55. Potent Novel Mutagens Produced by Broiling Fish under Normal Conditions

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Introduction. Mutagenicity has been demonstrated in smoke condensates from broiling fish and beefsteak and in the charred surface of fish and beefsteak (Nagao *et al.*, 1977; Sugimura *et al.*, 1977; Rappaport *et al.*, 1979). Further studies showed that pyrolysis products of proteins and amino acids contained strong mutagens (Sugimura and Nagao, 1979a; Matsumoto *et al.*, 1977), and active compounds were isolated from pyrolysates of various amino acids (Sugimura *et al.*, 1977; Yamamoto *et al.*, 1978; Wakabayashi *et al.*, 1978; Kosuge *et al.*, 1978) and proteins (Kosuge *et al.*, 1978; Yoshida *et al.*, 1978). Among these compounds, 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) from tryptophan (Sugimura *et al.*, 1977), and 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-2) from glutamic acid (Yamamoto *et al.*, 1978) are very strong mutagens, especially towards *Salmonella typhimurium* TA98 with metabolic activation. The *in vitro* (Takayama *et al.*, 1977; 1978) and *in vivo* (Ishikawa *et al.*, 1979; unpublished data of our laboratory) carcinogenicities of Trp-P-1 and Trp-P-2 were already demonstrated.

For evaluation of the effects of mutagens in cooked foods on humans, it is important to determine the properties of mutagenic substances that are actually present in foods. Mutagenicity has been demonstrated in fried hamburgers (Commoner *et al.*, 1978; Pariza *et al.*, 1979; Spingarn and Weisburger, 1979) and fried potatoes (Spin-

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garn *et al.*, 1980b), but the compounds responsible for this activity have not yet been isolated and identified. We previously detected Trp-P-1 and Trp-P-2 in broiled sardine, but these compounds accounted for only 1.5% of the total mutagenicity present (Yamaizumi *et al.*, 1980). It strongly suggests that most of the mutagenicity in broiled sardine is due to compounds other than the series of newly described heterocyclic amines, and in fact we found that the basic fraction of broiled sardine contained an unidentified mutagenic substances (Kasai *et al.*, 1979).

This communication describes two new potent mutagens isolated from the neutral fraction of broiled sardines. They are novel mutagens with a six-six-five membered ring system and their structures are 2-amino-3-methylimidazo[4,5-*d*]quinoline (IQ) and 2-amino-3,5-dimethylimidazo[4,5-*d*]quinoline (methyl-IQ). This is a first report of elucidation of the structures of new mutagens isolated from ordinarily cooked food.

Isolation of pure mutagens from the neutral fraction of broiled sardines. A sample of 3 kg of sun-dried sardines (named "Maruboshi" in Japanese) were broiled in the ordinary way as described previously (Kasai *et al.*, 1979). Then they were ground up and extracted with methanol. The extract was evaporated and the residue, which showed strong mutagenicity to *S. typhimurium* TA98 in the presence of S9 mix, was further fractionated as described previously (Kasai *et al.*, 1979). The neutral fraction thus obtained, which contained 40% of the original mutagenicity, was subjected successively, to Diaion HP-20 column chromatography, chloroform-methanol-water partitioning, and Sephadex LH-20 column chromatography. The active fraction was then chromatographed on a column of silica gel with 10% methanol in chloroform as solvent. The mutagens were roughly separated into two components (compound 1 and compound 2) by this procedure. Final purification of the two components was achieved by successive application of high pressure liquid chromatographies as described in Fig. 1. Approximately 0.06 mg of each purified compound was obtained from 3 kg of sardines. Actually 200 μ g of each material, obtained from 10 kg of sardines, was used for structural studies. Table I shows the increase in specific activity at each step of purification. The final purified samples of compounds 1 and 2 showed high mutagenic activities to TA98 strain of *Salmonella* of 8,000 and 18,500 revertants/ μ g, respectively.

Elucidation of the structures of the mutagens. The structure of compound 1 was proposed as **I** based mainly on its 270 MHz ^1H -NMR spectrum, and low- and high-resolution mass spectra. The mass spectrum of compound 1 showed a high intensity molecular ion (M^+)

Fig. 1. Purification of the mutagens by high pressure liquid chromatography (HPLC). (a) The mutagenic fractions eluted later (Fr. II, 1.6 mg) from silica gel were dissolved in 0.2 ml of 40% methanol and injected. Conditions for HPLC were as follows: Instrument, Shimazu LC-3A; column μ Bondapak C₁₈ (Waters 7.9 $\times$ 300 mm); flow rate, 2.0 ml/min; eluent, 40%–70% methanol, linear gradient. The profiles of A₂₅₄ (upper figure) and mutagenic activity (revertants/plate/min, lower figure) are shown. The second minor peak of activity was that of compound 2. (b) The mutagenic fraction obtained by HPLC-a (fraction between dotted lines, 0.13 mg) was dissolved in 0.2 ml of 60% methanol and injected. Column, μ Bondapak C₁₈ (Waters 4 $\times$ 300 mm, two columns connected in series); flow rate, 1.0 ml/min; eluent, 60% methanol. (c) The mutagenic fractions eluted earlier (Fr. I, 3 mg) from silica gel were injected. Conditions were as for (a). (d) The mutagenic fraction (0.18 mg) obtained by HPLC-c was injected. Conditions were as for (b) except that the solvent system was 40% methanol, 0.04% formic acid.

Table I. Purification of compounds 1 and 2 from broiled sardines

Purification steps	Weight (mg)	Specific activity (revertants/mp)	Total activity (revertants $\times 10^{-4}$)
MeOH extract	817,000	289	23,600
Et ₂ O-H ₂ O partition	537,000	178	9,560
HP-20	54,000	974	5,260
CHCl ₃ -MeOH-H ₂ O partition	16,600	3,520	5,840
LH-20	443	84,400	3,740
LH-20 (rechromato.)	89.4	243,000	2,170
Silica Gel-Fr. I	3.0	622,000	187
HPLC-c	0.18	9,720,000	175
HPLC-d, compound 2	0.06	18,500,000	103
Silica Gel-Fr. II	1.6	549,000	88
HPLC-a	0.13	4,920,000	64
HPLC-b, compound 1	0.06	8,000,000	50

at m/e 198 and fragment ion at m/e 183 (M^+-15), suggesting that compound 1 has a stable heterocyclic nucleus and methyl group. The elemental composition of compound 1 was determined to be $C_{11}H_{10}N_4$ by exact mass measurement (calculated mass, 198.0906; observed mass, 198.0900). The 270 MHz 1H -NMR spectrum of compound 1 measured in $CDCl_3$ showed five aromatic protons, methyl protons and broad amino protons. The chemical shifts (ppm) and coupling constants (Hz) are shown in Fig. 2. Three protons in the aromatic region

Fig. 2. 270 MHz 1H -NMR data for compound 1 in $CDCl_3$. Chemical shifts (ppm) and coupling constants (Hz) were shown by arrows.

(8.87, 8.70 and 7.46 ppm) were assigned to three protons, H-7, H-9 and H-8, respectively, based on their chemical shifts and coupling constants. Another set of protons at 7.88 and 7.57 ppm, which were coupled to each other ($J=9.2$ Hz), can be assigned to H-4 and H-5. Long-range coupling ($J=1$ Hz) was observed between H-5 and H-9. A methyl signal found at a considerably low field (3.70 ppm) can be assigned to the N-methyl group. NOE was observed only for H-4 (13%) when methyl protons were irradiated. The broad signal at 5.52 ppm was assigned to protons of the amino group because this signal disappeared by the saturation transfer from the proton resonance of water in sample solution. The presence of a free amino group was confirmed by the mass spectral analysis of the trimethylsilyl deri-

vative (M^+ : 270).

The structure of compound 2 was proposed as **II** by comparing the 270 MHz ^1H -NMR and high resolution mass spectrum data for compound 2 with those for compound 1. The mass-spectrum of compound 2 shows a molecular ion at m/e 212 and fragment ion at m/e 197 ($M^+ - 15$). Exact mass measurement of compound 2 gave the elemental composition $\text{C}_{12}\text{H}_{12}\text{N}_4$ (calculated mass, 212.1063; observed mass, 212.1046). From these results it was concluded that an extra methyl group is present in compound 2. The presence of an extra methyl group in compound 2 is also supported by the fact that the UV spectra of compound 1 and compound 2 are similar except that the λ_{max} of the former shows a blue shift of 2 nm relative to that of the latter (λ_{max} of compound 1 in methanol, 264 nm; λ_{max} of compound 2 in methanol, 266 nm). The location of the extra methyl group was determined by comparing the ^1H -NMR spectrum of compound 2 with that of compound 1 (Fig. 3). In the spectrum of compound 2, there was

Fig. 3. 270 MHz ^1H -NMR data for compound 2 in CDCl_3 . Chemical shifts (ppm) and coupling constants (Hz) were shown by arrows.

no H-5 signal, the H-4 signal appeared as a singlet and an extra methyl signal appeared at 2.86 ppm. From these results we concluded that compound 2 has structure **II**, in which the extra methyl group is attached at position 5.

Discussion. Two mutagens present in the neutral fraction of broiled sardines were concluded to be 2-amino-3-methylimidazo[4,5-*d*]quinoline and 2-amino-3,5-dimethylimidazo[4,5-*d*]quinoline. They are hereafter abbreviated as IQ and methyl-IQ, respectively. They should be new mutagens which are commonly present in ordinary cooked foods. It is interesting that a mutagen recently isolated from heated beef extract seems to be identical to IQ, judging from its mass-, NMR- and UV-spectra and chromatographic behavior (Spingarn *et al.*, 1980a). Thus it is likely that IQ is widely distributed in a variety of broiled foods. It is possible that the mutagenic component previously detected in the basic fraction of broiled sardines (Kasai *et al.*, 1979) is the same compound as IQ or methyl-IQ, because it behaves similarly as IQ or methyl-IQ in HPLC (Kasai *et al.*, 1979), and IQ from beef extract was isolated from the basic fraction (Spingarn *et al.*, 1980b).

IQ and methyl-IQ have a six-six-five membered ring system, and they are the first mutagens of this type. It seems likely that in ultimate active forms in cells the amino group of IQ and methyl-IQ is converted to hydroxylamino group, as in the case of Trp-P-1 and Trp-P-2 (Sugimura and Nagao, 1979b), because S-9 mixture treatment is required for their mutagenic action.

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