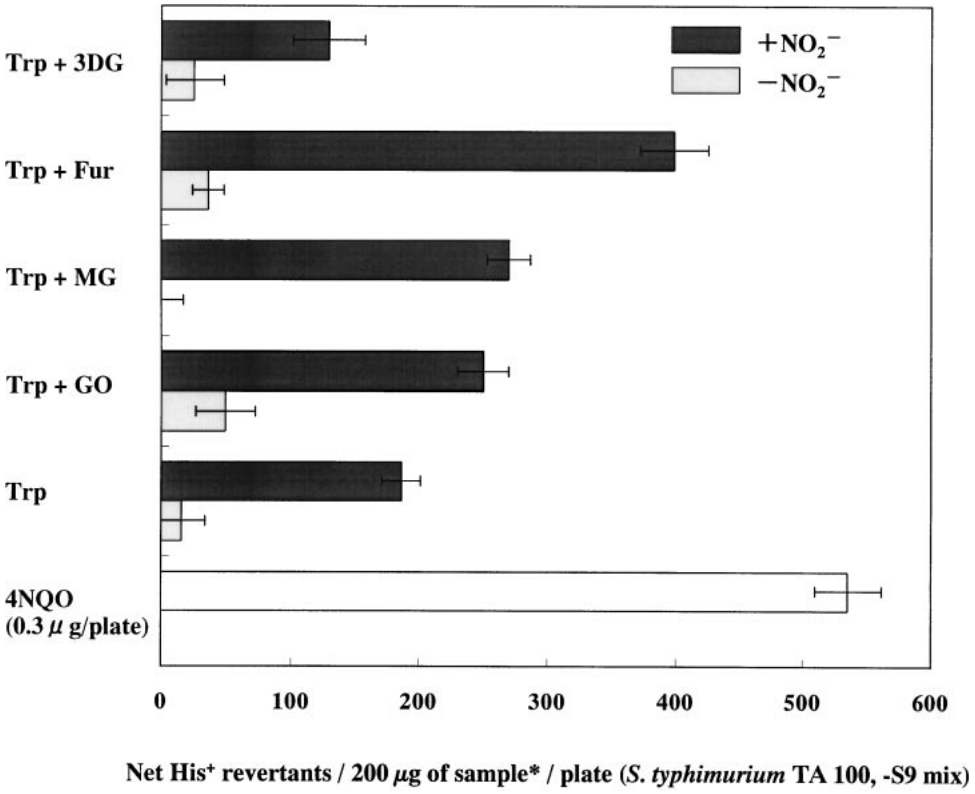


Fig. 1. Mutagenic Activities of Reaction Mixtures from the Carbonyl Compounds and L-Tryptophan.
3DG, 3-deoxy-D-glucosone; Fur, furfural; MG, methylglyoxal; GO, glyoxal; Trp, L-tryptophan; 4NQO, 4-nitroquinoline 1-oxide. *Each sample was the lyophilized Maillard reaction mixture after a nitrite treatment. 4NQO was used as a positive control.

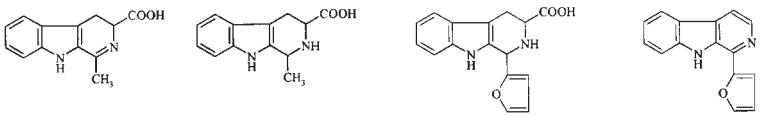
water (45/55, v/v), which was isocratically eluted. The HPLC analysis enabled us to isolate 3,4-dinitro-1-methyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid (I) and 1-methyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid (II) from the solution of methylglyoxal and L-tryptophan (I, 2.6 μg/200 μg of sample; II, 7.8 μg/200 μg of sample). 1,2,3,4-Tetrahydro-1-(5′-hydroxymethylfuryl)-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid (III) and 1,2,3,4-tetrahydro-1-furyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid (IV) were identified from the reaction mixture of furfural and L-tryptophan (III, 18.6 μg/200 μg of sample; IV, 8.7 μg/200 μg of sample). These β-carboline derivatives have been identified in some Maillard reaction mixtures, which had been respectively treated with L-ascorbic acid and L-tryptophan,[3] and 2-furaldehyde and L-tryptophan,[4] as well as in soy sauce.[6] We examined the mutagenicity of these products by a nitrite treatment under acidic conditions. They all showed high mutagenic activity (I, 3100; II, 12100; III, 28750; IV, 7700 Net His^+ revs./mg) after the nitrite treatment (Table 1). Compounds II and III exhibited equal mutagenicity to that of the reference compound. The nitrite treatment of these β-carboline derivatives might have produced the *N*-nitroso compounds. These results indicate that compounds I, II, III and IV were formed in the reaction mixtures of the carbohydrate decomposition products such as furfural or methylglyoxal and L-tryptophan, and that the mutagenic activities of these compounds were increased by the nitrite treatment under acidic conditions.

We next quantified the premutagens in three daily foodstuffs. We lyophilized 100 ml of soy sauce and 300 ml of beer and extracted the residue with methanol. Bean paste (50 g) was concentrated to dryness under reduced pressure and then applied to a Sephadex LH-20 column. Elution was carried out with methanol at a flow rate of 45 ml/hr, the eluate being separated into 3–4 fractions by measuring the optical density at 254 nm. The eluate corresponding to the retention time of each standard compound was fractionated and rechromatographed under the same conditions. Table 2 shows the content of premutagens in soy sauce, bean paste and beer. Compounds I, II and IV were respectively contained in amounts of 1724.1, 5268.9, 293.2 μg/100 ml in soy sauce. The respective contents of compounds II, III and IV were 253.8, 1226.0, 232.8 μg/100 g in bean paste, while the concentrations of these compounds in beer were lower than in two other foodstuffs.

We also investigated the contents of these premutagens in the urine of subjects taking a normal diet. The experimental procedure used in this study was approved by the Research Ethics Committee of University of Shizuoka. Urine samples were collected for 18 hr after consumption of the diet. Acetic acid was added to each sample to adjust to pH 3.0, before filtrating thorough an Amberlite XAD-2 column and washing the resin in

Table 1. Determination of Mutagenic β-Carbolines in Maillard Reaction Mixtures

Product	Maillard reaction mixture	Concentration (μg/200 μg of sample*)	Mutagenic activity toward TA 100, (Net His$^+$ revs./mg)	
			Untreated	Treated with nitrite
I	MG + Trp	2.6	0	3100
II	MG + Trp	7.8	0	12100 (10,400)[6]**
III	Fur + Trp	18.6	0	28750 (22,750)[4]**
IV	Fur + Trp	8.7	2400	7700



I II III IV

MG, methylglyoxal; Fur, furfural; Trp, L-tryptophan.
*, Samples were obtained by lyophilizing Maillard reaction mixtures after a nitrite treatment.
**, Values in parentheses were cited from references.
HPLC conditions: A UV wavelength of 254 nm was used. Each sample was chromatographed in a YMC-Pack A-314 column at 40 °C. The flow rate was 0.8 ml min. The mobile phase was a mixture of methanol and water (45/55, vv), which was eluted isocratically.

Table 2. Content of β-Carbolines in Daily Foodstuffs and Urine Samples

Sample		Contents			
		I	II	III	IV
Foodstuffs	Soy sauce	1724.1	5268.9	ND	293.2
	Bean paste	<0.1	253.8	1226.0	232.8
	Beer	52.1	12.2	<0.1	5.1
Urine	Subject A	ND	66.7	ND	6.8
	B	ND	328.9	ND	ND
	C	37.1	36.2	ND	4.5
	D	26.6	201.2	ND	3.6

Contents: Foodstuffs (μg/100 ml or 100 g) ND, not detected.
Urine (μg/l)

HPLC conditions: A UV wavelength of 254 nm was used. Each sample was chromatographed in a YMC-Pack A-314 column at 40 °C. The flow rate was 0.8 ml/min. The mobile phase was a mixture of methanol and water (45/55, v/v), which was eluted isocratically.

methanol. The methanol extract was evaporated under reduced pressure to give a sample for the HPLC analysis. Table 2 shows the contents of premutagens in the urine samples. Compound II was contained at the highest concentration (36.2–328.9 μg/l). Compounds I and IV were each contained at a low concentration, while compound III could not be detected in any sample.

Several β-carboline derivatives other than the premutagens isolated in our study have been found in tobacco tar,[7] sake,[8] alcoholic beverages,[9] fruits,[10] and cheese.[11] These β-carboline derivatives, such as norharman, harman, harmol and harmalol, exhibited mutagenic activity after a nitrite treatment.[12] We isolated four premutagens from the reaction mixtures of carbohydrate decomposition products of methylglyoxal and furfural with L-tryptophan kept under mild conditions (pH 7.0, 37 °C), and have demonstrated that these premutagens showed mutagenicity after a nitrite treatment. We also revealed that these products were present in daily foodstuffs and in human urine. These results indicate the possibility that the reaction might be developed in food-making processes and *in vivo*. Therefore, these premutagens would play an important role in the induction of gastric cancer in Japan. We need to identify the products from the reaction between the premutagens and nitrite, and further details will be reported in the near future.

References

1) Cuzzoni, M. T., Stoppini, G., Gazzani, G., and Mazza, P., Effect of storage on mutagenicity, absorbance and content of furfural in the ribose-lysine Maillard system. *Food Chem. Toxicol.*, **27**, 209–213 (1989).
2) Glosl, S., Wagner, K. H., Draxler, A., Kaniak, M., Lichtenecker, S., Sonnleitner, A., Somoza, V., Erbersdobler, H., and Elmadfa, I., Genotoxicity and mutagenicity of melanoidins isolated from a roasted glucose–glycine model in human lymphocyte cultures, intestinal Caco-2 cells and in *Salmonella typhimurium* strains TA98 and TA102 by applying the Ames test. *Food Chem. Toxicol.*, **42**, 1487–1495 (2004).
3) Kanamori, H., Morimoto, K., Kinae, N., and Tomita, I., Mutagenicity of the reaction products of L-ascorbic acid and L-tryptophan. *Yakugaku Zasshi* (in Japanese), **101**, 596–604 (1981).
4) Kanamori, H., Kinae, N., Saito, M., and Tomita, I., Isolation and identification of 1-furyl-β-carboline derivatives that are mutagenic after nitrite treatment. *Chem. Pharm. Bull.*, **32**, 1980–1986 (1984).
5) McCann, J., Spingarn, N. E., Kobori, J., and Ames, B. N., Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 979–983 (1975).
6) Wakabayashi, K., Ochiai, M., Saito, H., Tsuda, M., Suwa, Y., Nagao, M., and Sugimura, T., Presence of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, a precursor of mutagenic nitroso compounds, in soy

sauce. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 2912–2916 (1983).
7) Pfau, W., and Skog, K., Exposure to beta-carbolines norharman and harman. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **802**, 115–126 (2004).
8) Tomita, I., Kinae, N., Nakamura, Y., Takenaka, H., Kanamori, H., Hashizume, H., and Yokoyama, T., Mutagenicity of various Japanese foodstuffs treated with nitrite. II. Directly-acting mutagens produced from *N*-containing compounds in foodstuffs. *IARC Sci. Publ.*, **57**, 33–41 (1984).
9) Bosin, T. R., and Faull, K. F., Harman in alcoholic beverages: pharmacological and toxicological implications. *Alcohol Clin. Exp. Res.*, **12**, 679–682 (1988).
10) Kartal, M., Altun, M. L., and Kurucu, S., HPLC method for the analysis of harmol, harmalol, harmine and harmaline in the seeds of Peganum harmala L. *J. Pharm. Biomed. Anal.*, **31**, 263–269 (2003).
11) Herraiz, T., and Sanchez, F., Presence of tetrahydro-beta-carboline-3-carboxylic acids in foods by gas chromatography–mass spectrometry as their *N*-methoxycarbonyl methyl ester derivatives. *J. Chromatogr. A*, **765**, 265–277 (1997).
12) Lin, J. K., Wu, S. S., and Chen, J. T., Mutagenicities of nitrosated carboline derivatives. *Proc. Natl. Sci. Counc. Repub. China B*, **10**, 280–286 (1986).