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Improved Synthesis of the Food Mutagen 2-Amino-3,7,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline and Activity in a Mammalian DNA Repair System

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A simplified, direct synthesis of 2-amino-3,7,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (7,8-Me_2IQ_x) is described. Reaction of butane-2,3-dione with 4-nitro-1,2-phenylenediamine yielded 87% 2,3-dimethyl-6-nitroquinoxaline, which in three convenient steps gave 6-(methylamino)-2,3-dimethylquinoxaline (51% yield). Nitration and separation of the two nitrated isomers provided the needed 5-nitro derivative (42%) that upon catalytic reduction and reaction with cyanogen bromide gave 7,8-Me_2IQ_x in 65% yield. 7,8-Me_2IQ_x has about 50% of the mutagenicity of 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ) in *Salmonella typhimurium* TA98 with biochemical activation and also in the Williams test, the induction of unscheduled DNA synthesis in rat hepatocytes. Thus, 7,8-Me_2IQ_x is genotoxic, and most likely carcinogenic, as are most chemicals with reliable activity in both tests.

The process of frying or broiling meat or fish under realistic conditions produces a series of chemicals generally belonging to the class of 2-amino-3-methyl-3*H*-imidazo-[4,5-*f*]quinolines or -quinoxalines (Figure 1). Depending on structure, some of these chemicals demonstrate high mutagenic activity in the Ames *Salmonella typhimurium* assay system (Hatch et al., 1986; Sugimura et al., 1986). In addition, those heterocyclic amines that have been tested in other bacterial or mammalian in vitro test systems uniformly exhibit activity. This includes activity in the selective and critical unscheduled DNA synthesis test (UDS) in freshly explanted rodent liver cells, the Williams test (Barnes et al., 1985). Hatch (1986) has reviewed the genotoxicity data base of these new heterocyclic amines.

The production of this class of mutagens has been thought to occur via Maillard-type reactions. The groups of Matsushima (1982), Jägerstad et al. (1986), and Taylor et al. (1986) have utilized in vitro systems to study mechanistic aspects and have found that the chemicals isolated from the surface of fried fish or meat could also be formed under these in vitro conditions. One of the chemicals so formed is 2-amino-3,7,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (7,8-Me_2IQ_x) (Negishi et al., 1984; Jägerstad et al., 1986; Sugimura et al., 1986).

A number of syntheses for this class of chemicals have been reported (Kasai et al., 1980a,b, 1981; Adolfsson and

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Table I. Analytical Characterization of Chemicals Produced

			spectral data					
			IR, cm^{-1} (KBr)		NMR, δ		MS, m/e	
no.	compound	formula						
3	2,3-dimethyl-6-nitroquinoxaline	$C_{10}H_9N_3O_2$	NO_2	1520, 1340	2,3-CH_3	2.70	M^+	203
					ring H	7.9–8.8	$M^+ - 16$	157
4	6-amino-2,3-dimethylquinoxaline	$C_{10}H_{11}N_3$	NH_2	3340, 3320, 1640, 1500	2,3-CH_3	2.6	M^+	173
					NH_2	4.1		
					ring H	6.9–7.8		
5	6-(succinimidomethylamino)-2,3-dimethylquinoxaline	$C_{14}H_{16}N_4O_2$	NH	3360	2,3-CH_3	2.62		
			CO	1690	CH_2	5.00		
					ring H	7.2–7.9		
6	6-(methylamino)-2,3-dimethylquinoxaline	$C_{11}H_{13}N_3$	NH	3295, 1625	2,3-CH_3	2.65	M^+	188
					N-CH_3	2.95 (d, J = 6 Hz)	$M^+ - 1$	187
					ring H	6.9–7.7	$M^+ - 2$	186
7	6-(methylamino)-5-nitro-2,3-dimethylquinoxaline	$C_{11}H_{12}N_4O_2$	NH	3390	2,3-CH_3	2.70, 2.78	M^+	232
			NO_2	1520, 1382, 1360	*N*-CH_3	3.15 (d, J = 6 Hz)	$M^+ - 1$	231
					ring H	7.28 (d, J = 7 Hz)		
						7.98 (d, J = 7 Hz)		
9	2-amino-3,7,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (7,8-Me_2IQ_x)	$C_{12}H_{13}N_5$	NH_2	3410 (s), 3020, 1620, 3300 (br), 3100, 1665	3-CH_3	3.65	M^+	227
					ring H	7.45	$M^+ - 82$	145
						7.65 (d, J = 6 Hz)		

Olsson, 1983; Grivas and Olsson, 1985; Jägerstad and Grivas, 1985; Kaiser et al., 1986). An early synthesis of 7,8-Me_2IQ_x was achieved by the radical methylation of 7-$MeIQ_x$, a low-yield reaction using as starting material 7-$MeIQ_x$, itself obtained in a long series of steps in low overall yield (Negishi et al., 1984). A more general feasible procedure depended on an interesting, useful intermediate, 5-chloro-4-nitro-2,1,3-benzoselenadiazole, derived from 4-chloro-1,2-benzenediamine (Grivas, 1986). A later synthesis (Olsson and Grivas, 1986) based on the preparation of 6-amino-2,3-dimethylquinoxaline according to Le Bris (1976), N-methylation (via a prior quaternary base formation), nitration, separation of isomers, reduction, and imidazo ring formation yielded 7,8-Me_2IQ_x in a calculated yield of 39% from the nitration step, on a 0.5-g scale.

We have developed an improved fairly direct synthesis of 7,8-Me_2IQ_x that is capable of scale-up to produce a larger quantitity. In addition, we report the activity of this compound as a genotoxin in the Ames and the Williams tests.

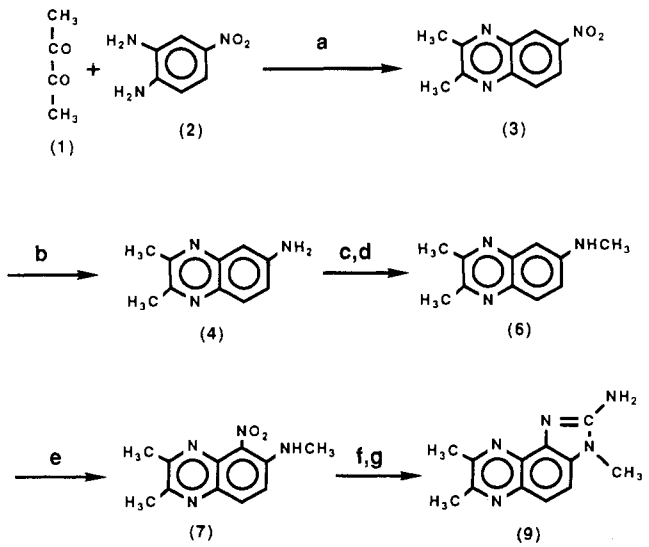

Figure 1. Seven-step synthesis of 2-amino-3,7,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (7,8-Me_2IQ_x). Reaction conditions: (a) reflux 2 h in ethanol; (b) catalytic reduction in Parr shaker with 10% Pd/C; (c) reflux 1 h in ethanol with formaldehyde and succinimide, followed by (d) $NaBH_4$ and aqueous NaOH; (e) HNO_3, H_2SO_4, glacial acetic acid, at room temperature; (f) like (b) and followed by (g) reflux 1 h CNBr in methanol.

EXPERIMENTAL SECTION

Syntheses. The general strategy involved constructing the quinoxaline ring with substituents appropriately located for the final ring-closure step, thereby assuring the correct site of all substituents in the final product (Figure 1).

2,3-Dimethyl-6-nitroquinoxaline (*3*). 4-Nitro-1,2-phenylenediamine (**2**; 134 g, 0.88 mol) was dissolved in 50% aqueous ethanol (1.5 L), and butane-2,3-dione (1; 86.1 g, 1 mol) was added and the solution heated under reflux for 1.5 h. Cooling to room temperature afforded a copious amount of buff needles. Recrystallization from ethanol (95%) gave off-white needles of **3**: 116.4 g (87%); mp 129 °C. Compound **3** was characterized by spectral means (see Table I).

6-Amino-2,3-dimethylquinoxaline (*4*). Compound **3** (14.5 g, 0.07 mol) was suspended in 200 mL of cold 95% ethanol, and palladium–charcoal (0.25 g, 10%) was added. Hydrogenation was accomplished at slightly more than atmospheric overpressure in a Paar hydrogenation apparatus for a period of 4–6 h. Removal of the catalyst and concentration under reduced pressure afforded brown irregular prisms of **4**: 10.6 g (73%); mp 122 °C dec; spectral data, Table I. Compound **4** was reasonably stable toward oxidation by air when stored at 0 °C in a tightly sealed container.

6-(Methylamino)-2,3-dimethylquinoxaline (*6*). Compound **4** (18.5 g, 0.06 mol), *succinimide* (6.0 g, 0.061 mol), and *formaldehyde* (50 mL, 37%) in methanol were refluxed for 1.5 h according to Kadin (1973). After cooling, the solvent was removed under reduced pressure, affording an oil that was triturated with water, producing brown irregular crystals of the succinimide derivative **5**. This crude material was dissolved, without further purification, in warm dimethyl sulfoxide (150 mL) and treated with a solution of 1.5 g of sodium borohydride dissolved in 50 mL of aqueous NaOH (0.5 g). After addition of water (100 mL) (*Caution*! gas evolution), the solution was warmed to 60–70 °C and then stirred for 1 h while the solution cooled to 60–70 °C and then stirred for 1 h while the solution cooled to room temperature. Extraction with diethyl ether (5 × 25 mL) and removal of the solvent under reduced pressure followed by cooling induced the crystallization of a brown-yellow crude product. Recrystallization from absolute ethanol afforded pale yellow plates: 9.5 g (51% from **4**); mp 147 °C dec; spectral evidence for the structure of **6**, Table I.

Table II. Instrumental Analysis of 2-Amino-3,7,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline

parameter	this work	lit.[a]
UV spectrum, nm	216/273/338	215/273/340
MS, molec ion	227, 211, 199, 185, 171, 145, etc.	227, 212, 199, 185, 169, 157, 145, etc.

[a] Negishi et al. (1984).

Table III. Comparison of Mutagenicity Experiments for IQ and 7,8-Me_2IQ_x Using *S. typhimiurium* TA98 with Metabolic Activation[a]

compd	test data	lit.[b] data
7,8-Me_2IQ_x	192000	163000
IQ	360000	433000

[a] Average revertants/microgram. [b] From Sugimura et al. (1986).

6-(Methylamino)-5-nitro-2,3-dimethylquinoxaline (*7*). Compound **6** (5.0 g, 0.02 mol) was dissolved in glacial acetic acid (25 mL) and fuming nitric acid (*d* 1.49, >90%, 2.5 mL) added dropwise with stirring at 0 °C. After the addition was complete, the ice bath was removed and the solution was permitted to warm to room temperature in about 30 min. The reaction mixture was poured over 80 g of crushed ice, the pH adjusted to 8.5 with ammonium hydroxide, and the resulting solution extracted with 5 × 50 mL portions of dichloromethane. Evaporation of the dichloromethane solution afforded a yellow powder containing two new compounds, in addition to unreacted starting material, as demonstrated by thin-layer chromatography (silica; chloroform–ether (1:1)). Column chromatography (Baker flash silica gel system; J. T. Baker Chemical Co., Phillipsburg, NJ 08865; chloroform–ethyl acetate (1:1) solvent mixture) gave compound **7**, mp 238–240 °C, whose structure was confirmed by spectroscopic means (see Table I) in 42% yield.

The second compound was identified by proton NMR, as the 7-nitro isomer. Thus, singlets of δ 2.72 and 2.82 were assigned the ring methyl resonances and a doublet at δ 3.22 the *N*-methyl signal. A doublet (J = 2.5 Hz) at δ 7.22 and a second doublet at δ 8.13 (J = 2.5 Hz) were characteristic of the para relationship of the remaining ring protons. The yield of this isomer was 28%.

2-Amino-3,7,8-trimethyl-3H-imidazo[4,5-f]quinoxaline (*9*). Compound **7** (2.8 g, 0.01 mol) was dissolved in methanol (100 mL), palladium–charcoal (0.25 g, 10%) was added, and the resultant mixture was hydrogenated as described previously. The resulting diamine **8** was not isolated, but after removal of the catalyst cyanogen bromide (0.015 mol) was added and the resulting solution was refluxed for 2 h. Removal of the solvent and recrystallization of the crude product afforded the final product **9**: 1.5 g (65%); mp >300 °C. Spectral properties are listed in Table I, and Table II represents a comparison of mass spectral and spectroscopic data for **9** and an authentic sample, made available by Dr. T. Sugimura, National Cancer Center, Tokyo. In addition, **9** (100 mg) was warmed with acetic anhydride (2 mL) and after cooling afforded the 2-acetamido derivative (IR (KBr) NH at 3150, CO at 1695 cm^{-1}.

Separation Techniques. HPLC. 7,8-Me_2IQ_x in methanol was injected onto a Whatman Partisil 5/ODS-3 (C_{18}) analytical column (0.46 × 25 cm) and chromatographed isocratically at 0.6 mL/min with a mobile phase of 60:40 1 mM ammonium formate–methanol (pH 5.8, adjusted with 1 N HCl). Retention time for 7,8-Me_2IQ_x was 36 min vs. 26 min for standard IQ (courtesy of Dr. T. Sugimura, Tokyo, and also purchased from Toronto Research Chemicals, Toronto, Canada). The peak eluting at 36 min showed a characteristic spectral pattern by scanning UV spectrophotometry, with maxima at 216/273/338 nm for 7,8-Me_2IQ_x (Table II).

Table IV. Hepatocyte Primary Culture/DNA Repair Assay Results for IQ and 7,8-Me_2IQ

compd	concn, mM	av autoradiographic grains/nucleus ± SD
7,8-Me_2IQ_x	2.5×10^{-6}	43.2 ± 16.0
	1×10^{-6}	49.6 ± 14.0
	5×10^{-7}	27.3 ± 14.4
	2.5×10^{-7}	12.6 ± 8.0
	1×10^{-7}	7.6 ± 6.1
IQ	2.5×10^{-6}	55.5 ± 12.6
	1×10^{-6}	101.6 ± 26.3
	5×10^{-7}	55.1 ± 14.9
	2.5×10^{-7}	39.4 ± 12.4
	1×10^{-7}	11.4 ± 8.7
DMAB[a] (pos control)	5×10^{-6}	118.9 ± 31.4
Me_2SO (neg control)	1%	0.0 ± 0.1

[a] 3,2′-Dimethyl-4-aminobiphenyl.

In Vitro Tests for Genotoxicity. The procedures for these tests were performed as described previously (Barnes et al., 1985).

RESULTS

Biological Properties. 7,8-Me_2IQ_x is considerably less mutagenic than IQ in the *S. typhimurium* TA98 system with metabolic activation, displaying about half the activity of IQ (Table IV). Published results (Sugimura et al., 1986; Jägerstad et al., 1986; Hatch, 1986) present data of the same order of magnitude, allowing for the variability of test results from laboratory to laboratory (Ashby and de Serres, 1981; Kier et al., 1986).

In the UDS assay in primary explants of rat hepatocytes, the synthetic 7,8-Me_2IQ_x is quite active, but again less so than the reference compounds IQ and 2′,3-dimethyl-4-aminobiphenyl (Barnes et al., 1985; Howes et al., 1986).

DISCUSSION

With the discovery that bacterial mutagens are produced during certain cooking processes, especially frying and broiling of meats and fish, a new, highly relevant field of nutritional carcinogenesis was opened (Hatch et al., 1986; Knudsen, 1986). With application of modern analytical techniques, it has been possible to separate pure chemical entities from the complex mixture present. These chemicals display a high mutagenic activity in the *S. typhimurium* test systems of Ames, and also in the DNA repair test of Williams. A positive result in both tests indicates probable carcinogenicity (Weisburger and Williams, 1984). To document carcinogenicity, it has been essential to develop synthetic methods to produce each chemical in sufficient amounts for bioassays. This synthesis has been accomplished for IQ and MeIQ, and several laboratories have found that these and related chemicals are carcinogenic (Tanaka et al., 1985; Sugimura et al., 1986; Ohgaki et al., 1986). We have postulated that this entire class of chemicals, although present in very small amounts, is consumed frequently and regularly by many people and might be the genotoxic carcinogens for target organs such as the breast, colon, or pancreas, which are major types of human cancers in the Western World (Tanaka et al., 1985; Weisburger, 1986). Results obtained so far from carcinogenicity bioassays are consonant with this hypothesis, especially since carcinogenesis in these target organs, but not in liver, is highly susceptible to promotion by other elements in the typical Western diet such as the total amount of dietary fat (Wynder et al., 1983).

In this paper, a feasible procedure has been presented for the synthesis of 7,8-Me_2IQ_x, a chemical that is formed

in in vitro systems by Maillard-type reactions and has genotoxic properties. Availability of a good synthetic method will facilitate generating additional data on this particular chemical as a potential carcinogen arising during cooking.

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Nitroxides Derived from Ethoxyquin and Dihydroethoxyquin as Potent Anti-Nitrosamine Agents for Bacon

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Attempted preparation of ethoxyquin nitroxide from ethoxyquin according to the procedure of Lin and Olcott resulted in dealkylation to give the corresponding hydroxy nitroxide as a dimer. The same behavior was also observed with dihydroethoxyquin. These nitroxides derived from ethoxyquin and dihydroethoxyquin were shown to be the most potent anti-nitrosamine agents for bacon, yet discovered with complete inhibition of nitrosamines at 20 ppm and in some instances as low as 10 ppm. Significant reduction was also observed at 1 ppm.

Earlier work from these laboratories (Bharucha et al., 1985) has shown that ethoxyquin, dihydroethoxyquin, and their analogues are excellent inhibitors of nitrosamine formation in bacon. As exemplified by ethoxyquin, it was postulated that they function in the case of nitrosopyrrolidine, by competing with proline for the available nitrosating species, the initially formed *N*-nitrosoethoxyquin undergoing rearrangement to 8-nitrosoethoxyquin prior to (air) oxidation to 8-nitroethoxyquin. The possibility was entertained that ethoxyquin may also function by first undergoing oxidation to ethoxyquin nitroxide (I), which would then trap nitric oxide (the nitrosating species) to give ethoxyquin nitrite (II). The latter could then undergo thermal rearrangement directly to give nitroethoxyquin (III) or alternatively undergo thermal dissociation to give N_2O_4 and ethoxyquin radical, which could then be converted to the nitroxide and thus carry on the chain. These transformations are depicted schematically in Figure

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