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Isolation and Identification of 1-Furyl-β-carboline Derivatives That are Mutagenic after Nitrite Treatment

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When a mixture of 2-furaldehyde (I) and L-tryptophan dissolved in 0.1 M phosphate buffer (pH 7.0) was kept at 37 °C for 4 weeks, a browning reaction gradually occurred. The reaction mixture after treatment with nitrite at pH 4.0, was mutagenic to *S. typhimurium* TA100 in the absence of S9 mix. The browning solution was fractionated by applying high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), and two 1-furyl-β-carboline derivatives were isolated. They were compound A, (1*R*,3*S*)-1-(2-furyl)-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid, and compound B, (1*S*,3*S*)-1-(2-furyl)-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid. These compounds had similar mutagenic potency toward *S. typhimurium* TA100 without metabolic activation after treatment with nitrite.

Keywords——2-furaldehyde; L-tryptophan; browning reaction; nitrite; mutagenicity; 1-furyl-β-carboline

It is well known that several pyrolyzates of amino acids,[1] proteins,[2] and sugars[3] are mutagenic to certain bacteria and other microorganisms. Recently, it has been shown that some of them are carcinogenic to mammals.[4] The browning solutions obtained from the reaction of reducing sugars with amino acids or other nitrogenous compounds also exhibit mutagenic,[5] comutagenic,[6] and antimutagenic activities.[7]

In our previous paper,[8] we reported that the browning reaction solution containing equimolar amounts of L-ascorbic acid and L-tryptophan showed mutagenic activity on *Salmonella typhimurium* TA100 in the presence and the absence of S9 mix. Two mutagenic β-carboline derivatives, 1-(2-furyl)-9*H*-pyrido[3,4-*b*]indole and 1-(2-furyl)-9*H*-pyrido[3,4-*b*]-indole-3-carboxylic acid, were isolated from the reaction mixture. Furthermore, we observed an increase of their mutagenic potency after treatment with nitrite under acidic conditions.[9]

In this paper, we report the formation of two chemical substances from the reaction mixture of 2-furaldehyde (I) and L-tryptophan. They are new compounds, and both of them become mutagenic after treatment with nitrite. The isolation and identification of these compounds are described.

Experimental

Materials and Chemicals——2-Furaldehyde (I) was purchased from Tokyo Kasei Kogyo Co. (Tokyo) and before use, it was distilled under reduced pressure. L-Tryptophan was from Wako Pure Chemical Industries (Tokyo). All other solvents and chemicals used were of reagent-grade.

Apparatus and Operating Conditions——High-performance liquid chromatography (HPLC) was carried out at 25 °C using a Waters M-6000A type chromatograph equipped with a variable-wavelength ultraviolet (UV) detector and a stainless steel column (ϕ4 mm × 30 cm) packed with μBondapak C_{18}. Thin-layer chromatography (TLC) was performed on a silica-gel plate (Kieselgel 60 F-254, 2 mm, Merck) by developing with benzene–EtOH–H_2O mixture

(6:4:1) and the spots were detected under UV light (2537 Å).

Infrared (IR) and UV spectra were measured with spectrometers, JASCO A 202 and Hitachi 557, respectively. Mass spectra (MS) were recorded on a JEOL D-300 machine. Proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were measured with a JNM-PFT-100 spectrometer and chemical shifts are expressed in δ value using tetramethylsilane as an internal standard. All melting points are uncorrected.

Amino-carbonyl Reaction of I with L-Tryptophan——Compound I (0.3 mol) was dissolved in 500 ml of 0.1 M phosphate buffer (pH 7.0) containing L-tryptophan (0.15 mol), and the mixture was kept at 37 °C for 4 weeks in an incubator. An aliquot of the reaction mixture was taken out every week and tested for mutagenicity.

On the other hand, 0.3 mol of I or 0.15 mol of L-tryptophan was separately dissolved in 500 ml of the same buffer and incubated under the conditions mentioned above.

Treatment of the Reaction Products with Nitrite——The reaction mixtures or the reaction products were diluted or dissolved at appropriate concentrations using 0.3 M acetate buffer (pH 4.0). The test solution (0.4 ml) was mixed with 0.3 ml of $NaNO_2$ solution (containing 207 mg of $NaNO_2$ in 10 ml of 0.3 M acetate buffer, pH 4.0) and incubated at 37 °C for 60 min. Then, 0.3 ml of ammonium sulfamate solution (containing 343 mg of $H_2NSO_3NH_4$ in 10 ml of 0.3 M acetate buffer, pH 4.0) was added to the solution to stop the reaction with nitrite. After 10 min, 0.1 ml aliquots of the solutions obtained here were used for mutagenic assay.

Mutagenic Assay——A histidine-dependent strain of *S. typhimurium* TA100 (a base substitution mutant) was kindly provided by Dr. Bruce Ames of the University of California. The method was essentially that of Ames *et al.*[10] with some modifications.[11] The test samples were dissolved in 0.3 M acetate buffer (pH 4.0) at an appropriate concentration and incubated for 15 min after treatment with or without nitrite as described above. A test strain was cultured overnight in Difco broth medium. Aliquots of 100 μl each of the cell suspension and the sample solution, and 500 μl of 100 mM phosphate buffer (pH 7.4) in the absence of S9 were mixed and then incubated at 37 °C for 15 min. Then 2 ml of soft agar containing 0.7% Difco agar, 0.6% NaCl, 0.5 mM histidine and 0.5 mM biotin was added to the above mixture and the whole was poured on top of agar plates with Vogel Bonner essential minimum culture medium containing 2% D-glucose. The plates were incubated at 37 °C for 48 h and the numbers of his$^+$ revertant colonies were counted. 4-Nitroquinoline-1-oxide (4NQO) was used as a positive reference compound. The numbers of revertant colonies given in figures are the averages of three plates.

Isolation of Compounds A and B——A solution of 60 g (0.625 mol) of I and 20.4 g (0.1 mol) of L-tryptophan in 600 ml of distilled water (pH 5.6) was kept at 20 °C for 5 d in a dark room. The reaction mixture was fractionated to isolate compounds A and B as shown in Fig. 2.

The reaction mixture was filtered and the filtrate was extracted 3 times with 100 ml each of $CHCl_3$ to exclude unreacted I. The aqueous solution was lyophilized to obtain a brown powder (fraction 1, 13.1 g). The fraction was treated 3 times with 100 ml each of acetone and the acetone extract was evaporated under reduced pressure. The residue was subjected to TLC on a plate (Kieselgel 60 F-254) using benzene–EtOH–H_2O mixture (6:1:1). The zone of *Rf* 0.5 was taken from the plate and extracted 3 times with 100 ml each of acetone–H_2O mixture (50:4). The extract was evaporated *in vacuo* and then lyophilized. The yellow powder (fraction 2, 1.2 g) was subjected to HPLC on a μBondapak C_{18} column and eluted with MeOH–H_2O mixture (25:75). Two fractions which eluted at the retention times of 11.0 min and 17.0 min were evaporated to dryness to give crystalline products. The former was named compound A (380 mg, 0.47%) and the latter compound B (430 mg, 0.53%).

(1*R*,3*S*)-1-(2-Furyl)-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic Acid (Compound A)——Light yellow prisms, mp 109 °C, *Anal.* Calcd for $C_{16}H_{14}N_2O_3$: C, 68.07; H, 5.00; N, 9.92. Found: C, 68.12; H, 4.98; N, 9.84. IR ν_{max}^{KBr} cm^{-1}: 3430 (NH), 1630 (CO). UV λ_{max}^{MeOH} nm: 211, 226 (sh), 270, 280. ^{1}H-NMR (CD_3OD) δ: 3.04 (1H, d, J=16.12 Hz, 4-H), 3.47 (1H, d, J=16.6 Hz, 4-H), 4.02 (1H, d, J=12.6 Hz, 3-H), 6.54 (1H, q, J=2 Hz, 4′-H). ^{13}C-NMR: Table I. MS *m/z*: 282 (M^+).

(1*S*,3*S*)-1-(2-Furyl)-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic Acid (Compound B)——Light yellow prisms, mp 180 °C, *Anal.* Calcd for $C_{16}H_{14}N_2O_3$: C, 68.07; H, 5.00; N, 9.92. Found: C, 67.98; H, 5.04; N, 10.05. IR ν_{max}^{KBr} cm^{-1}: 3430 (NH), 1630 (CO). UV λ_{max}^{MeOH} nm: 211, 225 (sh), 270, 280. ^{1}H-NMR (CD_3OD) δ: 3.07 (1H, d, J=16.12 Hz, 4-H), 3.41 (1H, d, J=16.6 Hz, 4-H), 4.06 (1H, d, J=12.6 Hz, 3-H), 6.35 (1H, q, J=2 Hz, 4′-H). ^{13}C-NMR: Table I. MS *m/z*: 282 (M^+).

Results and Discussion

A mixture of I (0.3 mol) and L-tryptophan (0.15 mol) was dissolved in 0.1 M phosphate buffer (pH 7.0, 500 ml) and the solution was kept at 37 °C for 4 weeks. Every week, an aliquot of the reaction mixture was taken out and the absorbance was determined at 470 nm. Then, these browning solutions were tested for mutagenicity towards *S. typhimurium* TA100 in the absence of S9 mix with and without nitrite treatment. To confirm that the mutagenic effects

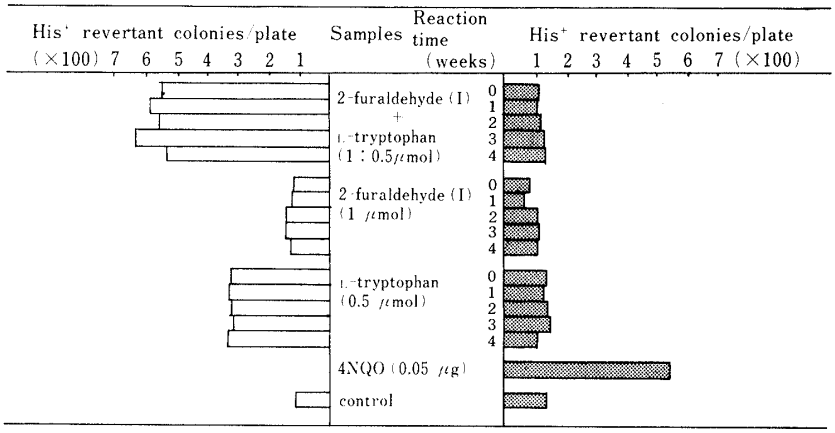


Fig. 1. Mutagenic Potency on *S. typhimurium* TA100 of the Reaction Mixture Obtained from 2-Furaldehyde (I) and L-Tryptophan

A mixture of I (0.3 mol) and L-tryptophan (0.15 mol) was dissolved in 500 ml of 0.1 M phosphate buffer (pH 7.0) and incubated at 37 °C for 4 weeks. Every week, 0.1 ml of the reaction mixture was subjected to the Ames test. Solutions containing I or L-tryptophan were also kept under similar conditions and were similarly tested. The revertant colonies of *S. typhimurium* TA100 strain are shown as the averages of three plates.
□, treated with nitrite; ▨, untreated.

were due to the amino-carbonyl reaction products, a solution containing I or L-tryptophan was separately kept under the same conditions at 37 °C. The time course of the mutagenic activity of the reaction mixture of I and L-tryptophan on *S. typhimurium* TA100 is shown in Fig. 1.

The mixture was not mutagenic even after prolonged reaction, but when the browning solution was treated with nitrite at pH 4.0, it induced his^+ revertant colonies (1.7 times as many as in the case of nitrite-treated L-tryptophan). This change was rapid, and no further change in mutagenicity occurred during 4 weeks. 2-Furaldehyde (I) was not mutagenic at up to 3 mg per plate and the solution kept at 37 °C for 4 weeks was also nonmutagenic. L-Tryptophan (0.5 μmol) treated with nitrite induced 315 revertant colonies per plate at the beginning of the reaction and this value did not change with reaction time. The mutagenic activity of nitrite-treated L-tryptophan has already been reported by Natake *et al.*[12] and our result is consistent with theirs.

We tried to isolate and identify the mutagen(s) from the reaction mixture after nitrite treatment. The fractionation procedure is shown in Fig. 2.

The untreated 2-furaldehyde in the reaction mixture was removed by extraction with chloroform, and the remaining solution was lyophilized to obtain a brown powder (fraction 1). The powder was extracted with acetone and the concentrate obtained from the extract was subjected to TLC on silica-gel with benzene–EtOH–H_2O mixture (6 : 4 : 1). The spot at *Rf* 0.50 was fluorescent, and the acetone extract showed mutagenic activity after nitrite treatment. The zone having *Rf* 0.50 was taken from the plate (20 × 20 cm) and extracted with acetone–H_2O mixture (50 : 4). The extract was evaporated under reduced pressure at 20 °C and then lyophilized to yield a yellow powder (fraction 2). This treatment was repeated until a single spot was observed on a TLC plate.

The dose–response curves of mutagenic activity for the reaction mixture, and fractions 1 and 2 after nitrite treatment are shown in Fig. 3.

The mutagenicity of fraction 2 treated with nitrite was about 2- and 6-fold higher than those of the fraction 1 and the reaction mixture, respectively, at the amount of 1 μg per plate. None of these fractions was mutagenic in the test system without nitrite treatment. The ^{13}C-NMR spectrum of fraction 2 suggested that this fraction contained two isomers A and B,

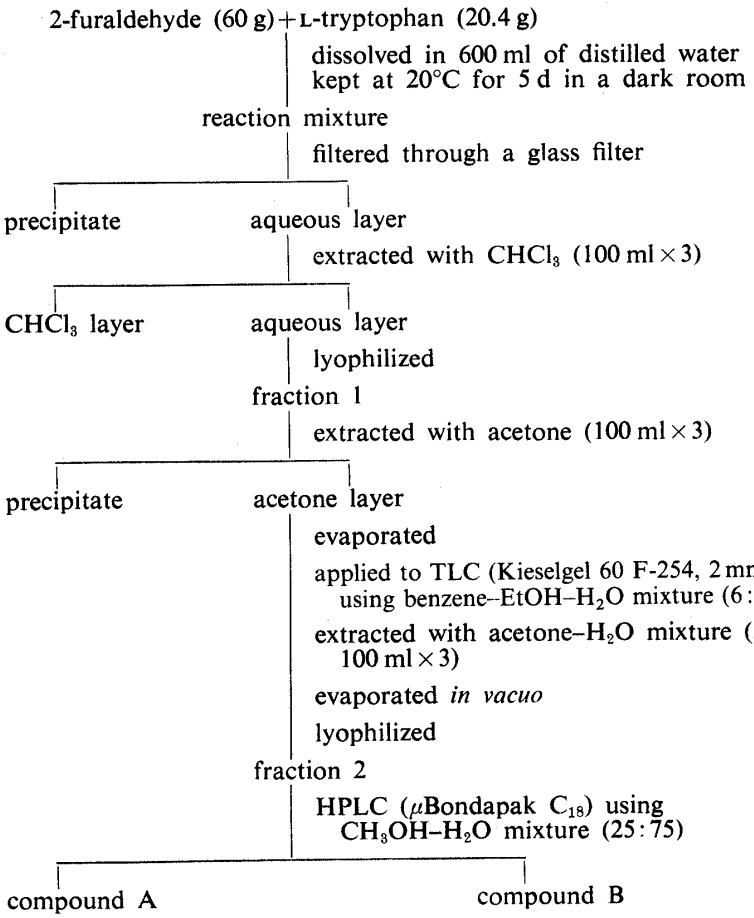


Fig. 2. Isolation of Compounds A and B from the Reaction Mixture of 2-Furaldehyde (I) and L-Tryptophan

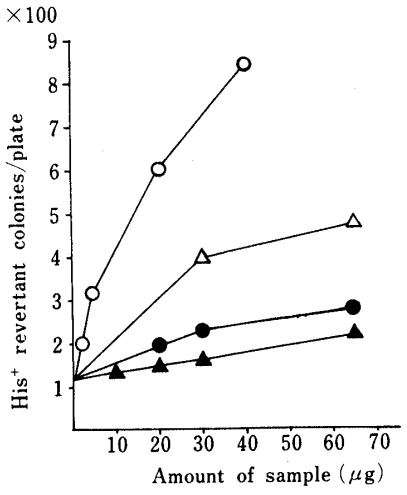


Fig. 3. Mutagenic Potency of the Reaction Products from 2-Furaldehyde (I) and L-Tryptophan after Nitrite Treatment

▲—▲, L-Tryptophan; △—△, fraction 1; ●—●, reaction mixture; ○—○, fraction 2.

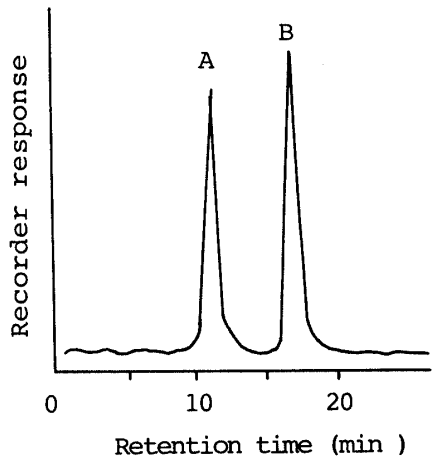


Fig. 4. HPLC Profile of Compounds A and B from the Reaction of 2-Furaldehyde (I) and L-Tryptophan

Conditions:
Column: μBondapak C_{18}. Column size: 4 mm i.d. × 30 cm. Mobile phase: CH_3OH–H_2O (25:75). Flow rate: 1 ml/min. Detector: 270 nm (range 0.16). Temperature: 25 °C.

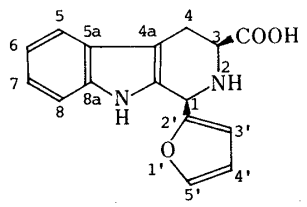
which could be separated by HPLC with a C_{18} reversed-phase column; the chromatogram is shown in Fig. 4. Compounds A and B were eluted at retention times of 11.0 and 17.0 min, respectively. These two peaks were detected from all browning solutions obtained from I and L-tryptophan kept at 37 °C for 0—4 weeks.

The lyophilysates of both compounds were yellowish crystals. The UV and IR spectra of these isomers were very similar and the molecular weights were both obtained as 282 (M^+) from the MS. However, the NMR spectra of A and B were somewhat different. In the ^{1}H-NMR spectra, these compounds showed signals due to 2 protons at C-4, 1 proton at C-3 and vinyl protons in the furan ring. By comparison of the signals at 52.6 and 59.9 (δ) in the ^{13}C-NMR spectrum of A with the data of Bobbit *et al.*,[13] A was assigned as a 1,3-*cis*(1*R*,3*S*) form. Therefore, the other compound B exhibiting signals at 50.2 and 55.3 (δ) was concluded to be a 1,3-*trans*(1*S*,3*S*) form, a stereoisomer of A. Furthermore, by comparing the signals of C-5 through C-8 in the ^{13}C-NMR spectra with those of 1-(2-furyl)-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid obtained from the reaction of L-ascorbic acid with L-tryptophan as shown in our previous paper,[8] we concluded that compound A is (1*R*,3*S*)-1-(2-furyl)-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid (Table I, Fig. 5).

TABLE I. ^{13}C-NMR Data for Compounds A and B

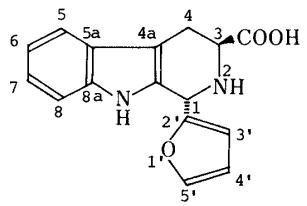
No. of position	δ (OFR)	
	A	B
1	52.6 (d)	50.2 (d)
3	59.6 (d)	55.3 (d)
4	24.5 (t)	24.4 (t)
3′	112.1 (d)	111.9 (d)
4′	112.3 (d)	112.3 (d)
8	113.2 (d)	111.3 (d)
6	119.1 (d)	119.2 (d)
5	120.4 (d)	120.4 (d)
7	123.4 (d)	123.5 (d)
4a	109.7 (s)	108.9 (s)
1a	127.5 (s)	127.3 (s)
5a	127.9 (s)	127.5 (s)
8a	138.5 (s)	138.4 (s)
2′	149.0 (s)	150.0 (s)
5′	145.6 (d)	145.6 (d)
–COO–	174.2 (s)	174.6 (s)

^{13}C-NMR spectra were measured in 10% CD_3OD solution at 22.5 MHz.
The symbols in parenthesis indicate the multiplicity (s, singlet; d, doublet; t, triplet).



(1*R*, 3*S*)-1,2,3,4-tetrahydro-1-furyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid

(A)



(1*S*, 3*S*)-1,2,3,4-tetrahydro-1-furyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid

(B)

Fig. 5. Structures of Compounds A and B

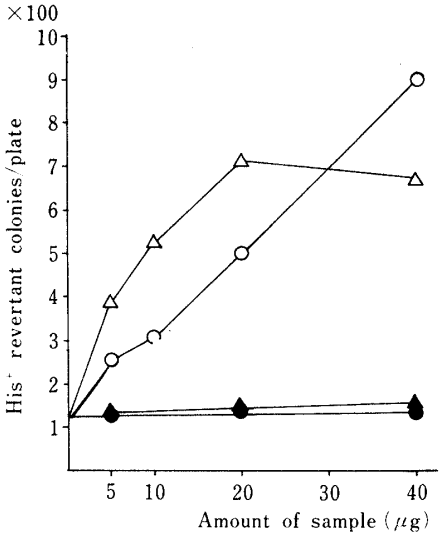


Fig. 6. Dose–Response Curves of Mutagenic Potency on *S. typhimurium* TA100 of Compounds A and B

○---○, compound A treated with nitrite; ●---●, untreated compound A; △---△, compound B treated with nitrite; ▲---▲, untreated compound B.

On the other hand, as the signals of C-1 and C-3 of compound B appeared at higher magnetic field than those of A, B was identified as (1*S*,3*S*)-1-(2-furyl)-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid.

Compounds A and B have not previously been reported, and they showed no mutagenic activities at up to 1 mg per plate. The dose–response curves of the mutagenic potency of these compounds after nitrite treatment are shown in Fig. 6.

The nitrite-treated compounds A and B were strongly mutagenic; the former gave 18.1 and the other 29.4 net his^+ revertant colonies per 1 μg per plate, respectively. Compound B, which has the *trans* relation between the carboxyl group and furan ring, showed higher mutagenic activity at the dose of 20 μg, but the activity leveled off with increasing dose of the sample.

Recently, Wakabayashi *et al.*[14] reported that two organic compounds were isolated from soy sauce after nitrite treatment. They were (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid and its stereoisomer, (1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid and were derived from acetaldehyde and L-tryptophan during preparation or storage of the seasoning.

The mutagenic precursors which we isolated and identified in this work appear to be formed by reaction between the amino group of L-tryptophan and the aldehyde group of 2-furaldehyde followed by cyclization. Treatment of these compounds with nitrite may result in nitrosation at the imino group of the β-carboline ring as described in the literature.[14] Foodstuffs containing L-tryptophan and pentose may also produce these two compounds on cooking and storage. We are currently investigating the presence of compounds A and B in soy sauce, other seasonings and some beverages. We also identified chemical substances from the reaction mixture of 5-hydroxymethyl-2-furaldehyde and L-tryptophan and found that they were mutagenic after nitrite treatment. These results will be published in the next paper.

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