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Identification of 2-Amino-1,7-dimethylimidazo[4,5-g]quinoxaline: An Abundant Mutagenic Heterocyclic Aromatic Amine Formed in Cooked Beef

Robert J. Turesky^{*,#}, Angela K. Goodenough^{#,†}, Weijuan Ni[#], Lynn McNaughton[§], David M. LeMaster[§], Ricky D. Holland[‡], Rebekah W. Wu[¶], and James S. Felton[¶]

[#] Division of Environmental Disease Prevention, Wadsworth Center, New York State Department of Health, Albany, NY 12201

[§] Division of Molecular Medicine, Wadsworth Center, New York State Department of Health, Albany, NY 12201

[‡] Division of System Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079

[¶] Chemistry, Materials and Life Sciences Directorate, P.O. Box 808, Lawrence Livermore National Laboratory, Livermore, CA 94551

Abstract

A previously unknown isomer of the carcinogenic heterocyclic aromatic amine (HAA) 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (8-MeIQx) was recently discovered in urine of meat-eaters and subsequently detected in cooked ground beef [Holland, R.D. et al. (2004) *Chem. Res. Toxicol.* 17, 1121 – 1136]. In this current investigation, the identity of the analyte was determined through comparison of its chromatographic t_R by HPLC, and through UV and mass spectral comparisons to the synthesized isomers of 8-MeIQx. Angular tricyclic isomers of 8-MeIQx were excluded as potential structures of the newly discovered HAA, based upon dissimilar t_R and product ion mass spectral data. The linear tricyclic isomers 2-amino-1,6-dimethylimidazo[4,5-g]quinoxaline (6-MeIgQx) and 2-amino-1,7-dimethylimidazo[4,5-g]quinoxaline (7-MeIgQx) were postulated as plausible structures. Both compounds were synthesized from 4-fluoro-5-nitro-benzene-1,2-diamine in five steps. The structure of the analyte was proven to be 7-MeIgQx, based upon co-injection of the compound with the synthetic isomers, and corroborated by comparisons of the UV and mass spectral data of the analyte and MeIgQx isomers. 7-MeIgQx induced 348 revertants/ μ g in the *S. typhimurium* tester strain YG1024, when liver S-9 homogenate of rats pretreated with polychlorinated biphenyls (PCBs) was used for bioactivation. This newly discovered 7-MeIgQx molecule is one of the most abundant HAAs formed in cooked ground beef patties and pan-fried scrapings. The human health risk of 7-MeIgQx requires investigation.

Introduction

More than 20 HAAs have been identified in cooked beef, poultry, and fish, as well as in tobacco smoke condensate (1,2). The formation of HAAs is dependent upon the type of meat and method of cooking, and the concentrations can range from less than 1 part-per-billion (ppb),

*To whom correspondence should be addressed: Division of Environmental Disease and Prevention, Wadsworth Center, NYS Dept of Health, Empire State Plaza, P.O. Box 509, Albany, NY 12201-0509. Phone: 518-474-4151. Fax: 518-486-1505. E-mail: rturesky@wadsworth.org.

[†]Formerly Angela K. Brock

up to 500 ppb in meats or poultry that are cooked well-done (2). Many HAAs are potent bacterial mutagens and induce tumors in multiple tissues of experimental laboratory animals during long-term feeding studies (1). The frequent consumption of red meats has been reported to be associated with an elevated risk of colon cancer in humans (3). Moreover, a markedly increased risk of colorectal cancer has been observed among individuals who frequently consume meats that are cooked well-done and who are both rapid cytochrome P450 1A2-mediated *N*-oxidizers and rapid *N*-acetylators (4,5); both of these phenotypes are associated with enzymes capable of bioactivating HAAs. Several studies have reported the detection of HAA-DNA adducts in human tissues, even though the concentrations of HAAs in the diet are generally at the low ppb level (6-9). Thus, some of the epidemiological data reported on frequent meat consumption, putative HAA exposure, and genetic polymorphisms in xenobiotic enzymes implicate HAAs as causal risk factors for human cancers.

The mechanisms of formation and biochemical toxicology of HAAs have been studied for more than 20 years. Much of the research has been devoted to 8-MeIQx and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), since these compounds are two of the most abundant HAAs formed in cooked meats. It is noteworthy that the known HAAs only account for about 30% of the total mutagenicity attributed to HAAs in well-done cooked beef (10), suggesting that other uncharacterized, genotoxic HAAs are likely present. We recently discovered, by LC/MS analysis, a previously unknown isomer of 8-MeIQx in urine of meat-eaters (11), and we subsequently identified the compound in grilled meats (11,12). In this investigation, we have isolated the novel HAA from pan-fried scrapings of cooked beef, and have characterized the molecule by UV and MS. The spectral data were compared to those of several synthesized angular and linear tricyclic isomers of 8-MeIQx. The spectral data support the assigned structure of the molecule as 7-MeIQx. This newly identified HAA is one of the most abundant HAAs formed in cooked ground beef and pan-fried scrapings.

Materials and Methods

Caution

HAAs are carcinogenic. They should be handled in a well-ventilated hood with extreme care, and with use of appropriate protective clothing and equipment.

Chemicals

The following chemicals were purchased from Toronto Research Chemicals (Downsview, Ontario, Canada): 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and trideuterated 3-[²H₃C]-IQ (isotopic purity >99%), 2-amino-1-methylimidazo[4,5-*b*]quinoline (IQ[4,5-*b*]), 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (8-MeIQx) and the trideuterated 3-[²H₃C]-8-MeIQx (isotopic purity >96%), 2-amino-3,7-dimethylimidazo[4,5-*f*]quinoxaline (7-MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and trideuterated 1-[²H₃C]-PhIP (isotopic purity >99%), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC). 2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), 3-[²H₃C]-4,8-DiMeIQx and 3-[²H₃C]-7,8-DiMeIQx (isotopic purity >99%) were synthesized as previously described (13). 1-[²H₃C]-IQ[4,5-*b*] (isotopic purity >99%) was synthesized from 1-[²H₃C]-creatinine and 2-aminobenzaldehyde (14), and 2-amino-1,8-dimethylimidazo[4,5-*f*]quinoxaline was synthesized as previously described (11). [4b,5,6,7,8,8b-¹³C₆]-2-AαC (isotopic purity >99%) was a kind gift of Dr. D. Doerge, Jefferson, NCTR, AR. 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline (4-MeIQx) was kindly provided by Dr. M. Knize, Lawrence Livermore National Laboratory, Livermore, CA. 2-Amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline (7,9-DiMeIQx) was kindly provided by Dr. K. Wakabayashi, National Cancer Center Research Institute, Tokyo, Japan. CNBr,

pyruvaldehyde (40%), hydrazine hydrate (50% v/v), and palladium/carbon (10%) were purchased from Sigma-Aldrich (Milwaukee, WI). 4-Fluoro-5-nitro-benzene-1,2-diamine was purchased from Maybridge through Ryan Scientific Inc. (Isle of Palms, SC). Oasis MCX LP extraction (500 mg) and borosilicate glass total recovery capLC vials were purchased from Waters (Milford, MA). Extrelut-20 resin was obtained from EMD Chemicals (Gibbs Town, NJ). C₁₈ solid phase extraction (SPE) resins (500 or 1000 mg) were purchased from J.T. Baker (Phillipsburg, NJ) and glass-back TLC plates were purchased from Whatman (Florham Park, NJ).

General Methods

Mass spectra of synthetic derivatives were obtained on a Finnigan™ TSQ Quantum Ultra™ triple quadrupole (TSQ) mass spectrometer (Thermo Electron, San Jose, CA). Typical instrument tune parameters used were as follows: capillary temperature 275 °C, source spray voltage 4.0 kV, sheath gas setting 35, tube lens offset 95, capillary offset 35, source fragmentation 15 V. Argon, set at 1.5 mTorr, was used as the collision gas. Analyses were conducted in positive ionization mode. NMR spectra were collected on a Bruker 600 MHz DRX Spectrometer (Bruker BioSpin Corporation, Billerica, MA) using solutions in CDCl₃ or DMSO-*d*₆. Selected NMR spectra are provided in the Supplementary Information. HPLC separations were done with an Agilent (Palo Alto, CA) model 1100 HPLC system equipped with a photo-diode array detector equipped with a Rheodyne 7725i (Rheodyne, CA) manual injector.

Syntheses

N⁴-Methyl-5-nitro-benzene-1,2,4-triamine (2)—A mixture of 4-fluoro-5-nitro-benzene-1,2-diamine (**1**) (50 mg, 0.29 mmol), methylamine-HCl (194 mg, 2.9 mmol), and anhydrous sodium acetate (237 mg, 2.9 mmol) in anhydrous DMSO (10 mL) was stirred at 120 °C for 4 h. After cooling, water (125 mL) was added and the mixture was applied to a Baker C₁₈ SPE resin (1 g), prewashed with CH₃OH and then water. After the application of the sample, the cartridge was washed with water, and the desired product was eluted with 10 bed volumes of CH₃OH, which was evaporated under vacuum to give **2** (48 mg, 0.26 mmol, 90%). ¹H NMR (DMSO-*d*₆): δ 8.43 (d, *J* = 4.8 Hz, 1H, N⁴-H), 7.18 (s, 1H, H-6), 6.47 (2H, 2-NH₂), 5.83 (1H, H-3), 4.52 (2H, 1-NH₂), 2.85 (d, *J* = 5.0 Hz, 3H, N⁴-CH₃). Positive ESI-MS (relative intensity) *m/z* 183.0 [M + H]⁺, MS/MS of 183.0 (100%), 166.1 (20%), 148.0 (35%), 137.0 (25%).

N,3-Dimethyl-7-nitro-quinoxalin-6-amine (3a)—A solution of **2** (40 mg, 0.22 mmol) and methylglyoxal (17.3 mg, 0.24 mmol) in 1:1 H₂O:C₂H₅OH (10 mL) was heated at 50 °C for 1 h. The mixture was placed under a stream of argon to evaporate the C₂H₅OH, and the desired product, which precipitated from solution, was collected by centrifugation, washed with cold water, and dried under vacuum to give **3a** (38 mg, 0.18 mmol, 82%). ¹H NMR (CDCl₃): δ 8.93 (1H, 8-H), 8.53 (1H, H-2), 7.58 (br, H, N⁶-H), 7.17 (1H, H-5), 3.15 (d, *J* = 5.2 Hz, 3H, N⁶-CH₃), 2.73 (3H, 3-CH₃). ¹³C NMR (CDCl₃): 168.147 (C3), 146.184 (C4a), 144.855 (C6), 144.627 (C2), 136.062 (C7), 131.789 (C8a), 129.098 (C8), 110.229 (N⁶-CH₃), 107.472 (C5), 103.131 (3-CH₃). Positive ESI-MS (relative intensity) *m/z* 219.1 [M + H]⁺, MS/MS of 219.1 (60%), 189.0 (10%), 173.1 (90%), 144.1 (100%).

N⁷,2-Dimethylquinoxaline-6,7-diamine (4)—A solution of **3a** (20 mg, 0.092 mmol) in anhydrous THF (3 mL) was reduced with H₂ at atmospheric pressure using Pd/C (5 mg) as a catalyst. After 4 h, the Pd/C was removed by centrifugation, the charcoal was washed with C₂H₅OH (2 × 1 mL), and the pooled organic extracts were dried under vacuum to give **4** (14 mg, 0.074 mmol, 82%). Due to the instability of compound **4**, the product was characterized by LC-ESI-MS/MS only and used without further purification. Positive ESI-MS (relative

intensity) m/z 189.2 $[M + H]^+$, MS/MS of 189.2 (45%), 174.1 (100%), 157.8 (5%), 147.1 (35%).

2-Amino-1,7-dimethylimidazo[4,5-g]quinoxaline (5a)—Compound **4** (14 mg, 0.074 mmol) was treated with CNBr (11 mg, 0.10 mmol) in C_2H_5OH (2 mL) under argon for 18 h at room temperature. Concentrated NH_4OH (26% v/v) (0.1 mL) was added and the mixture was dried under vacuum. The crude residue was purified by preparative silica TLC (20% CH_3OH , 0.2% NH_4OH , 79.8% CH_2Cl_2) to give **5a** (0.5 mg, 0.002 mmol, 3%). 1H NMR ($DMSO-d_6$): δ 8.58 (s, 1H, H-6), 7.58 (s, 1H, H-9), 7.55 (s, 1H, H-4), 7.20 (br, 2H, NH_2), 3.61 (s, 3H, N1- CH_3), 2.63 (s, 3H, 7- CH_3). ^{13}C NMR ($DMSO-d_6$): δ 159.073 (C2), 148.523 (C7), 145.887 (C3a), 141.844 (C6), 139.207 (C9a), 137.098 (C4a), 136.658 (C8a), 118.29 (N1- CH_3), 111.259 (7- CH_3), 108.886 (C4), 101.767 (C9). Positive ESI-MS (relative intensity) m/z 214 $[M + H]^+$, MS/MS of 214.1 (15%), 199.1 (100%), 172.2 (20%), 130.9 (75%).

5-Fluoro-6-nitro-1H-benzimidazole-2-amine (6)—A solution of compound **1** (100 mg, 0.58 mmol) in C_2H_5OH (5 mL) was reacted with CNBr (184 mg, 1.74 mmol) under argon for 18 h. Concentrated NH_4OH (26% v/v) (0.5 mL) was added, and the mixture was dried under vacuum. The residue was resolubilized with warm water, allowed to precipitate on ice, collected by filtration, and then dried by rotary evaporation, to give compound **6** (100 mg, 0.51 mmol, 88%). 1H NMR ($DMSO-d_6$): δ 7.87 (d, $J = 6.9$ Hz, 1H, 7-H), 7.28 (br, 3H, NH -1 and 2- NH_2), 7.15 (d, $J = 12.8$ Hz, 1H, H-4). Positive ESI-MS (relative intensity) m/z 197.1 $[M + H]^+$, MS/MS of 197.1 (5%), 151.0 (100%), 124.1 (185), 97.1 (5%).

6-Nitro-1H-benzoimidazole-2,5-diamine (7)—A solution of compound **6** (50 mg, 0.26 mmol) in $DMSO$ (3.5 mL) and concentrated NH_4OH (26%) (1.25 mL) was heated at 130 °C for 5 days. After cooling, the reaction mixture was diluted with water (100 mL) and applied to a Baker C_{18} SPE resin (1 g), prewashed with CH_3OH , followed by water. Following application of the sample, the cartridge was washed with water, and the product was eluted with CH_3OH (10 mL). The CH_3OH was evaporated under vacuum and the resulting residue was purified by preparative silica TLC (20% CH_3OH in CH_2Cl_2 containing 0.2% NH_4OH) to give **7** (40 mg, 0.2 mmol, 78%). 1H NMR ($DMSO-d_6$): δ 7.68 (br, 1H, NH -1), 7.08 (s, 1H, H-7), 6.95 (br, 2H, 2- NH_2), 6.51 (s, 1H, H-4). Positive ESI-MS (relative intensity) m/z 194 $[M + H]^+$, MS/MS of 194.1 (10%), 164.0 (5%), 148.0 (100%), 121.0 (35%).

1H-Benzimidazole-2,5,6-triamine (8)—A solution of compound **7** (10 mg, 0.052 mmol) in CH_3OH (1 mL) was treated with $NaBH_4$ (4 mg, 0.11 mmol) in water (1 mL) that was premixed with 5 mg Pd/C in CH_3OH (1 mL) for 15 min at room temperature (15). Then, the Pd/C was removed by centrifugation and the charcoal was washed with C_2H_5OH (2×1 mL). The excess $NaBH_4$ in the combined organic extracts was inactivated by the drop-wise addition of 20% acetic acid (1 mL). The LC-ESI-MS analysis of the reaction by infusion in full scan mode revealed that the reduction of **7** was complete. Positive ESI-MS (relative intensity) m/z 164.0 $[M + H]^+$, MS/MS of 164.0 (15%), 147.1 (100%), 120.1 (90%). Due to instability of the reduced diamine compound **8**, the product was characterized by LC/ESI-MS only and used without further purification.

2-Amino-6-methyl-1H-imidazo[4,5-g]quinoxaline (Compound 9)—The solution of the diamine **8** (8 mg, 0.05 mmol) was immediately reacted with methylglyoxal (4.3 mg, 0.06 mmol) and heated at 50 °C for 15 min. After cooling, the mixture was diluted with water (50 mL) and applied to a Baker C_{18} solid phase extraction cartridge (1 g), prewashed with CH_3OH and then by water. Following application of the sample, the cartridge was washed with water, and product was eluted with CH_3OH (10 mL). The eluent was evaporated under vacuum to give **9** (8.5 mg, 0.04 mmol, 80%). 1H NMR ($DMSO-d_6$): δ 8.58 (s, 1H, H-7), 7.58 (br, 1H,

NH-1), 7.50 (br, 2H, NH₂), 6.98 (s, 2H, H-9, H-4), 2.65 (s, 3H, 6-CH₃). Positive ESI-MS (relative intensity) *m/z* 200.1 [M + H]⁺, MS/MS of 200.1 (50%), 173.2 (45%), 159.1 (20%), 132.3 (100%).

2-Amino-1,7-dimethyl-1*H*-imidazo[4,5-*g*]quinoxaline (Compound 5a) and 2-amino-1,6-dimethyl-1*H*-imidazo[4,5-*g*]quinoxaline (Compound 5b)—CH₃I (30 μmol) was added to a mixture of compound **9** (5 mg, 25 μmol) and K₂CO₃ (4 mg, 30 μmol) in anhydrous DMSO (0.5 mL) (16). The reaction mixture was stirred for 2 h at room temperature to produce 2-amino-1,7-dimethylimidazo[4,5-*g*]quinoxaline (7-MeIgQx) (**5a**) and 2-amino-1,6-dimethylimidazo[4,5-*g*]quinoxaline (6-MeIgQx) (**5b**) in ~1:1 ratio, based upon HPLC, using System 1 (*vide infra*). Following purification by HPLC and evaporation of solvent under high vacuum, compounds **5a** and compound **5b** were recovered (2.4 mg of each compound; 11 μmol; ~44% yield per compound). The NMR and LC-ESI-MS/MS spectral data of 7-MeIgQx (**5a**) were identical to those data acquired on the molecule synthesized by reaction of compound **4** with CNBr (*vide supra*). 6-MeIgQx (**5b**) ¹H NMR (DMSO-*d*₆): δ 8.56 (s, 1H, H-7), 7.61 (s, 1H, H-9), 7.50 (s, 1H, H-4), 7.35 (br, 2H, NH₂), 3.62 (3H, N1-CH₃), 2.63 (3H, 6-CH₃). ¹³C NMR (DMSO-*d*₆): δ 160.089 (C2), 150.077 (C6), 147.616 (C3a), 141.991 (C7), 139.355 (C9a), 139.179 (C4a), 136.454 (C8a), 119.140 (N1-CH₃), 112.197 (6-CH₃), 109.033 (C4), 103.233 (C9). Positive ESI-MS (relative intensity) *m/z* 214 [M + H]⁺, MS/MS of 214.1 (15%), 199.1 (100%), 172.2 (20%), 130.9 (75%).

Tandem Solvent-Solid Phase Extraction (SPE) of HAAs from Cooked Meat

Grilled meat samples or pan-fried scrapings (4 g) were spiked with isotopically labeled HAAs (4 ng for meat and 40 ng for pan-fried scrapings), homogenized in 1 N NaOH (15 mL), mixed thoroughly with Extrelut-20 powder (18 g), and placed into a cartridge holder as previously described (17), except that an MCX cartridge, instead of independent C₁₈ and sulfonic acid SPE resins, was used for simultaneous collection of both apolar and polar HAAs (12). The eluents from the MCX cartridge were concentrated by vacuum centrifugation, resuspended in 0.1% HCO₂H (30 μL), and assayed by HPLC-ESI-MS or further purified by HPLC as described below.

HPLC conditions for purification of synthetic MeIgQx isomers and cooked meat mutagens

System 1—HPLC separation of MeIgQx isomers, on a Waters XBridge™ phenyl column (5 μm particle size; 4.6 × 250 mm). The A buffer was 15 mM ammonium acetate (pH 6.8) and the B solvent was CH₃CN. A linear gradient was employed, starting at 8% CH₃CN and arriving at 12% CH₃CN over 60 min at a flow rate of 1 mL/min.

System 2—HAAs in grilled pan scrapings (4 g, not spiked with isotopically labeled internal standards), were partially purified by tandem SPE and further purified by HPLC using an Aquasil C₁₈ reversed-phase column (4.5 × 250 mm; 5 μm particle size) and Javelin precolumn, Thermo Electron Corp. (Bellefonte, PA). The A buffer was 0.1% HCO₂H and the B solvent was CH₃CN. The meat components were separated by means of a linear gradient starting at 100% A and ending at 100% B, over 60 min, at a flow rate of 1 mL/min. One-minute fractions were collected and infused by LC-ESI-MS/MS (*vide infra*), to identify the fractions that contained 7-MeIgQx and 8-MeIQx.

System 3—Further HPLC purification of HAAs in cooked meats was done with the same Aquasil C₁₈ reversed-phase column and Javelin precolumn, and employed the same gradient and flow conditions of System 2, except that solvent A consisted of 50 mM ammonium acetate (pH 6.8) containing 10% CH₃CN.

System 4—Analyses of HAAs in cooked meat by LC-ESI-MS/MS. Chromatography was performed on an Agilent Technologies 1100 series capillary LC system. The separation of HAAs was done with an Aquasil C₁₈ reversed-phase column (1 × 250 mm, 5 μm particle size) and Javelin precolumn. The flow rate was set at 50 μL/min with a linear gradient over 30 min, starting from 100% solvent A (0.1% HCO₂H containing 0.5% CH₃CN) and ending at 100% solvent B (90% CH₃CN:9.9% H₂O: 0.1% HCO₂H).

LC-ESI-MS/MS Analyses

The MS analyses were done by ESI-MS/MS with either the TSQ MS or a Waters/Micromass Quattro Ultima triple quadrupole MS (Manchester, UK). Quantitative analysis was done in positive ionization mode using the SRM transitions $[M+H]^+ > [M+H - 15]^+$ (loss of CH₃[•]) for IQ, IQ[4,5-*b*], IQx, 8-MeIQx, 4,8-DiMeIQx, 7,9-DiMeIQx, and PhIP, and $[M+H]^+ > [M+H - 18]^+$ (loss of CD₃[•]), for the respective trideuterated internal standards (12,18-20). For AαC, MeAαC, and 2-[¹³C₆]-AαC, the SRM transition was $[M+H]^+ > [M+H - 44]^+$, which is attributed to the loss of NH₃, followed by loss of HCN. The dwell time for each transition was set at 0.1 s. Individual instrument parameters were optimized by infusion the HAAs with a syringe pump into the MS source through a mixing tee, at a flow rate of 10 μL/min, with the LC solvent (1:1 A:B) (A = 0.1% HCO₂H containing 0.5% CH₃CN) and B = 90% CH₃CN: 9.9% H₂O: 0.1% HCO₂H) flowing at 50 μL/min. The optimized instrument tune parameters for the TSQ MS were those conditions described in General Methods (*vide supra*).

For the Waters/Micromass Quattro Ultima triple quadrupole MS, the capillary voltage was set at 3.5 kV, the cone voltage was set at 50 V, hexapoles 1 and 2 were set at 18 and 1 V, respectively. The collision energy was optimized for each HAA with values between 29 and 32 eV. The source and desolvation temperatures were 120 and 350 °C, respectively. The N₂ cone gas flow rate was set at 95 L/h, and the desolvation gas flow rate was 500 L/h. Argon, set at a pressure of 2.5 mTorr, was used as the collision gas. The LC solvent conditions were the same as those described above. Product ion spectra of the analytes were obtained on the protonated molecules $[M + H]^+$ scanning from *m/z* 100 to 250 at a scan speed of 150 Da/s, using the same acquisition parameters described above for the two instruments.

Quantitation of HAAs was done with an external calibration curve, injecting 266 pg of internal standards (2 μL) containing unlabeled HAAs at nine calibrant levels ranging from 2.66 to 4,000 pg injected on column, or the equivalent of 0.01 to 15 ppb of HAA in cooked meat. Since isotopically labeled internal standards were not available for IQx, 7,9-DiMeIQx, and MeAαC, the 3-[²H₃C]-8-MeIQx, 1-[²H₃C]-PhIP, and [¹³C₆]-AαC were used as the respective internal standards. The coefficient of determination (*r*²) of all HAA calibration curves exceeded 0.998.

Bacterial Mutagenesis Assays

The genotoxicity of HAAs was conducted in the Ames reversion assay, which measures revertants that are protrophic and functional in histidine biosynthesis (21). Both tester strains *Salmonella typhimurium* TA98 or YG1024 (0.1 ml, 1 × 10⁹ cells/ml) were employed. The Standard Pour Plate method (21) in which the test chemical, bacteria and metabolic activating enzymes (S-9 mix) were added directly to the top agar without preincubation. After mixing, the entire content was poured onto the minimal glucose agar Petri plates. The plates were then incubated for 48 h at 37 °C. To construct a dose response curve, the cells were exposed to non-toxic concentrations of HAAs, and the revertant colonies were quantified as previously reported (22). The activating enzymes (S-9 mix) consisted of cofactors: NADP (4 mM), glucose-6-phosphate (5 mM), MgCl₂ (8 mM), KCl (33 mM), sodium phosphate buffer (100 mM at pH 7.4), and the S-9 fraction of rat liver homogenate (2 mg of protein concentration per plate). To prepare the S-9 fraction, six-week-old male Sprague Dawley rats were used. They

were injected with a single dose of Aroclor 1254 at 500 mg /kg body weight. Five days later, the rats were euthanized and the liver were removed for processing (the procedure was approved by the Institute of Animal Care and Utilization Committee (IACUC)) at the Lawrence Livermore National Laboratory).

Results

Identification of Known HAAs and Detection of Novel HAAs in Cooked Meat by LC-ESI-MS

The chemical structures of many HAAs formed in cooked meat, including those HAAs investigated in this study are depicted in Figure 1. The LC-ESI-MS/MS analyses of HAAs formed in ground beef patties pan-fried at ~165 °C for 10 min/side are presented in Figure 2. 8-MeIQx is the most abundant of the known HAAs formed in ground beef cooked at 165 °C, followed by 4,8-DiMeIQx, IQx, and PhIP. Lesser amounts of 7,9-DiMeIQx were also detected, along with trace amounts of IQ, its linear tricyclic isomer, IQ[4,5-*b*], and the pyrolysis mutagens, AαC and MeAαC (11,12). These HAAs were also detected in the scrapings of the pan-fried ground beef (12). The extracts of both fried meat samples and pan-fried scrapings were analyzed by LC-ESI-MS/MS in the product ion scan mode, which provided characteristic mass fragmentations of the protonated molecules $[M + H]^+$, for corroboration of the analytes' identities (MS data shown in references (12,19))

In addition to these known compounds, five other putative HAAs were detected and labeled as peaks a – e (Figure 2). These analytes underwent ESI-MS/MS fragmentation to produce products ions attributed to the IQx skeleton (12). One prominent analyte, peak b, was identified as an isomer of 8-MeIQx (11,12). The spectroscopic characterization of this compound is described below. The identities of the other putative HAAs, putative isomers of IQx (peak a) and DiMeIQx (peaks c, d, and e) remain to be determined.

The protonated molecule $[M + H]^+$ of peak b and that of 8-MeIQx occur at m/z 214, and the two compounds undergo the same MS/MS transition ($[M + H]^+ > [M + H - 15]^+$ (due to loss of CH_3^+)). The product ion spectra of peak b and 8-MeIQx are presented in Figure 3. The spectra are similar: many of the same fragment ions are observed, although with differing relative abundances. The accurate mass measurement of peak b in full scan and product ion scan modes by ESI-quadrupole-time-of-flight-MS proved that the molecule was an isomer of 8-MeIQx (11). The angular tricyclic isomers 4-MeIQx, 7-MeIQx, and the *N*1-methyl-isomer of 8-MeIQx were excluded as plausible structures of the analyte, based upon t_R values and product ion spectra that differed from those of the analyte in grilled meat (data not shown) (11). The exclusion of these molecules suggested that a linear tricyclic ring compound was a plausible structure of the isomer.

The similarities in the product ion mass spectra of peak b and 8-MeIQx provided insight into the structure of the isomer. The proposed mechanisms of mass fragmentation of 8-MeIQx are based upon isotopically unlabeled compound, and labeled 3- $[^2H_3C]$ -8-MeIQx and 2- $[^{14}C]$ -8-MeIQx derivatives (19). The initial, principal cleavage of 8-MeIQx occurs at the *N*3- CH_3 bond, to produce the radical cation species at m/z 199 $[M + H - CH_3]^+$, which subsequently undergoes cleavage of the pyrazine ring to produce fragment ions at m/z 172 $[M + H - CH_3 - HCN]^+$, m/z 158 $[M + H - CH_3 - CH_3CN]^+$, and m/z 131 $[M + H - CH_3 - HCN - CH_3CN]^+$ (19). The product ion spectrum of the labeled 2- $[^{14}C]$ -8-MeIQx analogue displayed the same pattern of fragmentation, but the product ions were up-shifted by 2 Da, proving that the ^{14}C -labeled imidazole moiety remains intact under these LC-ESI-MS/MS conditions (19). The product ion spectrum of the novel analyte is similar to the spectrum of 8-MeIQx, except that the fragment ion at m/z 131 is more abundant, suggesting that the analyte contains an *N*-methylimidazo[4,5-*g*]quinoxaline ring system and not an *N*-methylimidazole[4,5-*b*]

quinoxaline skeleton; the latter ring structure would not be expected to undergo fragmentation to produce the fragment ion at m/z 131 as the base peak in the spectrum.

Syntheses of 6-MeIgQx and 7-MeIgQx

The syntheses of 6-MeIgQx and 7-MeIgQx were undertaken to determine whether either compound was the novel analyte formed in cooked beef. The chemical syntheses are described in Schemes 1 and 2. The ^1H NMR spectra, and HMBC and ROESY experiments for many of the key synthetic intermediates are presented in the Supplementary Information. The stereoselective synthesis of 7-MeIgQx (Scheme 1) was done in five steps starting from commercially available 4-fluoro-5-nitro-benzene-1,2-diamine. The key intermediate was *N*⁴-methyl-5-nitro-benzene-1,2,4-triamine (**2**). The electron-withdrawing, *para*-directed effect of the nitro substituent resulted in the selective reaction of the *meta* 1-NH₂ group of compound **2** with the aldehydic carbon of methylglyoxal, to form *N*,3-dimethyl-7-nitro-quinoxalin-6-amine (**3a**) (23). Based upon ^1H NMR, there was no evidence for the formation of the isomeric *N*,2-dimethyl-7-nitro-quinoxalin-6-amine (**3b**) (Supporting Information, Figure 1). The position of the newly formed CH₃-group on the pyrazine portion of the quinoxaline ring of compound **3a** was determined through HMBC experiments (Supporting Information, Figure 2). A long-range correlation was observed between the C4a carbon and H-8. If the compound **3b** isomer had formed, an additional correlation between C4a and H-3 would have been expected. H-2 displays only one long-range correlation to C8a, and establishes that H-2 and H-8 are on the same edge of the ring system.

The final ring-closure step of the diamine **4a** with CNBr produced 7-MeIgQx (**5a**) (Supporting Information, Figure 3). The through space ROEs corroborated that the 7-CH₃ and *N*-CH₃ groups were on the same edge of the molecule: ROEs were observed between the *N*1-CH₃ and the H-9 (peak-to-peak S/N ~40) and between the 7-CH₃ and the H-9 (peak-to-peak S/N ~10) (Supporting Information, Figures 4 and 5). There were no ROEs observed between either CH₃ group and the H-4. Long-range through-bond couplings, in the HMBC experiments, were observed from H-6 to C4a, from H-9 to C4a, and from H-4 to C8a and C9a (Supporting Information, Figure 6).

However, compound **5a** was obtained in only several percent yield and numerous unwanted products were formed. We investigated different reaction conditions, by usage of alternative solvents and temperatures, in attempts to improve the efficiency of the CNBr-ring-closure step, but we attained no appreciable increase in the yield of **5a**. The use of activated di(imidazole-1-yl)methanimine (24) or *S*-methyl(isothioureia) (25) derivatives as cyclo condensing reagents, instead of CNBr, also resulted in poor yields of compound **5a**. Low product yields have similarly been reported for the CNBr-ring-closure step of the diamine precursors of PhIP (26) and several other HAAs (25).

Because of the low yields of 7-MeIgQx by Scheme 1, we devised an alternative scheme for the synthesis of both isomers (Scheme 2). 4-Fluoro-5-nitro-benzene-1,2-diamine was reacted with CNBr to produce 5-fluoro-6-nitro-1*H*-benzimidazole-2-yl amine (**6**) in near-quantitative yield. The ring closure with CNBr also served as a protective group for the *o*-diamines, which undergo oxidation during the direct amination of compound **1** with concentrated NH₄OH (R.T., unpublished observations). Following amination of compound **6**, reduction of the nitro moiety was achieved with NaBH₄, with Pd/C used as a catalyst (15). The resultant diamine **7**, was condensed with methylglyoxal to produce compound **8** at high yield. The treatment of compound **8** with CH₃I resulted in formation of 6-MeIgQx (**5b**) and 7-MeIgQx (**5a**) in a 1:1 ratio, based upon ^1H NMR analysis (data not shown).

The 6-MeIgQx and 7-MeIgQx isomers were not resolved by either silica TLC or by C₈ or C₁₈ reversed-phase HPLC, using a variety of different solvents, buffers, and pH conditions.

However, baseline resolution of the two isomers was achieved by HPLC, using a Waters XBridge™ Phenyl column (HPLC System I). The ¹H NMR spectrum of 6-MeIgQx is shown in Supporting Information, Figure 7. The positions of the CH₃ groups on the 6-MeIgQx molecule were determined primarily through space ROESY NMR experiments (Supporting Information, Figures 8 and 9), which showed ROEs between 6-CH₃ and H-7 (peak-to-peak S/N ~60) and to a lesser extent between the 6-CH₃ and H-4 (peak to peak S/N ~10). An ROE was also observed between the N1-CH₃ to the H-9 (peak-to-peak S/N ~ 50); however, no ROE was observed between 6-CH₃ to H-9. HMBC experiments revealed long-range coupling between H-7 to C8a, H-4 to C8a, and between H-9 to C4a and C3a (Supporting Information, Figure 10). These three-bond couplings were more intense than the corresponding two-bond couplings and had intensities of ~50 for peak to peak S/N. Hence, H-9 and H-7 are on the same edge of the molecule, and the two CH₃ groups are on opposite edges of the molecule.

Comparisons of Chromatographic (*t_R*) and UV and LC-ESI-MS/MS Spectra of the Analyte in Grilled Meat and Synthetic 6-MeIgQx and 7-MeIgQx Isomers

Kilogram quantities of grilled meat or pan-scrappings would be required for us to obtain the MeIgQx isomer (peak b, Figure 2) in quantities sufficient to conduct structural characterization by NMR spectroscopy. Therefore, in order to establish the identity of the positional MeIgQx isomer in cooked meat, we compared the online-UV and LC-ESI-MS/MS mass spectral data for the analyte to those for the synthetic MeIgQx isomers. The LC/MS chromatogram of pan-fried meat scrapings, unspiked, synthetic 6-MeIgQx and 7-MeIgQx standards, and meat-scrappings spiked with MeIgQx isomers monitored in the full product ion scan mode, are shown in Figures 4 A-C. In the unspiked pan-fried meat scrapings, there are two peaks with protonated molecules [M+H]⁺ at *m/z* 214.1. The isomer of 8-MeIQx elutes at a *t_R* 41.3 min, while 8-MeIQx elutes at a *t_R* 44.1 min. The pan-fried meat scrapings was spiked with comparable amounts of the synthetic 6-MeIgQx and 7-MeIgQx isomers. 7-MeIgQx was found to co-elute with the analyte in cooked meat at *t_R* 41.3 min, while 6-MeIgQx eluted at *t_R* 42.6 min. The use of the characteristic *t_R* as a means of identification of the analyte was crucial for identification purposes, since the product ion spectra of the analyte and of the synthetic 6- and 7-MeIgQx isomers are identical (data not shown).

UV spectroscopy was used to corroborate the identity of the analyte as 7-MeIgQx. The UV spectra of synthetic 6-MeIgQx and 7-MeIgQx, and of the analyte in pan-fried meat scrapings, acquired online by HPLC, are shown in Figure 4D. The UV spectra of the synthetic 6- and 7-MeIgQx compounds are very similar; however, subtle differences are discernible in the absorbance maxima centered about 255 and 360 nm for both the two molecules. There is a ~3-nm bathochromic shift in the maximum of 6-MeIgQx relative to 7-MeIgQx (363 vs. 360 nm), and the second maximum at 255 nm in the spectrum of 6-MeIgQx is less intense than the maximum observed for 7-MeIgQx. The UV spectrum of the analyte purified from the pan-fried scrapings of beef is a perfect match to the spectrum of 7-MeIgQx. The UV spectra of synthetic 8-MeIQx and the 8-MeIQx analyte isolated from cooked beef, shown in Figure 4B, differ notably from the spectra of the linear MeIgQx isomers. Thus, on the basis of co-chromatography experiments and UV spectroscopy, the structure of the novel 8-MeIQx isomer in grilled meat was proven to be 7-MeIgQx.

LC/ESI-MS Product Ion Spectra of 7-MeIgQx and 7,9-DiMeIgQx

7,9-DiMeIgQx was previously identified, at low concentrations, in cooked beef extract (27) and fried ground beef (12). At the outset of our investigation, 6- and 7-MeIgQx were dismissed as plausible structures for the analyte, because their mass fragmentations were vastly different from that of the dimethylated 7,9-DiMeIgQx homologue, under the ESI-MS/MS conditions employed. 7,9-DiMeIgQx is a stable molecule and undergoes little fragmentation, while the pyrazine ring of 7-MeIgQx readily undergoes cleavage (Figure 5). Prominent fragment ions

of 7,9-DiMeIgQx $[M + H]^+$ (m/z 228) are observed at m/z 213 and 212 and are respectively attributed to $[M + H - CH_3]^+$ and $[M + H - CH_4]^+$. We attribute the loss of CH_4 to abstraction of a $H^\bullet$ from the 9- CH_3 , which is *epi* to the *N1*- CH_3 group (11,20) (Scheme 3). The resulting product ion at m/z 212 $[M + H - CH_4]^+$ is a stable 2-amino-6-methyl-4-methyleneimidazo[4,5-*g*]quinoxaline/2-amino-4,6-dimethylimidazo[4,5-*g*]quinoxalin-2-amine cationic species; this fragment ion is the base peak in the product ion spectrum of 7,9-DiMeIgQx even at high CID energy conditions. 7-MeIgQx $[M + H]^+$ (m/z 214) also undergoes fragmentation at the *N1*- CH_3 bond to form a radical cation species at m/z 199 $[M + H - CH_3]^\bullet+$; however, the 7- CH_3 group of 7-MeIgQx is not in close proximity to the *N1*- CH_3 group and is thus unable to donate a $H^\bullet$ to produce CH_4 as a neutral fragment. Therefore, secondary fragmentation occurs at the pyrazine ring to produce the 2-amino-benzimidazole radical cation species (m/z 131 $[M + H - C_4H_7N_2]^\bullet+$) as the base peak in the product ion spectrum.

Estimates of 7-MeIgQx and other HAAs in Fried Beef

The concentration of 7-MeIgQx and other known HAAs formed in ground beef patties fried at various temperatures were quantitated; the results are depicted in Figure 6 A – C. 7-MeIgQx and 8-MeIQx are the two most abundant HAAs formed during the cooking of ground beef patties at a pan-frying temperature of $\sim 165^\circ\text{C}$ for 10 min per side, and 7-MeIgQx is the most abundant of all the HAAs recovered in the pan-fried meat scrapings after 10 min of cooking (Figure 6B) 7-MeIgQx and PhIP are formed in the maximal amounts in ground beef patties prepared at higher cooking temperatures (Figure 6C).

Bacterial Mutagenicity of PhIP, 8-MeIQx Isomers, and 7,9-DiMeIgQx

The Ames reversion assay with tester strains TA98 and YG1024 was conducted to assess the genotoxic potencies of HAAs, with liver S-9 fraction of rats pretreated with PCBs used for bioactivation. The YG1024 strain is derived from strain TA98 (*hisD3052*, Δ *uvrB*, *rfa*, *pKM101*) and contains high *O*-acetyltransferase (OAT) activity that enhances the mutagenicity of some HAAs, presumably through the formation of the reactive *N*-acetoxy intermediates, which adduct to DNA (28,29). The estimated mutagenic potencies of 8-MeIQx, 6-MeIQx, 7-MeIQx, 7,9-DiMeIgQx, and PhIP in TA98 and YG1024 tester strains are summarized in Table 1. The dose-response curves of the mutagenicity are shown in Supporting Information, Figure 11. 8-MeIQx is potent in both tester strains and induces mutations at <1 ng per plate. All of the other HAAs assayed are weaker in potency than 8-MeIQx by a factor of ≥ 100 -fold in both tester strains. All of the putative *N*-hydroxy-HAA metabolites were substrates for OAT and were more potent mutagens in tester strain YG1024 than in strain TA98. 7-MeIgQx elicited the highest differential fold induction of revertants in YG1024 over tester strain TA98.

Discussion

Even though HAAs have been monitored in cooked meats for more than 20 years (1,30,31), our studies are the first to report the formation of 7-MeIgQx in cooked ground beef. 7-MeIgQx is one of the most mass-abundant HAAs formed in ground beef patties cooked over a wide range of temperatures ($165 - 300^\circ\text{C}$). Early investigations that sought to identify HAAs in cooked meat employed multiple chromatography steps, and the mutagens were monitored, by the Ames bacterial mutagenesis assay, at each step of the purification (1,30,32). The purified mutagenic fractions were characterized by $^1\text{H-NMR}$ and MS for structural elucidation. These methods were extremely labor-intensive, and kg quantities of grilled meat were required to produce amounts of HAAs sufficient for spectroscopic measurements (30,32). Since the mutagenic potencies of HAAs vary over a $>1,000$ -fold range in bacterial assays, only HAAs possessing high mutagenic activity or present at great abundance were successfully isolated and characterized from cooked meats, when bacterial mutagenicity assays were employed for screening. 7-MeIgQx, which was initially discovered, by LC/MS, in the urine of meat-eaters

(11), was not detected in cooked meats by bacterial mutagenesis assays; its mutagenic potency is weak relative to the more mutagenic angular tricyclic HAAs, including 8-MeIQx, which are easily detected in the Ames assay.

Because the known HAAs have been reported to account for ~30% of the total mutagenicity attributed to HAAs in well-done cooked beef, other uncharacterized HAAs are likely present in cooked meat (10). Our characterization of analytes in grilled meats by LC-ESI-MS/MS has clearly shown that other putative HAAs containing the IQx skeleton are present in cooked beef and pan-fried scrapings, and that 7-MeIQx is the most prominent among these novel HAAs (12). We have also used the highly sensitive YG1024 tester strain to assay the mutagenicity of pan-fried scrapings of beef, following HPLC separation of the HAAs enriched by tandem-SPE (33). Numerous fractions displayed mutagenic activity and many of these fractions were not associated with the t_R of known HAAs (see Supplementary Information, Figure 12). Thus, both the MS analyses and mutagenicity assays provide evidence for the presence of other putative HAAs, of unknown genotoxic potential, in cooked beef.

Although short-term bacterial mutagenesis assays have been an effective screening tool for the identification of some mutagenic HAAs in complex food matrices (34), they cannot reliably predict carcinogenic potency in mammals. For example, the mutagenic potency of PhIP is about 100-fold lower than the potency of 8-MeIQx, under the same assay conditions (Table 1). However, the two compounds are strong carcinogens and induce tumors at multiple sites in experimental laboratory animals during long-term feeding studies at comparable doses (1, 35,36). The newly discovered 7-MeIQx (348 rev/ μ g) is approximately 10-fold lower in mutagenic potency than is PhIP in *S. typhimurium* strain YG1024 (3,015 rev/ μ g). It is notable that the potency of 7-MeIQx is comparable to the potency of 4-aminobiphenyl (4-ABP) in TA98 and YG1024 (frameshift-specific) and YG1029 (primarily point mutation-specific) tester strains (37,38): 4-ABP is a bladder carcinogen in the experimental dog model and a recognized human urinary bladder carcinogen (39). Two reported studies have directly compared the carcinogenic potencies of 4-ABP and HAAs in rodents. In the neonatal B6C3F₁ male mouse model, which is highly sensitive to the induction of liver tumors by exposure to chemical carcinogens during a period of rapid liver cell proliferation, the ranking in potency of tumorigenicity, for comparable doses of chemical, was: 4-ABP > Glu-P-1 > IQ ~ PhIP > MeIQx (40). A second study assessed the carcinogenic potencies of IQ and 4-ABP in female Sprague-Dawley rats (41). The two compounds were found to induce tumors of the mammary gland at high frequency and at comparable dose levels. Thus, the potency of a compound in a bacterial mutagenesis assay does not necessarily correlate with carcinogenic potency.

The strong mutagenic potencies of MeIQx and several other HAA of angular tricyclic structure in the *S. typhimurium* TA98 strain have been attributed to these HAA's ability to frequently induce reversions about 9 base pairs upstream of the original CG deletion in the *hisD*⁺ gene in a run of GC repeats (42): this sequence context may not be a "hot-spot" for PhIP or linear tricyclic HAAs. The weaker mutagenicity of PhIP or 7-MeIQx relative to 8-MeIQx, is not attributed to the inability of cytochrome P450s to efficiently carry out *N*-oxidation of the exocyclic amine groups (43). On the contrary, the rate of PhIP *N*-oxidation with rat liver microsomes obtained from untreated or PCB-pretreated rats is 5 to 10-fold greater than the rate of *N*-oxidation of 8-MeIQx (43). Our preliminary data on the LC-ESI-MS/MS characterization of 7-MeIQx metabolites produced by rat and human liver microsomes fortified with NADH and NADPH also reveal that both species catalyze the *N*-oxidation of 7-MeIQx to the putative, genotoxic *N*-hydroxy metabolite at rates that exceed the *N*-oxidation of 8-MeIQx (R.T., unpublished observations).

The 16-fold higher mutagenic potency of 7-MeIQx in the YG1024 bacterial tester strain expressing elevated OAT, relative to this compound's potency in the TA98 strain (Table 1), indicates that the putative *N*-hydroxy-7-MeIQx metabolite does undergo *O*-acetylation to form the *N*-acetoxy intermediate, as does 8-MeIQx. A requirement for other phase II enzymes, such as sulfotransferase (which is not expressed at appreciable levels in *S. typhimurium* TA 98 and YG1024 strains) may be necessary for the efficient bioactivation of *N*-hydroxy-7-MeIQx, as has been reported for PhIP and MeAaC (44,45). Given the elevated concentrations of 7-MeIQx, as compared to many other known, carcinogenic HAAs formed in cooked ground beef, further studies on the genetic damage and health risk of this novel HAA are clearly warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4-ABP

	4-aminobiphenyl
4-MeIQx	2-amino-3,4-dimethylimidazo[4,5- <i>f</i>]quinoxaline
6-MeIQx	2-amino-3,6-dimethylimidazo[4,5- <i>f</i>]quinoxaline
7-MeIQx	2-amino-3,7-dimethylimidazo[4,5- <i>f</i>]quinoxaline
8-MeIQx	2-amino-3,8-dimethylimidazo[4,5- <i>f</i>]quinoxaline
6-MeIgQx	2-amino-1,6-dimethylimidazo[4,5- <i>g</i>]quinoxaline
7-MeIgQx	2-amino-1,7-dimethylimidazo[4,5- <i>g</i>]quinoxaline
Glu-P-1	2-amino-6-methyldipyrido[1,2- <i>a</i> :3',2'- <i>d</i>]imidazole
IQx	2-amino-3-methylimidazo[4,5- <i>f</i>]quinoxaline
IQ[4,5-<i>b</i>]	2-amino-1-methylimidazo[4,5- <i>b</i>]quinoline
IQ	2-amino-3-methylimidazo[4,5- <i>f</i>]quinoline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
AαC	2-amino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
MeAαC	2-amino-3-methyl-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
4,8-DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5- <i>f</i>]quinoxaline
7,8-DiMeIQx	2-amino-3,7,8-trimethylimidazo[4,5- <i>f</i>]quinoxaline
7,9-DiMeIgQx	2-amino-1,7,9-trimethylimidazo[4,5- <i>g</i>]quinoxaline
CID	collision induced dissociation
HAA	heterocyclic aromatic amine
LC-ESI-MS/MS	liquid chromatography-electrospray ionization tandem mass spectrometry

MS	mass spectrometry
OAT	<i>O</i> -acetyltransferase
ppb	parts-per-billion
PCBs	polychlorinated biphenyls
SRM	selected reaction monitoring
SPE	solid phase extraction
TSQ	Finnigan™ TSQ Quantum Ultra™ triple quadrupole mass spectrometer
HMBC and ROESY refer to commonly used pulse programs	
HMBC experiments create two-dimensional NMR spectra that correlate the signals of neighboring protons with neighboring carbon atoms, and ROESY experiments establish the spatial proximity between protons

Figure 1.

Chemical structures of HAAs investigated in this study.

Figure 2.

LC-ESI-MS/MS analyses of HAAs formed in ground beef patties fried at 165 °C for 10 min/side. Peaks labeled a – e represent novel HAAs, based upon the product ion spectra, which show the presence of either an IQx or IgQx skeleton (12). The HAAs and respective t_R (min) are: IQ (9.37), IQx (10.21), 8-MelQx (10.96), IQ[4,5-b] (11.29), 7,8-DiMeIQx (11.55), 4,8-DiMeIQx (11.69), 7,9-DiMeIQx (12.15), PhIP (12.99), AαC (13.45), MeAαC (14.4). Internal standards are shaded in gray. Peak d contains two HAAs: 7,8-DiMeIQx and another putative DiMeIQx (12).

Figure 3. LC-ESI-MS/MS product ion spectra of (A) novel 7-MeIgQx isomer and (B) 8-MeIQx formed in cooked beef.

Figure 4.

HPLC-ESI-MS/MS and UV spectral characterization of 8-MeIQx and MeIQx isomers formed in cooked beef. (A) Unspiked pan-fried meat scrapings (upper panel), (B) synthetic 6- and 7-MeIQx isomers (1500 pg), and (C) pan-fried meat scrapings spiked with 1500 pg of 6- and 7-MeIQx. HPLC separation of the MeIQx isomers was done with HPLC System 1. The effluent was split 1:10 post-column prior to entering the MS source. (D) On-line HPLC UV spectra of synthetic HAAs and analytes isolated from pan-fried meat scrapings. The UV spectra of synthetic isomers are superimposed with the analytes. The 8-MeIQx and analyte (dashed line) are indistinguishable. The chromophores of 7-MeIQx and the analyte are nearly identical, while the 6-MeIQx isomer (dotted trace) displays a less intense maximum at 260 nm and a 3 nm bathochromic shift, relative to 7-MeIQx, at 363 nm.

Figure 5. LC-ESI-MS/MS product ion spectra of 7-MeIgQx and 7,9-DiMeIgQx at several different collision energies.

Figure 6.

Formation of 7-MeIgQx and other HAAs in grilled ground beef patties. (A) HAA formation as a function of cooking time; (B) HAA content in grilled meat scrapings from ground beef cooked at 165 °C for 10 min; and (C) HAA formation in grilled beef patties as a function of temperature. The limit of quantification (LOQ) was 0.03 ppb.

Scheme 1.
Regioselective synthesis of 7-MeIgQx.

Scheme 2.
Alternative synthesis of 6-MeIgQx and 7-MeIgQx.

Scheme 3.

Proposed mechanisms of mass fragmentation of 7-MeIgQx and 7,9-DiMeIgQx under LC-ESI-MS/MS conditions.

Table 1Mutagenic Potencies of HAAs in *S. typhimurium* TA98 and YG1024 Tester Strains

Compound	Revertants/ μ g		Response Ratio
	TA98	YG1024	YG1024 / TA98
6-MeIgOx	6	46	7.7
7-MeIgOx	22	348	15.8
7,9-DiMeIgOx	638	2,420	3.8
PhIP	931	3,110	3.3
8-MeIQx	117,000	1,400,000	12.0