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Synthesis and Mutagenic Activity of Alkyl Derivatives of 2-Amino-9*H*-pyrido[2,3-*b*]indole†

Takashi MATSUMOTO, Daisuke YOSHIDA and Hideo TOMITA

Central Research Institute, The Japan Tobacco & Salt Public Corporation, 6–2, Umegaoka, Midori-ku, Yokohama 227, Japan

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Two mutagenic compounds were isolated from the pyrolysate of soybean globulin: 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAC) and 2-amino-3-ethyl-9*H*-pyrido[2,3-*b*]indole (EtAC). These two and other 3-substituted derivatives were synthesized by the condensation of 2-aminoindole with enaminonitriles, and tested for their mutagenic activity. The bulkiness of the alkyl group at C-3 position adjacent to the amino group was related to the mutagenic strength. 2-Amino-3-butyl-9*H*-pyrido[2,3-*b*]indole and the bulkier alkyl derivatives were not mutagenic.

Two kinds of potent mutagenic principles toward *Salmonella typhimurium* TA98 and TA100 were isolated from the pyrolysates of protein and of tryptophan, and the structures were determined to be 2-amino-9*H*-pyrido-[2,3-*b*]indole (**1a**) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (**1b**) as shown in Fig. 1 by X-ray analysis.[1,2] The amounts of these compounds isolated were too small for the further study on several biological activities such as carcinogenicity in animal. Therefore, the synthesis of these compounds in large scale by a facile method was urgently desirable. We previously reported that a certain amino group was important for mutagenic activities of *N*-heterocyclic bases in the *Salmonella*/microsome test system.[3] The mutagenic activity of **1a** is higher than that of 3-methyl derivative (**1b**).[1] In connection with a research program on the structure-activity relationship of mutagenic compounds, the effect of the alkyl group adjacent to the amino group on the mutagenic activity should be investigated.

We previously reported on the synthesis of **1a**.[4] This paper deals with the synthesis and

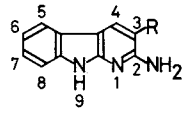

FIG. 1.

1a R=H 2-Amino-9*H*-pyrido[2,3-*b*]indole (AC)
1b R=CH_3 2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAC)
1c R=CH_2CH_3 2-Amino-3-ethyl-9*H*-pyrido[2,3-*b*]indole (EtAC)

the mutagenic activity of **1b** and the other 3-alkyl derivatives, and the identification of 2-amino-3-ethyl-9*H*-pyrido[2,3-*b*]indole (**1c**) in the pyrolysate of soybean globulin.

Synthesis of alkyl derivatives of 2-amino-9H-pyrido[2,3-b]indole

The synthesis of 3-alkyl derivatives (**1b**～**g**) of 2-amino-9*H*-pyrido[2,3-*b*]indole by the condensation of enaminonitriles (**5b**～**g**) with 2-aminoindole (**6**) was carried out as shown in Fig. 2. Five kinds of the alkyl derivatives (**2b**～**g**) of diethyl malonate were treated with a saturated solution of ammonia in methanol to give the amides (**3b**～**g**).[5] The mixture of the amides and phosphorus pentoxide placed in a flask was heated to dehydrate. After vaccume distillation, the nitriles (**4b**～**g**) were obtained.[6] The nitriles were reduced with lithium aluminum hydride to give the corresponding enaminonitriles (**5b**～**g**).[7,8] The re-

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Abbreviations: AC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; EtAC, 2-amino-3-ethyl-9*H*-pyrido[2,3-*b*]indole.

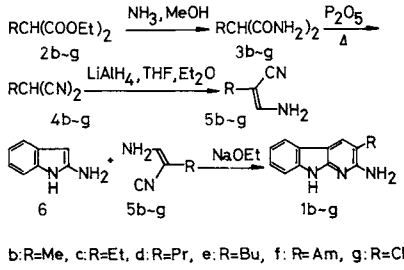

FIG. 2. Synthetic Route of MeAc (**1b**) and Other 3-Alkyl Derivatives (**1c**~**g**).

duction of *o*-nitrobenzylcyanide with tin and hydrochloric acid gave 2-aminoindole (**6**).[9] Six kinds of the 3-alkyl derivatives (**1b**~**g**) were prepared by the condensation of **5b**~**g** with **6** in sodium ethoxide solution.

The structures of the compounds prepared were confirmed by mass and NMR spectroscopy. Characteristic fragment peaks were observed in the mass spectra of **1b**~**g**. The peak at *m/z* 197 was parent one of **1b**. A high-intensity peak at *m/z* 196 was commonly found in the spectra of **1c**~**g**. This shows β-cleavage of the alkyl C–C bonds. In the ^{1}H-NMR spectrum of **1b**, the singlet at δ 2.21 was undoubtedly due to three protons of methyl group. Two single-proton doublets centered at δ 6.40 ($J_{3,4}$=8.3 Hz) attributed to C-3-H (pyridine β-proton) and δ 8.04 ($J_{3,4}$=8.3 Hz) attributed to C-4-H (pyridine γ-proton) in the spectrum of **1a** were not observed in that of **1b**. The single-proton singlet was observed at δ 7.90 corresponding to the latter chemical shift due to C-4-H. The signal for C-2-NH_2 (pyridine α-amino protons) of **1b** was observed as a singlet at δ 5.85. The *ind*-NH absorbed quite unexceptionally at δ 11.04. The signals of four protons on the benzene ring were observed from δ 7.00 to δ 7.84. Similarly, ^{1}H-NMR spectra of **1c**~**g** supported the presence of an ethyl, propyl, butyl, amyl, and benzyl group at C-3 position of pyridine moiety, respectively. The synthetic compounds **1b** and **1c** showed the same mass and NMR spectra as those of MeAC and EtAC obtained from globulin pyrolysate.

Mutagenic activity of alkyl derivatives of 2-amino-9H-pyrido[2,3-b]indole

Six kinds of the alkyl derivatives of **1a** were tested for their mutagenic activity by Ames test[10] and rec-assay.[11] **1b** and **1c** showed substantially the same mutagenic activity as those of MeAC and EtAC obtained from the pyrolysate of soybean globulin. Table I indicates the relationship between the bulkiness of the C-3 alkyl group and the strength of mutagenic activity of the 3-alkyl derivatives. Ames test and rec-assay gave the similar results. An increase in the number of carbons of the alkyl group was accompanied by a decrease in the mutagenic activity. The derivatives (**1e**~**g**) having the alkyl group composed of more than four carbons did not show any mutagenic activity.

These results suggested that the activation of the C-2 amino group by liver-homogenate

TABLE I. MUTAGENIC ACTIVITIES OF **1a**~**g**

Compound No.	R	TA98 revertants/10 nmol[a]	Lengths of inhibition zones (mm)/0.3 μmol[b]	
			H17 Rec$^+$	M45 Rec$^-$
1a	H	790	0	6.9
1b	CH_3	560	0	5.4
1c	C_2H_5	320	0	5.0
1d	C_3H_7	60	0	3.9
1e	C_4H_9	0	0	0
1f	C_5H_{11}	0	0	0
1g	CH_2Ph	0	0	0

[a] Tested by Ames test.
[b] Tested by rec-assay.

was hindered sterically by a substituent near by and/or so was the binding of DNA and the activated compound.

EXPERIMENTAL

All melting points and boiling points were uncorrected. NMR spectra were recorded at 100 MHz with TMS as an internal standard on JEOL JNM-FX100 spectrophotometer. IR spectra were recorded on JASCO IRA-3 spectrophotometer. Mass spectra were obtained at 70 eV using a Hitachi RM-50GC mass spectrometer. High performance liquid chromatography was carried out on a JASCO TRI ROTAR.

3-Amino-2-methylacrylonitrile (**5b**).[7] Diethyl methylmalonate (**2b**) (100 g) was dissolved in methanol (500 ml) and liquid ammonia (300 ml) was added. The mixture was allowed to stand in a stoppered flask at room temperature for a week. Deposited methylpropanediamide (**3b**) was collected by filtration in a yield of 66.4 g (99.7%), mp 210°C. A mixture of **3b** (66.4 g) and phosphorus pentoxide (120 g) was heated at 200°C. The reaction mass frothed and blackened. The product was distilled (15～30 mmHg) and sublimed. Methylpropanedinitrile (**4b**) was obtained in a yield of 32.0 g (70%). **4b** (6.9 g) dissolved in a mixture of ether (20 ml) and tetrahydrofuran (40 ml) was added in drops to a stirred solution of lithium aluminum hydride (5 g) in a mixture of ether (400 ml) and tetrahydrofuran (100 ml) at 20～25°C. After stirring for an additional 4 hr, water (10 ml) was added carefully. The organic layer was then washed thoroughly with 20% sodium hydroxide solution (10 ml) followed by water (30 ml). Filtration, dehydration (potassium carbonate), and fractional distillation gave **5b** in a yield of 1.05 g (15%), bp 65～68°C (1 mmHg). MS *m/z*: M^+ 82 (44%), 81 (44), 55 (42), 54 (83), 28 (100).

3-Amino-2-ethylacrylonitrile (**5c**).[8] Metallic sodium (11.5 g) cut into small pieces was added to absolute ethanol (250 ml). To the stirred solution of sodium ethoxide, diethyl malonate (82.5 g) was added and then ethylbromide (55 g) gradually added. After heating for an additional 2 hr under reflux, the sodium bromide was filtered and the major part of ethanol was then removed *in vacuo*. The residue was distilled *in vacuo* and diethyl ethylmalonate (**2c**) (78 g, 80.6% yield) was obtained, bp 100～102°C (16 mmHg). Compound **5c** was prepared using **2c** according to the procedures similar to those of **5b**. Ethylpropanediamide (**3c**) (49.6 g) obtained from **2c** (78 g) using a saturated solution of ammonia in methanol was dehydrated by phosphorus pentoxide to give ethylpropanedinitrile (**4c**) (27.9 g, 77.7% yield). The reduction of **4c** (8.1 g) with lithium aluminum hydride gave **5c** (2.2 g, 27.0% yield), bp 72°C (1 mmHg). MS *m/z*: M^+ 96 (17%), 81 (100), 54 (47), 28 (53).

Compounds **5d～g** were prepared similarly using propylbromide, butylbromide, amylbromide, and benzylbromide, respectively.

3-Amino-2-propylacrylonitrile (**5d**). Diethyl propylmalonate (85 g), bp 109～114°C (16 mmHg), obtained from diethyl malonate (82.5 g) and propylbromide (61.5 g) was converted into propylpropanediamide (**3d**) (58.7 g, 96.9% yield) using a saturated solution of ammonia in methanol. **3d** (58.7 g) was dehydrated by phosphorus pentoxide to give propylpropanedinitrile (**4d**) (30.9 g, 70.1% yield). The reduction of **4d** (9.3 g) with lithium aluminum hydride gave **5d** (2.9 g, 30.6% yield), bp 75°C (1 mmHg). MS *m/z*: M^+ 110 (15%), 81 (100), 54 (47), 28 (51).

3-Amino-2-butylacrylonitrile (**5e**). Diethyl butylmalonate (**2e**) (64 g), bp 121～129°C (17 mmHg), obtained from diethyl malonate (82.5 g) and butylbromide (68.5 g) was converted into butylpropanediamide (**3e**) (45.4 g, 97% yield) using a saturated solution of ammonia in methanol. **3e** (45.4 g) was dehydrated by phosphorus pentoxide to give butylpropanedinitrile (**4e**) (27.6 g, 78,7% yield). The reduction of **4e** (10.5 g) with lithium aluminum hydride gave **5e** (1.6 g, 15.0% yield), bp 95～102°C (1 mmHg). MS *m/z*: M^+ 124 (10%), 81 (100), 54 (37), 28 (40).

3-Amino-2-amylacrylonitrile (**5f**). Diethyl amylmalonate (**2f**) (75 g), bp 136～143°C (16 mmHg), obtained from diethyl malonate (82.5 g) and amylbromide (75.5 g) was converted into amylpropanediamide (**3f**) (54.6 g, 97.5% yield) using a saturated solution of ammonia in methanol. **3f** (54.6 g) was dehydrated by phosphorus pentoxide to give amylpropanedinitrile (**4f**) (36.6 g, 84.6% yield). The reduction of **4f** (11.7 g) with lithium aluminum hydride gave **5f** (6.4 g, 53.9% yield), bp 110～115°C (1 mmHg). MS *m/z*: M^+ 138 (6%), 81 (100), 54 (25), 28 (23).

3-Amino-2-benzylacrylonitrile (**5g**). Diethyl benzylmalonate (**2g**) (62 g), bp 123～130°C (0.1 mmHg) obtained from diethyl malonate (82.5 g) and benzylbromide (85.5 g) was converted into benzylpropanediamide (**3g**) (45.8 g, 96% yield) using a saturated solution of ammonia in methanol. **3g** (45.8 g) was dehydrated by phosphorus pentoxide to give benzylpropanedinitrile (**4g**) (13.4 g, 36.1% yield). The reduction of **4g** (13.4 g) with lithium aluminum hydride gave **5g** (2.6 g, 19.2% yield), bp 138～145°C (0.27 mmHg). MS *m/z*: M^+ 158 (96%), 130 (100), 91 (55), 81 (62), 51 (52), 28 (65).

2-Aminoindole (**6**). To a mixture of *o*-nitrobenzylcyanide (16 g), tin (20 g) and ethanol (200 ml) was slowly added hydrochloric acid (90 ml) with stirring at about 30°C for 3 hr. The solution was concentrated *in vacuo* to a volume of approximately 50 ml. After cooling with an ice bath, tin salt (28 g) was collected by filtration and dissolved in cold water (280 ml). After filtration, 30% sodium hydroxide solution (80 ml) was added to the yellow filtrate. The crystalline precipitate was filtered off, washed

with water and air-dried to give **6** (6.8 g, 52% yield), decomp. 197~200°C. MS *m/z*: M^+ 132 (100%), 131 (53), 104 (78), 77 (51), 51 (42). IR ν_{max}^{KBr}: 3200, 1665, 1590, 1460, 1385, 1350, 1300, 1265, 1190, 1175, 1045, 1000, 935, 740. ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 5.22 (2H, s), 5.23 (1H, s), 6.72 (1H, m), 7.04 (2H, m), 10.02 (1H, s).

2-Amino-3-methyl-9H-pyrido[2,3-b]indole (**1b**). To absolute ethanol (50 ml) was added sodium (2.3 g), **5b** (400 mg) and **6** (660 mg). The mixture was refluxed for 29 hr. After evaporation of ethanol, cold water (50 ml) was added to the reaction mixture and the basic product was extracted with ethyl acetate. The extracts were dried over anhydrous sodium sulfate. After evaporation *in vacuo*, the residue was purified by Sephadex LH-20 column chromatography using methanol, followed by high performance liquid chromatography (column: μBondapak C18, mobile phase: 70% methanol/water, flow rate: 1.5 ml/min, UV : λ=345 nm) to give **1b** as colorless needles (16.1 mg, 1.7% yield), mp 215~218°C. MS *m/z*: M^+ 197 (100%), 196 (60), 179 (17), 169 (24), 141 (17). ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 2.21 (3H, s, CH_3), 5.85 (2H, s, NH_2), 7.00~7.42 (3H, m), 7.81 (1H, d) } (C_6H_4), 7.90 (1H, s, C-4-H), 11.04 (1H, s, *ind*-NH).

Compounds **1c~g** were prepared similarly using **5c~g** respectively.

2-Amino-3-ethyl-9H-pyrido[2,3-b]indole (**1c**). To absolute ethanol (25 ml) was added sodium (1.2 g), **5c** (176 mg) and **6** (290 mg). The mixture was refluxed for 72 hr. After evaporation of ethanol, cold water (50 ml) was added to the reaction mixture and the basic product was extracted with ethyl acetate. After purification as mentioned above, **1c** was obtained as colorless needles (5.6 mg, 1.5% yield), mp 184~187°C. MS *m/z*: M^+ 211 (51%), 197 (17), 196 (100), 179 (13), 169 (12), 141 (11). ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 1.23 (3H, t, CH_3), 2.61 (2H, quartet, $C\underline{H}_2$–CH_3), 5.74 (2H, s, NH_2), 6.97~7.36 (3H, m), 7.80 (1H, d) } (C_6H_4), 7.90 (1H, s, C-4-H), 10.92 (1H, s, *ind*-NH).

2-Amino-3-propyl-9H-pyrido[2,3-b]indole (**1d**). To absolute ethanol (25 ml) was added sodium (1.2 g), **5d** (200 mg) and **6** (330 mg). The mixture was refluxed for 72 hr. After evaporation of ethanol, cold water (50 ml) was added to the reaction mixture and the basic product was extracted with ethyl acetate. After purification, **1d** was obtained as colorless needles (1.3 mg, 0.3% yield), mp 200~203°C. MS *m/z*: M^+ 225 (33%), 197 (20), 196 (100), 179 (16), 169 (11). ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 0.97 (3H, t, CH_3), 1.63 (2H, m, CH_2–$C\underline{H}_2$–CH_3), 2.55 (2H, t, $C\underline{H}_2$–CH_2–CH_3), 5.72 (2H, s, NH_2), 7.02~7.42 (3H, m), 7.84 (1H, d) } (C_6H_4), 7.93 (1H, s, C-4-H), 10.96 (1H, s, *ind*-NH).

2-Amino-3-butyl-9H-pyrido[2,3-b]indole (**1e**). To absolute ethanol (25 ml) was added sodium (1.2 g), **5e** (214 mg) and **6** (300 mg). The mixture was refluxed for 72 hr. After evaporation of ethanol, cold water (50 ml) was added to the reaction mixture and the basic product was extracted with ethyl acetate. After purification, **1e** was obtained as colorless needles (5.2 mg, 1.3% yield), mp 189~192°C. MS *m/z*: M^+ 239 (30%), 197 (21), 196 (100), 179 (12), 169 (10). ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 0.93 (3H, t, CH_3), 1.21~1.78 (4H, m, CH_2–$C\underline{H}_2$–$C\underline{H}_2$–CH_3), 2.58 (2H, t, $C\underline{H}_2$–CH_2–CH_2–CH_3), 5.77 (2H, s, NH_2), 7.03~7.48 (3H, m), 7.84 (1H, d) } (C_6H_4), 7.92 (1H, s, C-4-H), 11.04 (1H, s, *ind*-NH).

2-Amino-3-amyl-9H-pyrido[2,3-b]indole (**1f**). To absolute ethanol (25 ml) was added sodium (1.2 g), **5f** (320 mg) and **6** (300 mg). The mixture was refluxed for 72 hr. After evaporation of ethanol, cold water (50 ml) was added to the reaction mixture and the basic product was extracted with ethyl acetate. After purification, **1f** was obtained as colorless needles (6.7 mg, 1.2% yield), mp 183~185°C. MS *m/z*: M^+ 253 (26%), 197 (22), 196 (100), 179 (12), 169 (10). ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 0.88 (3H, t, CH_3), 1.32~1.59 (6H, m, CH_2–$C\underline{H}_2$–$C\underline{H}_2$–$C\underline{H}_2$–CH_3), 2.55 (2H, t, $C\underline{H}_2$–CH_2–CH_2–CH_2–CH_3), 5.70 (2H, s, NH_2), 7.02~7.42 (3H, m), 7.84 (1H, d) } (C_6H_4), 7.92 (1H, s, C-4-H), 10.95 (1H, s, *ind*-NH).

2-Amino-3-benzyl-9H-pyrido[2,3-b]indole (**1g**). To absolute ethanol (25 ml) was added sodium (1.2 g), **5g** (380 mg) and **6** (300 mg). The mixture was refluxed for 72 hr. After evaporation of ethanol, cold water (50 ml) was added to the reaction mixture and the basic product was extracted with ethyl acetate. After purification, **1g** was obtained as colorless needles (5.0 mg, 0.8% yield), mp 216~219°C. MS *m/z*: M^+ 273 (100%), 196 (27). ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 2.60 (2H, s, CH_2–Ph), 5.75 (2H, s, NH_2), 7.02~7.47 (8H, m), 7.80 (1H, d) } (C_6H_4 and C_6H_5), 7.93 (1H, s, C-4-H), 11.10 (1H, s, *ind*-NH).

2-Amino-9H-pyrido[2,3-b]indole (**1a**).[4] MS *m/z*: M^+ 183 (100%), 156 (61), 155 (50), 129 (32), 128 (30). ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 6.10 (2H, s, NH_2), 6.40 (1H, d, J=8.3 Hz, C-3-H), 7.00~7.46 (3H, m), 7.82 (1H, d) } (C_6H_4), 8.04 (1H, d, J=8.3 Hz, C-4-H), 11.18 (1H, s, *ind*-NH).

Isolation of 2-amino-3-ethyl-9H-pyrido[2,3-b]indole in pyrolysate of soybean globulin. Soybean globulin (1 kg) was placed in a Pyrex glass flask and subjected to strong heat of a gas stove. The volatile pyrolysis product was collected in a cold trap. Approximately 400 g of tar was obtained and separated into acidic, basic and neutral fractions according to the conventional liquid-liquid partition method, and 40.3 g of the basic fraction was obtained. A 1 g aliquot of the basic fraction was chromato-

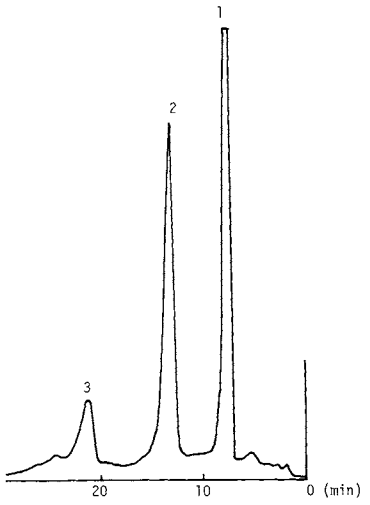

FIG. 3. Separation of the Mutagenic Compounds by HPLC.

Column, μBondapak C18; mobile phase, 50%methanol/water; flow rate, 1.5 ml/min; UV, $\lambda = 345$ nm; 1, AC; 2, MeAC; 3, EtAC.

graphed on a silica gel column (2 × 15 cm) using benzene, 10% ethyl acetate/benzene, 30% ethyl acetate/benzene, 50% ethyl acetate/benzene, and ethyl acetate as solvents successively. The eluate with 30%~50% ethyl acetate/benzene was subjected to chromatography on Sephadex-LH20 column (4 × 40 cm) using methanol as solvent. Further purification by high performance liquid chromatography (Fig. 3) gave purified mutagenic compounds **1** (1.0 mg), **2** (2.1 mg) and **3** (1.3 mg). The mass and NMR spectra of **1** and **2** were identical with those of AC[4] and MeAC,[1] respectively. **1**, MS *m/z*: M^+ 183 (100%), 156 (46), 155 (38), 129 (20), 128 (20); ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 6.10 (2H, s), 6.40 (1H, d, $J = 8.3$ Hz), 7.00~7.46 (3H, m), 7.82 (1H, d), 8.04 (1H, d, $J = 8.3$ Hz), 11.18 (1H, s). **2**, MS *m/z*: M^+ 197 (100%), 196 (50), 169 (32), 141 (32); ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 2.21 (3H, s), 5.85 (2H, s), 7.00~7.42 (3H, m), 7.81 (1H, d), 7.90 (1H, s), 11.04 (1H, s). The structure of **3** was elucidated as 2-amino-3-ethyl-9*H*-pyrido[2,3-*b*]indole (EtAC) by the mass and NMR spectra. **3**, MS *m/z*: M^+ 211 (82%), 196 (100); ^{1}H-NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 1.23 (3H, t), 2.61 (2H, quartet), 5.74 (2H, s), 6.97~7.36 (3H, m), 7.80 (1H, d), 7.90 (1H, s), 10.92 (1H, s).

Ames test. To a test tube containing 2 ml of molten top agar (0.6% agar, 0.6% NaCl, 0.05 mM L-histidine, 0.05 mM biotin) were added: 0.15 ml DMSO solution of a test compound, 0.1 ml of an overnight nutrient broth culture of *Salmonella typhimurium* TA98 and 0.5 ml of a solution (S-9Mix) that contained 0.02 ml of rat liver microsomal fraction (S-9), 4 μmol $MgCl_2$, 20 μmol KCl, 2.24 μmol glucose-6-phosphate, 1.74 μmol NADPH, and 50 μmol sodium phosphate buffer (pH 7.4). The ingredients of the tube were mixed and poured onto a plate containing 1.5% agar, 2% glucose and minimal inorganic salts. After incubation at 37°C for 48 hr, histidine-revertant colonies were scored. The results are expressed as the average of replicate plates from which the number of spontaneous revertants (30) were subtracted. S-9 was prepared from rats that had been injected preliminarily with PCB. The strongest mutagenic activity of each compound (10 nmol) was attainable with 0.5 ml of S-9 Mix containing 0.02 ml of S-9.

Rec-assay. To an empty plate were added 0.1 ml of suspension containing 2×10^6 spores of the strain H17 Rec^+ or M45 Rec^-, 0.2 ml of S-9, and then 10 ml of the medium (kept at 40°C) containing 0.8% agar, 0.8% nutrient broth, and 0.5% NaCl. After mixing and caking, on the surface of a spore agar plate was subsequently placed a paper disk (8 mm in diameter, 1 mm in thickness) impregnated with 0.01 ml of DMSO solution of a test compound and 0.04 ml of the cofactor solution containing 0.32 μmol $MgCl_2$, 1.36 μmol KCl, 0.18 μmol glucose-6-phosphate, 0.14 μmol NADPH, and 4 μmol sodium phosphate buffer (pH 7.4). After incubation at 37°C for 24 hr, lengths of inhibition zones were measured and compared between the strains H17 Rec^+ and M45 Rec^-.

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