A Potent Mutagen in Broiled Fish. Part 1. 2-Amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline

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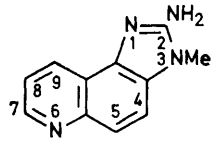
A potent mutagen isolated from a methanol extract of sardines broiled under normal domestic conditions was strongly mutagenic to *Salmonella typhimurium* TA98. Its identity has been established as 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline on the basis of spectral evidence and by chemical synthesis.

MUTAGENICITY has been demonstrated in smoke condensates from broiling fish and beef-steak and in the charred surface of fish and beef-steak.[1-3] Further studies showed that the pyrolysis products of proteins and amino-acids contain strong mutagens [4,5] and active compounds have been isolated from pyrolysates of various amino-acids [6-9] and proteins.[10] Among these compounds, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole (Trp-P-2) from tryptophan,[6] and 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-amino-dipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) from glutamic acid [7] are very strong mutagens, especially towards *Salmonella typhimurium* TA98 with microsomal (S-9 mixture) metabolic activation. The *in vitro* [11,12] and *in vivo* [13,14] carcinogenicities of Trp-P-1 and Trp-P-2 have been 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. Mutagenic activity has been demonstrated in fried hamburgers [15-17] and fried potatoes,[18] but the compounds responsible for this activity have not yet been isolated and identified. These active components are present in cooked foods in very small amounts, and this has made it difficult to obtain enough of the pure compounds for structural characterization. We have previously detected Trp-P-1 and Trp-P-2 in broiled sardine by means of g.c.–mass spectrometry, but these compounds accounted for only 1.5% of the total mutagenicity present.[19] This low value might be partly explained by low recoveries of these compounds, but 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 recently found that the basic fraction of broiled sardine contained an unidentified mutagenic substance.[20]

This paper describes a new potent mutagen isolated from the neutral fraction of broiled sardines. A pure, active component was obtained by using several fractionation procedures including high pressure liquid chromatography (h.p.l.c.). The structure was assigned principally by means of mass and n.m.r. spectral data. It is a novel mutagen with a six-six-five-membered ring-system and its structure is 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (1). Its specific mutagenicity



(1)

towards *S. typhimurium* TA98 was much higher than those of Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, and aflatoxin B_1. This is a first report, apart from preliminary accounts,[21,22] of elucidation of the structure of a new mutagen isolated from ordinarily cooked food.

RESULTS AND DISCUSSION

Sun-dried sardines (10 kg) (Japanese name, Maruboshi) were broiled in the ordinary way, as described previously.[20] They were then ground up and extracted with methanol; after solvent evaporation, the residue, which showed strong mutagenicity to *S. typhimurium* TA98 in the presence of an S-9 mixture, was further fractionated, as described previously [20] (for details, see Experimental section). The neutral fraction thus obtained, which contained 40% of the original mutagenicity, was subjected successively to 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. The mutagens were roughly separated into two components by this procedure. Final purification of compound (1) was achieved by successive h.p.l.c. procedures, as described in the Figure. Approximately 0.2 mg of purified compound (1) was obtained from 10 kg of sardines.

The proposed structure of compound (1) was based on its 270-MHz ^{1}H n.m.r. spectrum, low- and high-resolution mass spectra, f.t.i.r. spectrum, and pK_a values. The

mass spectrum of compound (1) showed a high intensity molecular ion (M^+) at *m/e* 198 and fragment ion at *m/e* 183 ($M^+ - 15$), suggesting that compound (1) has a stable heterocyclic nucleus and a methyl group. The elemental composition of compound (1) was established as $C_{11}H_{10}N_4$ on the basis of an exact mass measurement (calculated mass, 198.0906; observed mass, 198.0900). The 270-MHz ^1H n.m.r. spectrum of naturally occurring compound (1) measured in $CDCl_3$ showed signals assigned to five aromatic protons, methyl protons, and amino-protons (broad). Three protons in the aromatic region (δ_H 8.87, 8.70, and 7.46) were assigned to three protons, 7-H, 9-H, and 8-H, respectively, based on their chemical shifts and coupling constants. The large difference in coupling constants between $J_{7,8}$ (4.2 Hz) and $J_{8,9}$ (8.4 Hz) can be accounted for by the difference of bond length in the quinoline ring. Another set of protons at δ 7.88 and 7.57, which were coupled to each other (J 9.2 Hz), can be assigned to 5-H and 4-H on the quinoline ring. Long-range coupling (J 1 Hz) was observed between 5-H and 9-H. A methyl signal found at low field (δ 3.70) can be assigned to the *N*-methyl group. A nuclear Overhauser effect (n.O.e.) was observed only for 4-H (13%) when methyl protons were irradiated. The broad signal at δ 5.52 was assigned to protons of the amino-group because this signal disappeared by saturation transfer from the proton resonance of water in the sample solution.

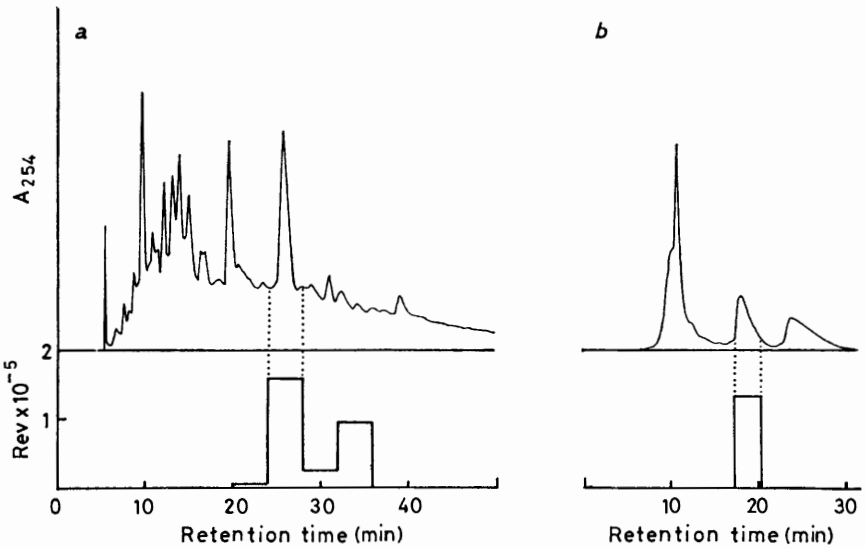
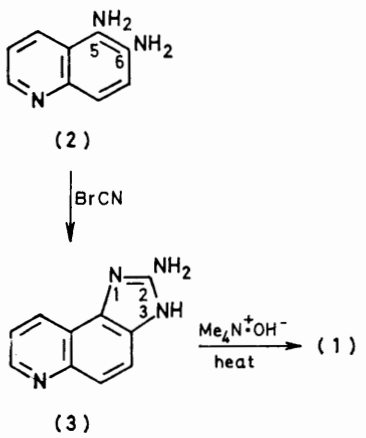


FIGURE Isolation of mutagenic compound (1) by h.p.l.c. The profiles of A_{254} (upper figure) and mutagenic activity [revertants (rev)/plate/min, lower figure] are shown; *a*, Fr. II by h.p.l.c.; *b*, final purification of compound (1) by h.p.l.c.



SCHEME

The presence of a free amino-group was confirmed by the mass spectral analysis of the trimethylsilyl derivative (M^+, 270) and acetyl derivative (M^+, 240). No structures which have a six-five-five- or six-five-six-membered fused-ring system fit the observed chemical shifts and coupling constants. Therefore, we propose structure (1) for the compound.

Fourier-transform i.r. (f.t.i.r.) and pK_a data also support this structure. In the f.t.i.r. spectrum, pertinent absorptions were observed at $\nu_{max.}$ 3 400, 3 350, and 3 250 (NH_2, stretching), 3 090 (=CH, stretching), 1 670 (C=N, stretching), 1 550 (C=C, stretching), 1 375 (Me, bending), and 800 cm^{-1} (=CH, out-of-plane bending); no C=N absorption was found in the region *ca.* 2 200 cm^{-1}. Two pK_a values, 3.5 and 6.6, obtained from the change in the u.v. spectra with pH change, are accounted for by protonation at the guanidinium group (pK_a, 6.6) and protonation at N-6 (pK_a 3.5).

The proposed structure of compound (1) was finally confirmed by its chemical synthesis from 5,6-diaminoquinoline (2) by a two-step reaction, as shown in the Scheme. 5,6-Diaminoquinoline (2), prepared by reduction of 6-amino-5-nitroquinoline according to the method of Kaufmann and Zeller,[23] was treated with cyanogen bromide [24] to afford compound (3), which was converted into its 3-*N*-methyl derivative, compound (1), by heating the tetramethylammonium salt of compound (3) under

reduced pressure.[25] By this method, methylation occurred predominantly at the N-3 position. The crude product was isolated by sublimation and was further purified by silica-gel column chromatography. The mass, n.m.r., and u.v. spectra of the synthetic compound (1) were identical with those of the mutagen isolated from broiled fish, thus confirming the structure proposed.

Compound (1) showed strong mutagenic activity towards the *S. typhimurium* strain when tested by the Ames method [26] with a modification of pre-incubation.[27] This mutagen requires metabolic activation by microsomal enzymes (S-9). Specific activity of this compound towards TA98 and TA100 was 433 000 and 7 000 revertants/μg, respectively. It should be noted that the precursor of compound (1), compound (3), possessed a weaker mutagenicity (200 revertants/μg with TA98). The mutagenicity of compound (1) towards *S. typhimurium* TA98 (433 000 revertants/μg) is much higher than that of Trp-P-2, which is the strongest mutagen thus far found from amino-acid pyrolysis products.[4] It is interesting that a mutagen recently isolated from heated beef-extract seems to be identical to compound (1), judging from its mass, n.m.r., and u.v. spectra and chromatographic behaviour.[28] Thus it is likely that compound (1) is widely distributed in a variety of broiled foods suggesting that it may represent a risk to humans. It is possible that the mutagenic component previously detected in the basic fraction of broiled sardines [20] is compound (1), its h.p.l.c. being similar to compound (1).[20]

Compound (1) has a six-six-five-membered ring-system and is the first mutagen of this type to be discovered. Most mutagens isolated from amino-acid- or protein-pyrolysis products, such as Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, Lys-P-1, 2-amino-α-carboline, and 2-amino-3-methyl-α-carboline, are six-five-six-ring compounds.[6-10]

Compound (1) is a derivative of quinoline, which is known to be mutagenic [29,30] and carcinogenic.[31] Benzo[*f*]quinoline, a derivative of quinoline isolated from non-fat soy-bean tar, is also mutagenic [9] as are many other synthetic quinoline derivatives.[29,30] Their mutagenicities, however, are far weaker than that of compound (1) (3—4 order of magnitude lower), with the exception of 4-nitroquinoline 1-oxide. Why compound (1) possesses potent mutagenicity is not known yet. The amino-group may be converted into a hydroxyamino-group in the final forms, as in the case of Trp-P-1 and Trp-P-2.[27] Structure elucidation of the final reaction form of the quinoline (1) and the bound form of compound (1) with DNA components are being carried out. It is still uncertain whether compound (1) is carcinogenic, and studies on this problem are also under way.

EXPERIMENTAL

Isolation of Crude Mutagens from Broiled Sardines.—Ground, broiled sardines (3 kg) were extracted with methanol (20 l). The extract was evaporated and the residue (817 g) was dissolved in 1M HCl (4.8 l) and extracted 4 times with ether (4.8 l). This ether layer (acid–neutral fraction) contained no mutagenicity. The aqueous layer was adjusted to pH 10 by adding $NaHCO_3$ and NaOH, and then extracted 4 times with ether (5.6 l, basic fraction). The basic fraction contained *ca.* 20% of the mutagenicity of the original methanol extract [20] and the remaining aqueous layer contained *ca.* 40% of the mutagenicity (neutral fraction). This aqueous layer (5.5 l) was passed through a column of Dianion HP-20 (8.5 × 28 cm). The column was washed successively with water (10 l) and ethanol (6 l). No nutagenicity was eluted with water and the active materials were eluted with ethanol. The ethanol fraction was evaporated to dryness and the residue (54 g) was fractionated by liquid–liquid partitioning using chloroform–methanol–water (5 : 6 : 4) as solvent (4 × 1 l). The active materials were collected from the lower layer and evaporated to dryness. This fraction (16.2 g) was fractionated on a column of Sephadex LH-20 (5 × 120 cm) using methanol as solvent. After re-chromatography, using the same column, the active fraction obtained (89.4 mg) was further chromatographed on a column of silica gel (3 × 80 cm) with 10% methanol in chloroform as solvent. Mutagenic activity was roughly separated into two fractions (I and II).

Isolation of the Mutagenic Compound (1).—The mutagenic fractions which were eluted more slowly (fraction II, 1.6 mg) from a column of silica gel were dissolved in 0.2 ml of 40% methanol and injected into an h.p.l.c. column [Shimadzu LC-3A; μBondapak C_{18} (Waters, 7.9 × 300 mm); 2.0 ml min^{-1}; 40—70% methanol as eluant; linear gradient (Figure *a*)]. The mutagenic fraction (0.13 mg) represented by the dotted lines in Figure *a* was dissolved in 60% methanol (0.2 ml) and injected into another h.p.l.c. column [Waters; μBondapak C_{18}; two columns of 4 × 300 mm connected in series; flow rate, 1.0 ml min^{-1}, 60% methanol as eluant (Figure *b*)].

Mutagenesis Assay.—The mutagenicity of each fraction was assayed on strain TA98 of *S. typhimurium* with an S-9 mixture by a pre-incubation method [27] which is a modification of the method of Ames *et al.*,[26] using 30 μl of an S-9 preparation from PCB-treated rats.

Spectral Measurements.—^{1}H N.m.r. (270 MHz) spectra were recorded by using a Brucker WH 270 spectrometer at 23 °C. Chemical shifts were measured relative to the residual $CHCl_3$ signal (δ 7.25). Saturation transfer and n.O.e. were measured by the gated-decoupling method.

High- and low-resolution mass spectra were measured using a JEOL 01SG-2 instrument. Acetylation and trimethylsilylation of the mutagens for mass spectral measurement were made as previously reported.[32]

The f.t.i.r. spectrum was recorded using a Digilab FTS-20 B/D instrument. A micro-disk of KBr was prepared using *ca.* 20 μg of the sample. The spectrum was obtained after 400 scans, by subtracting the KBr spectrum from the sample spectrum.

U.v. spectra were obtained using a Shimadzu UV-300 spectrophotometer.

Synthesis of 2-Amino-3H-imidazo[4,5-f]quinoline (3).—6-Amino-5-nitroquinoline [23] (100 mg) was dissolved in concentrated HCl (1.5 ml) and reduced by adding Fe powder (200 mg). The reduction was completed after 15 min. The reaction mixture was filtrated and washed with water. The filtrate was neutralized with KOH solution, and aqueous BrCN (300 mg, 3 ml) was added during 2 h with stirring; the latter was continued for 2 h after the addition. The pH of the solution was adjusted to pH 10 with KOH

solution, and the reaction mixture was extracted with ethyl acetate (5 × 100 ml). The ethyl acetate layer was evaporated to dryness under reduced pressure and the residue was fractionated on a column of silica gel (1.5 × 30 cm) using 20%-methanol–chloroform as solvent to give compound (3) (35 mg), m.p. 276 °C (decomp.); *m/e* 184 (M^+); $\lambda_{max.}$ (MeOH) 202 (ε 25 300), 263 (48 300), and 352 nm (3 400); δ ($CDCl_3$) 8.84 (1 H, dd, *J* 4.2, 1.7 Hz, 7-H), 8.63 (1 H, m, 9-H), 7.83 (1 H, d, *J* 9.0 Hz, 5-H), 7.66 (1 H, d, *J* 9.0 Hz, 4-H), 7.42 (1 H, dd, *J* 8.3, 4.2 Hz, 8-H), and 4.61 (2 H, br s, NH_2).

Synthesis of 2-Amino-3-methyl-3H-imidazo[4,5-f]*quinoline* (1).—An aqueous solution of compound (3) (32 mg) and tetramethylammonium hydroxide (16 mg) was lyophilized in a sublimation apparatus, heated in an oil-bath, under reduced pressure (3 mmHg). At 200 °C evolution of trimethylamine was observed, followed by sublimation of the reaction product at 205—215 °C. The crude product thus obtained was purified on a column of silica gel (1.5 × 30 cm) using 20% methanol–chloroform as solvent. *Compound* (1) (18 mg) was finally crystallized from aqueous methanol, m.p >300 °C; *m/e* 198 (M^+) and 183 (M^+ — Me); $\lambda_{max.}$ (MeOH) 213 (ε 27 000), 264 (51 500), and 354 nm (4 000); δ ($CDCl_3$) 8.84 (1 H, dd, *J* 4.2, 1.8 Hz, 7-H), 8.72 (1 H, ddd, *J* 8.3, 1.8, and 0.8 Hz, 9-H), 7.84 (1 H, dd, *J* 9.0, 0.8 Hz, 5-H), 7.56 (1 H, d, *J* 9.0 Hz, 4-H), 7.43 (1 H, dd, *J* 8.3, 4.2 Hz, 8-H), 4.58 (2 H, br s, NH_2), and 3.70 (3-H, s, Me).

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REFERENCES

[1] M. Nagao, M. Honda, Y. Seino, T. Yahagi, and T. Sugimura, *Cancer Lett.*, 1977, **2**, 221.
[2] T. Sugimura, M. Nagao, T. Kawachi, M. Honda, T. Yahagi, Y. Seino, S. Sato, N. Matsukura, T. Matsushima, A. Shirai, M. Sawamura, and H. Matsumoto, in ' Origin of Human Cancer,' ed. H. H. Hiatt, J. D. Watson, and J. A. Winstein, Cold Spring Harbor Lab., Cold Spring Harbor, 1977, p. 1561.
[3] S. M. Rappaport, M. C. McCartney, and E. T. Wei, *Cancer Lett.*, 1979, **8**, 139.
[4] T. Sugimura and M. Nagao, *CRC Crit. Rev. Toxicol.*, 1979, **6**, 189.
[5] T. Matsumoto, D. Yoshida, S. Mizusaki, and H. Okamoto, *Mutat. Res.*, 1977, **48**, 279.
[6] T. Sugimura, T. Kawachi, M. Nagao, T. Yahagi, Y. Seino, T. Okamoto, K. Shudo, T. Kosuge, K. Tsuji, K. Wakabayashi, Y. Iitaka, and A. Itai, *Proc. Jpn. Acad.*, 1977, **53**, 58.
[7] T. Yamamoto, K. Tsuji, T. Kosuge, T. Okamoto, K. Shudo, K. Takeda, Y. Iitaka, K. Yamaguchi, Y. Seino, T. Yahagi, M. Nagao, and T. Sugimura, *Proc. Jpn. Acad.*, 1978, **54B**, 248.
[8] K. Wakabayashi, K. Tsuji, T. Kosuge, K. Takeda, K. Yamaguchi, K. Shudo, Y. Iitaka, T. Okamoto, T. Yahagi. M. Nagao, and T. Sugimura, *Proc. Jpn. Acad.*, 1978, **54B**, 569.
[9] T. Kosuge, K. Tsuji, K. Wakabayashi, T. Okamoto, K. Shudo, Y. Iitaka, A. Itai, T. Sugimura, T. Kawachi, M. Nagao, T. Yahagi, and Y. Seino, *Chem. Pharm. Bull.*, 1978, **26**, 611.
[10] D. Yoshida, T. Matsumoto, R. Yoshimura, and T. Matsuzaki, *Biochem. Biophys. Res. Commun.*, 1978, **83**, 915.
[11] S. Takayama, Y. Katoh, M. Tanaka, M. Nagao, K. Wakabayashi, and T. Sugimura, *Proc. Jpn. Acad.*, 1977, **53B**, 126.
[12] S. Takayama, T. Hirakawa, and T. Sugimura, *Proc. Jpn. Acad.*, 1978, **54B**, 418.
[13] T. Ishikawa, S. Takayama, T. Kitagawa, T. Kawachi, M. Kinebuchi, N. Matsukura, E. Uchida, and T. Sugimura, in ' Naturally Occurring Carcinogens-mutagens and Modulators of Carcinogenesis ed. E. C. Miller, J. A. Miller, J. Hirono, T. Sugimura, and S. Takayama, University Park Press, Baltimore, 1979, p. 159.
[14] Unpublished data from our laboratory.
[15] B. Commoner, A. J. Vithayathil, P. Dorala, S. Nair, P. Madyastha, and G. C. Cuca, *Science*, 1978, **201**, 913.
[16] M. W. Pariza, S. H. Ashoor, F. S. Chu, and D. B. Lund, *Cancer Lett.*, 1979, **7**, 63.
[17] N. E. Spingarn and J. H. Weisburger, *Cancer Lett.*, 1979, **7**, 259.
[18] N. E. Spingarn, L. A. Slocum, and J. H. Weisburger, *Cancer Lett.*, 1980, **9**, 7.
[19] Z. Yamaizumi, T. Shiomi, H. Kasai, S. Nishimura, Y. Takahashi, M. Nagao, and T. Sugimura, *Cancer Lett.*, 1980, **9**, 75.
[20] H. Kasai, S. Nishimura, M. Nagao, Y. Takahashi, and T. Sugimura, *Cancer Lett.*, 1979, **7**, 343.
[21] H. Kasai, Z. Yamaizumi, K. Wakabayashi, M. Nagao, T. Sugimura, S. Yokoyama, T. Miyazawa, N. E. Spingarn, J. H. Weisburger, and S. Nishimura, *Proc. Jpn. Acad.*, 1980, **56B**, 278.
[22] H. Kasai, S. Nishimura, K. Wakabayashi, M. Nagao, and T. Sugimura, *Proc. Jpn. Acad.*, 1980, **56B**, 382.
[23] A. Kaufman and O. Zeller, *Ber.*, 1917, **50**, 1626.
[24] D. J. Brown, *J. Chem. Soc.*, 1958, 1974.
[25] T. C. Myers and L. Zeleznick, *J. Org. Chem.*, 1963, **28**, 2087.
[26] B. N. Ames, J. McCann, and E. Yamasaki, *Mutat. Res.*, 1975, **31**, 347.
[27] T. Sugimura and M. Nagao, in ' Chemical Mutagens,' Vol. 6, ed. A. Hollaender and F. J. de Serres, Plenum Press, New York, 1979, p.41.
[28] N. E. Spingarn, H. Kasai, L. L. Vuolo, S. Nishimura, Z. Yamaizumi, T. Sugimura, T. Matsushima, and J. H. Weisburger, *Cancer Lett.*, 1980, **9**, 177.
[29] M. Nagao, T. Yahagi, Y. Seino, T. Sugimura, and N. Ito, *Mutat. Res.*, 1977, **42**, 335.
[30] M. Hollstein, R. Talcott, and E. Wei, *J. Natl. Cancer Inst.*, 1978, **60**, 405.
[31] K. Hirao, Y. Shinohara, H. Tsuda, S. Fukushima, M. Takahashi, and N. Ito, *Cancer Res.*, 1976, **36**, 329.
[32] H. Kasai, Z. Ohashi, F. Harada, S. Nishimura, N. J. Oppenheimer, P. F. Crain, J. G. Liehr, D. L. von Minden, and J. A. McCloskey, *Biochemistry*, 1975, **14**, 4198.