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MUTAGENICITY OF AMINO-α-CARBOLINES IN PYROLYSIS PRODUCTS OF SOYBEAN GLOBULIN

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SUMMARY : 2-amino-9H-pyrido[2,3-b]indole and 2-amino-3-methyl-9H-pyrido [2,3-b]indole (amino-α-carbolines), potent mutagens toward Salmonella typhimurium TA 98 and TA 100, have been isolated from the pyrolysis products of soybean globulin and the structures have been established by X-ray analysis. 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (amino-γ-carbolines) identified as the mutagenic principles in tryptophan pyrolyzate were not detected in the pyrolysis products of soybean globulin.

INTRODUCTION : We previously demonstrated that the pyrolyzate of tryptophan exhibits high mutagenic activity toward Salmonella typhimurium TA 98 (TA 98) as compared with those of the other amino acids(1). The mutagenic principles in the pyrolyzate of tryptophan were identified as 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (I) and 3-amino-1-methyl-5H-pyrido[4,3-b] indole (II) by Sugimura et al.(2). The pyrolyzates of proteins also showed mutagenic activity toward TA 98(3,4). However, mutagenic principles with high activity have not been known in the pyrolyzate of proteins yet(5). We now report on the absence of I and II, and the isolation, identification and the mutagenic activities of two novel compounds in the pyrolyzate of a protein. It has been reported that the charred material produced on the surface of broiled fish and meat showed high mutagenic potential and the mutagenic principles were formed by pyrolysis of proteins(4). Cigarette smoke condensate showed mutagenic activity toward TA 98 which was also correlated positively with the content of proteins in the tobacco shreds(6,7). The identification of the mutagenic principles appeared to be

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important for the development of safer cooking method as well as safer cigarettes.

MATERIALS AND METHODS

3 Kg of soybean globulin was heated in a Pyrex glass flask over a gas burner and the fume generated was collected in a cold trap. Approximately 450 g of tar was obtained and was separated into acidic, basic and neutral fractions according to the conventional liquid-liquid partition method. The basic fraction was subjected to the chromatography on a silica gel column(5 x 80 cm) using benzene, ethyl acetate, 30 % methanol in ethyl acetate and methanol as solvents successively. The eluate with 30 % methanol in ethyl acetate and the one with ethyl acetate were subjected to chromatography respectively on a CM-Sephadex C-25 column (3 x 15 cm) using the mixture of methanol and 2N acetic acid (3 : 1, v/v) as solvent. Mutagenic fractions obtained were further separated by gel filtration on a Sephadex LH-20 column (2 x 40 cm) using methanol as solvent. Aliquots of each fraction were spotted and aminocarbolines were separated by thin layer chromatography on Woelm silica gel plates (20 x 20 cm) with a mixture of chloroform and methanol (8 : 2, v/v) as the developing solvent.

Soybean globulin was purchased from Wako Pure Chemical Co. Reference compounds, I and II, were kindly provided by Professor T.Sugimura.

Mutagenic activities of isolated compounds were assayed according to the method of Ames et al.(8,9). The histidine-requiring strains of Salmonella typhimurium TA 98 and TA 100 (TA 100) were kindly supplied by Dr.B.N.Ames. Mixture of the tester strain, properly diluted sample and S-9 Mix were poured onto an agar over layer. After incubation at 37°C for 48 hours, histidine revertant colonies were counted. Liver microsomal fraction (S-9) was prepared from rats which had been injected with PCB as described by Ames et al.(9).

RESULTS AND DISCUSSION

The basic fraction of the pyrolyzate of soybean globulin was subjected to silica gel column chromatography. The eluate with 30 % methanol in ethyl acetate was purified by the chromatography on CM-Sephadex C-25 and followingly Sephadex LH-20 columns, since I and II should be contained in this fraction according to the isolation procedure of tryptophan pyrolyzate. From each subfraction, an aliquot was spotted and developed on the plate of thin layer chromatography to separate I and II. The spots corresponding to the reference compounds were scraped off under U.V.light, and the fluorescence spectra of the respective methanol extracts were measured. Neither I nor II could be detected in the pyrolyzate of soybean globulin, though tryptophan is a constituent of soybean globulin(10). It seemed most probable that I and II were not formed from the tryptophan moiety of the protein during the heat treatment in this experiment.

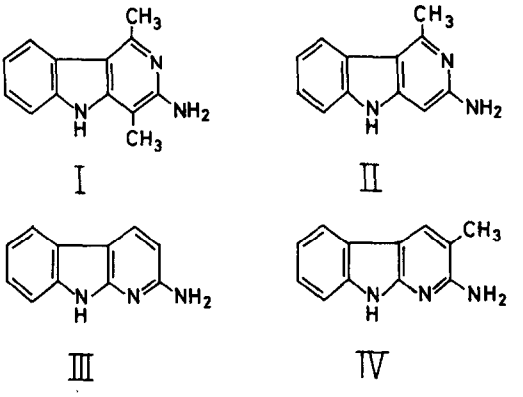

Fig. 1, Structures of I, II, III, and IV

The eluate with ethyl acetate on silica gel column chromatography was also purified similarly to the above mentioned eluate. Neither I nor II was, of course, detected but a fraction showing high mutagenic activity and fluorescence under U.V.light was obtained. The fraction from which crystalline material was obtained and used for X-ray analysis, was further separated into two subfractions having intense fluorescence by rechromatography with Sephadex LH-20 column using methanol as solvent. From two subfractions, 2.1 and 4.8 mg of crystalline materials (mp 186-189°C and 211-214°C) with high mutagenic activity were obtained respectively. The molecular formulas of two compounds were determined to be $C_{11}H_9N_3$ and $C_{12}H_{11}N_3$ respectively by a high resolution mass sepectrometry (183.0808; Calcd. 183.0797, and 197.0962; Calcd. 197.0912). The molecular structures were determined by X-ray analysis of the sample before the separation by rechromatography with Sephadex LH-20 column.

A crystal of the size of 0.08 x 0.08 x 0.25 mm was obtained by recrystallization in benzene and n-hexane solution and used for the experiment. The space group of the crystal is monoclinic $p2_1/a$ with 3 molecules in an asymetric unit. The cell dimentions are a = 26.351, b = 19.166, c = 5.579 Å, and β = 91.70°. The reflexion data were measured on an automated diffractometer by using Cu Kα radiation. The structure

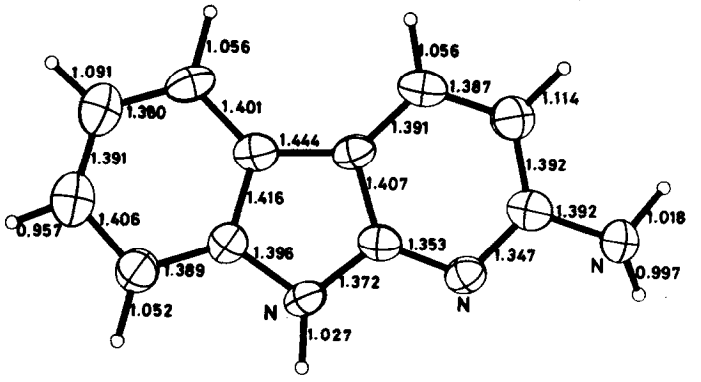

Fig. 2. Molecular structure of III

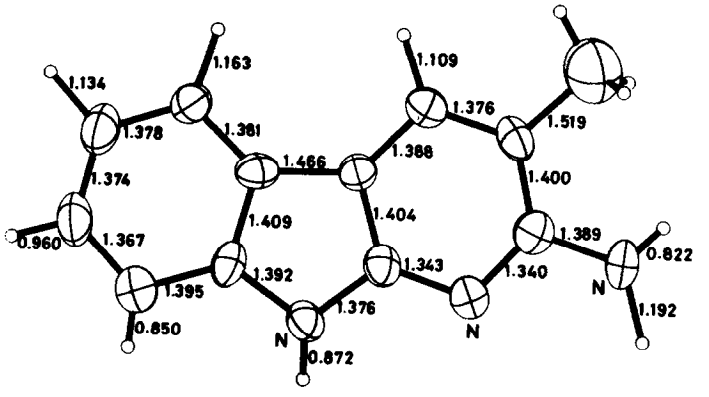

Fig. 3. Molecular structure of IV

C-H distances in the methyl group were assumed to be 1.00 Å.

was solved by the direct method and refined by the block-matrix least-squares method. The final R index for 1470 unique reflections was 0.058, assuming the anisotropic themal parameters for non-hydrogen atoms and the isotropic ones for hydrogens. The asymmetric unit contains two independent 2-amino-9H-pyrido[2,3-b]indole (III) molecules and one 2-amino-3-methyl-9H-pyrido[2,3-b]indole (IV). The structures of III and IV are shown in Fig. 2 and 3 with bond distances. The distances in Fig.2 are the mean values of the two crystallographically independent molecules. The molecules are nearly planer. The maximum deviation of the non-hydrogen atoms from the least-squares plane is 0.14 Å.

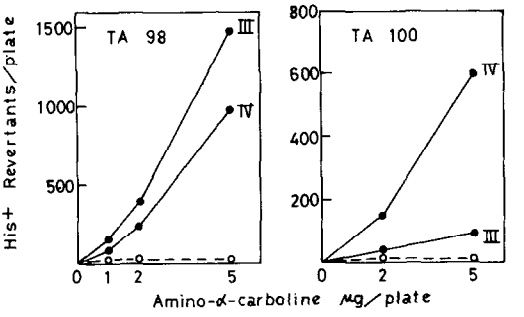

Fig. 4. Mutagenic activities of III and IV.
III and IV were tested for mutagenic activities using TA 98 and TA 100 in the presence (●) or absence (○) of S-9 Mix. Spontaneous revertant colonies have been subtracted (TA 98 ; +S-9 Mix, 42, - S-9 Mix,38, TA 100 ; +S-9 Mix,200, - S-9 Mix, 170).

Mutagenic activities of III and IV are shown in Fig.4. These compounds required a liver microsomal fraction, as representative of manmalian metabolism, to be detected as mutagens. Revertant numbers per μg of III and IV were higher than that of benzo(a)pyrene but lower than those of I and II(2). This is the first report on the mutagenic principles in the pyrolyzate of protein. The facts that tryptophan produces amino-γ-carbolines such as I and II, whereas soybean globulin produces amino-α-carbolines such as III and IV by pyrolysis seemed to be important for the study of mutagenic principles in the roasted food and tobacco smoke condensate. The content of free tryptophan in the food or tobacco is so small (11) that the formation of amino-γ-carbolines by roasting or smoking appeared to be negligible. However, any natural product including tobacco contains proteins to some extent, so it may not be strange if amino-α-carbolines are detected in such materials after heat treatment. Data on the extremely low content of I and II and the presence of III and IV in the cigarette smoke condensate will be published elsewhere. Organic synthesis of III and IV are in progress.

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