ISOLATION, STRUCTURE ELUCIDATION, AND SYNTHESIS OF THE MAJOR ANAEROBIC BACTERIAL METABOLITE OF THE DIETARY CARCINOGEN 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-*f*]QUINOXALINE (MeIQx)

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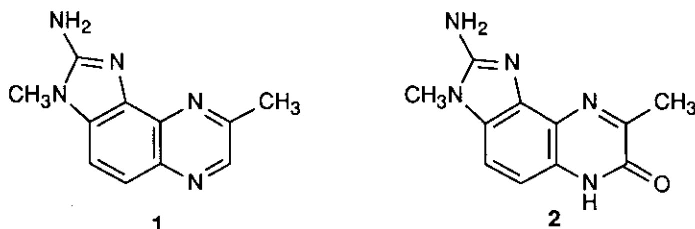
Abstract - Incubation of the heterocyclic cooked food mutagen 2-amino-3,8-dimethyl-3H-imidazo[4,5-*f*]quinoxaline (MeIQx, **1**) with mixed human fecal microflora under anaerobic conditions yielded 2-amino-3,6-dihydro-3,8-dimethylimidazo[4,5-*f*]-quinoxalin-7-one (7-HOMeIQx, **2**), as the major metabolite, but with low overall conversion. The metabolite **2** and its isomer **7** have been synthesized. The metabolite **2** is a direct-acting mutagen, but its isomer **7** is non-mutagenic in the absence of metabolic activation.

In continuation of our previously reported studies on the anaerobic metabolism of various heterocyclic food mutagens,[1] we elected to investigate the metabolism of the mutagenic quinoxaline 2-amino-3,8-dimethyl-3H-imidazo[4,5-*f*]quinoxaline, or MeIQx (**1**) as it is commonly known. MeIQx was isolated from cooked beef by Kasai et al.[2] and by Felton and his co-workers,[3] and its structure was confirmed by comparison with synthetic material.[3] It shows significant mutagenicity, giving approximately 100,000 revertants/μg in TA100 in the presence of the S9 liver fraction.[4]

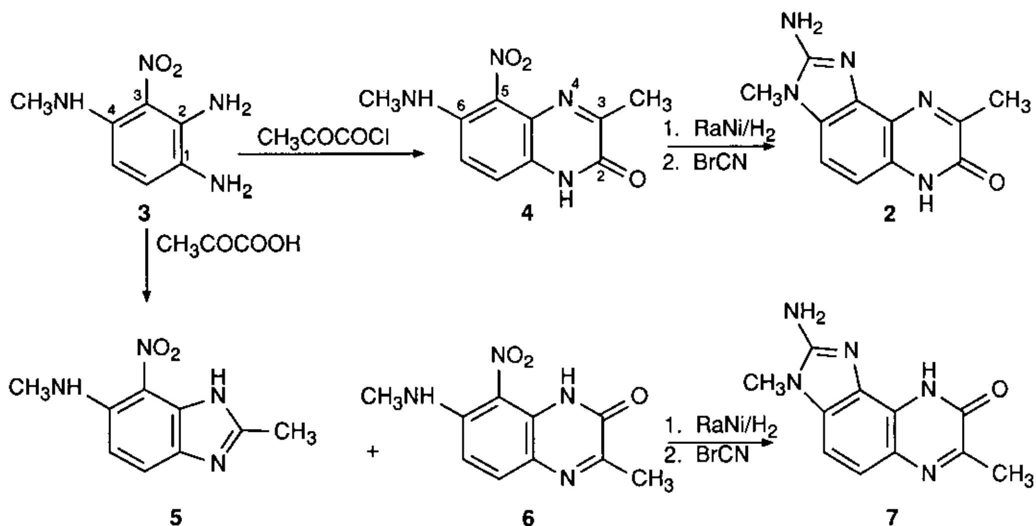
RESULTS AND DISCUSSION

Incubation of [2-^{14}C]MeIQx, prepared by the methods of Grivas,[5] with diluted human feces or pure cultures of *Eubacterium moniliforme* as previously described,[1a,1c] followed by extraction of aromatic metabolites with blue cotton,[1a,6] yielded one major metabolite as determined by radioautography. This metabolite was however only

formed to the extent of 1-3% of the starting material, depending on the conditions, and direct chemical characterization was thus not conveniently accomplished. In view of previous work on the anaerobic metabolism of compounds of this type,[1] it was anticipated that the metabolite would have the 7-oxo structure (**2**), and we thus elected to confirm this by synthesis of compound (**2**).



Treatment of 5-methylamino-4-nitro-2,1,3-benzoselenadiazole[5b] with ammonium sulfide yielded N^4-methyl-3-nitro-1,2,4-benzenetriamine (**3**), in which the 1-amino group is much more reactive than the 2- and 4-amino groups, since the latter are both ortho to the nitro group. Reaction of **3** with pyruvyl chloride thus yielded the quinoxalinone (**4**) as the only isolable product. Reduction of **4** with hydrogen over Raney nickel and reaction of the resulting diamine with cyanogen bromide yielded the imidazoquinoxalinone (**2**).



Confirmation of the structure of compounds (**4** and **2**) was achieved by the synthesis of the isomeric compounds (**6** and **7**). Reaction of the triamine (**3**) with pyruvic acid yielded two products, which were separated by chromatography. The more polar product was identified as the benzimidazole (**5**).[7] This must be formed by reaction of the 1- and 2-amino groups of (**3**) with the keto carbonyl group of pyruvic acid to form a benzimidazole carboxylic acid, which then undergoes oxidative decarboxylation. Formation of this product confirms that the keto carbonyl group of pyruvic acid does indeed react preferentially with the amino groups of compound **3** under these conditions.

The second and more polar product isolated was identified as the quinoxalinone (**6**), formed by reaction of the keto carbonyl group of pyruvic acid with the reactive 1-amino group of **3**, followed by cyclization. Reduction of **6** and treatment of the resulting diamine with cyanogen bromide yielded the imidazoquinoxalinone (**7**), isomeric with **2**.

Direct comparison of the anaerobic metabolite of MeIQx with the synthetic imidazoquinoxalinones (**2** and **7**) showed that the metabolite was identical (tlc, hplc) with the synthetic product (**2**), and was clearly differentiated from its isomer (**7**). The mutagenicity of MeIQx (**1**), its metabolite HOMeIQx (**2**), and the synthetic isomer iso-HOMeIQx (**7**) were determined by a standard preincubation assay against the Ames tester strain TA98. The results are shown below in Table 1.[8]

Table 1

Mutagenicity of MeIQx and derivatives against *S. typhimurium* TA98

Compound		Revertants / plate (100 ng) Control	Compound	Average net revertants
MeIQx	**1**	20, 28	20, 28	0
HOMeIQx	**2**	20, 28	600, 800	676
iso-HOMeIQx	**7**	20, 28	25, 34	6

The data of Table 1 show clearly that MeIQx and iso-HOMeIQx are both inactive as direct-acting mutagens in this system, but that HOMeIQx (**2**) is significantly active. This result is interesting in that it confirms the direct-acting mutagenicity of compounds of the same general type as **2**, such as HOIQ[1a] and HOMeIQ.[1e] In addition, the activity of **2** contrasts strongly with the inactivity of its isomer (**7**), and suggests that a precise relationship the newly formed amide carbonyl group and the imidazo ring is necessary for direct-acting mutagenicity in this series.

EXPERIMENTAL

General

General experimental procedures were as previously described.[1] ^{1}H-Nmr chemical shifts are reported in parts per million from internal tetramethylsilane.

Synthesis of [2-^{14}C]MeIQx (1)

[2-^{14}C]MeIQx (**1**) was prepared from 5-methylamino-4-nitro-2,1,3-benzoselenadiazole as described by Grivas.[5]

Incubation of [2-^{14}C]MeIQx with feces and pure cultures

[2-^{14}C]MeIQx (**1**) was incubated with fresh whole feces and pure cultures of *Eubacterium moniliforme* VPI 13480

as previouly described for [2-^{14}C]MeIQ.[1e] The extraction and isolation of the metabolite were also performed as described, but spectroscopic data were not obtained on the metabolite because of the low conversion observed. The metabolite has an R_F value of 0.55 on silica gel G tlc ($CHCl_3$:MeOH:NH_4OH, 80:20:1); under the same conditions MeIQx (**1**) had an R_F of 0.24.

3-Methyl-6-methylamine-5-nitro-2(1H)-quinoxalinone (4)

N^4-Methyl-3-nitro-1,2,4-benzenetriamine (**3**, 1g, 5.49 mmol) was prepared from 5-methylamino-4-nitro-2,1,3-benzoselenadiazole (1.33 g, 5.77 mmol) as described by Grivas.[5c] To this material in dry pyridine (20 ml) was slowly added freshly prepared pyruvoyl chloride (0.8 ml, 8 mmol) with stirring at room temperature under N_2. After 30 min tlc showed the absence of starting material; the solvent was removed *in vacuo* and the residue dissolved in $CHCl_3$. The solution was washed, dried (Na_2SO_4), and evaporated to afford crude **4** which was purified by flash chromatography ($CHCL_3$ to $CHCl_3$:MeOH, 98:2) to yield homogeneous material which was crystallized from MeOH. Yield 210 mg (16%); mp 278 - 280°C. ^{1}H Nmr ($CDCl_3/CD_3OD$) 2.56 (3H, s), 3.00 (3H, s), 7.14 (1H, d, J=9.3 Hz), 7.39 (1H, d, J=9.3 Hz); eims *m/z* (relative intensity) 234 ($M^{+\cdot}$, 100), 200 (20), 188 (20), 172 (22), 159 (25), 132 (30), 119 (23), 104 (20), 90 (20), 77 (15); uv λ_{max} 430 nm (ϵ 3,773), 365 (2,830), 245 (34,900); ir (KBr) 3450, 2950, 1680, 1540, 1380, 1280 cm^{-1}. *Anal.* Calcd for $C_{10}H_{10}N_4O_3$: N, 23.93. Found: N, 23.27.

2-Amino-3,6-dihydro-3,8-dimethylimidazo[4,5-*f*]quinoxalin-7-one (HOMeIQx, 2).

A stirred mixture of **4** (20 mg, 0.085 mmol) and 10% Pd/C (6 mg) in AcOH (8 ml) was hydrogenated for 20 min at room temperature. The catalyst was filtered off, the filtrate was evaporated to dryness, and the residue was dissolved in MeOH (5 ml) and treated with BrCN (18 mg, 0.169 mmol). After 12 h the solution was basified (10% NH_4OH) and evaporated, and the residue was purified by preparative tlc ($CHCl_3$:MeOH:NH_4OH, 80:20:1.5) to yield **2** (13.7 mg, 70%), mp > 300° (MeOH). ^{1}H Nmr (DMSO-d_6) 2.39 (3H, s), 3.52 (3H, s), 6.56 (2H, s), 6.77 (1H, d, J=8.4 Hz), 7.28 (1H, d, J=8.4 Hz), 12.07 (1H, s); eims *m/z* (relative intensity) 220 ($M^{+\cdot}$, 100), 200 (52), 186 (25), 173 (18), 159 (22), 132 (10), 119 (15), 105 (20), 91 (18), 77 (12); uv λ_{max} 350 nm (ϵ 18,400), 275 (29,900), 225 (64,400); ir (KBr) 3400, 2950, 1660, 1550, 1280 cm^{-1}. *Anal.* Calcd for $C_{11}H_{11}N_5O$: M 229.0963. Found: M 229.0953.

2-Methyl-6-methylamino-7-nitro-1H-benzimidazole (5) and 3-methyl-7-methylamino-8-nitro-2(1H)-quinoxalinone (6)

A solution of the triamine **3** (200 mg, 1.1 mmol) in anhydrous toluene (150 ml) was added dropwise over 1.5 h to a refluxing solution of pyruvic acid (100 mg, 1.14 mmol) in toluene (25 ml) in a Dean-Stark apparatus. After an additional reflux of 0.5 h the starting material was consumed (tlc), and the solvent was removed *in vacuo*. The residue was purified by flash chromatography ($CHCl_3$ to $CHCl_3$:MeOH, 98:2). Early fractions gave compound **6** (88 mg, 34%), mp 230-234°C (MeOH). ^{1}H Nmr ($CDCl_3/CD_3OD$) 2.67 (3H, s), 3.36 (3H, s), 7.10 (1H, d, J=9.4

Hz), 8.02 (1H, d, J=9.4 Hz); eims *m/z* (relative intensity) 234 ($M^{+\cdot}$, 95), 199 (20), 171 (18), 132 (32), 105 (100), 90 (20), 77 (45); uv λ_{max} 465 nm (ϵ 21,400), 345 (78,600), 265 (17,900), 235 (89,300); ir ($CHCl_3$) 3400, 3350, 2950, 1680, 1620, 1590, 1460, 1390, 1320, 1180 cm^{-1}. *Anal.* Calcd for $C_{10}H_{10}N_4O_3$: C, 51.28; H, 4.27; N, 23.93. Found: C, 50.99; H, 4.19; N, 23.62.

Later fractions afforded compound **5** (65 mg, 23%), mp 225-227° ($CHCl_3$). ^1H Nmr ($CDCl_3$) 2.60 (3H, s), 3.10 (3H, d, J=5.2 Hz), 6.64 (1H, d, J=9.1 Hz), 7.80 (1H, d, J=9.1 Hz), 8.72 (1H, br s), 10.44 (1H, br s); ^{13}C nmr (DMSO-d_6) 13.7 (2-Me), 26.2 (N-Me), 107.2 (C-5), 117.9 (C-3a), 129.0 (C-4), 129.7 (C-6), 134.8 (C-7a), 145.2 (C-7), 150.0 (C-2); eims *m/z* (relative intensity) 206 ($M^{+\cdot}$, 100), 172 (10), 159 (30), 132 (30), 118 (8), 103 (20), 90 (12), 63 (8); uv λ_{max} 460 nm (ϵ 21,100), 325 (47,400), 275 (57,900), 228 (184,000); ir ($CHCl_3$) 3475, 1640, 1590, 1510, 1415, 1320, 1285 cm^{-1}. *Anal.* Calcd for $C_9H_{10}N_4O_2$: C, 52.42; H, 4.85; N, 27.18. Found: C, 52.09; H, 4.86; N, 27.01.

2-Amino-3,9-dihydro-3,7-dimethylimidazo[4,5-*f*]quinoxalin-8-one (iso-HOMeIQx, 7)

Compound **6** (20 mg, 0.085 mmol) in EtOH (5 ml) was treated with Raney Ni (1/4 spatula) and hydrogenated for 20 min at room temperature. The catalyst was filtered off, the filtrate was evaporated, and the residue was treated with BrCN (18 mg, 0.169 mmol) at room temperature. After 12 h the solution was basified (10% NH_4OH) and evaporated to dryness *in vacuo*. Purification by preparative tlc ($CHCl_3$:MeOH:NH_4OH, 80:20:1) afforded **7** (15 mg, 77%), mp > 290°C (MeOH). ^1H Nmr (DMSO-d_6) 2.34 (3H, s), 3.55 (3H, s), 6.47 (2H, s), 7.11 (1H, d, J=8.5 Hz), 7.25 (1H, d, J=8.5 Hz), 11.95 (1H, s); eims *m/z* (relative intensity) 229 ($M^{+\cdot}$, 100), 201 (50), 188 (55), 173 (15), 159 (20), 145 (20), 132 (18), 117 (12), 91, (10), 76 (10); uv λ_{max} 345 nm (ϵ 13,000), 282 (34,400), 268 (22,100), 257 (19,100), 225 (47,300); ir (KBr) 3400, 3250, 1670, 1560, 1450, 1310, 1260, 1220 cm^{-1}. *Anal.* Calcd for $C_{11}H_{11}N_5O$: M 220.0963. Found: M 229.0956.

Comparison of HOMeIQx (7) and the MeIQx metabolite

The metabolite of MeIQx was compared with HOMeIQx (7) by silica gel G tlc ($CHCl_3$:MeOH:NH_4OH, 80:20:1), and by μBondapak C18 hplc (H_2O:MeOH, 90:10). The metabolite and the synthetic product had identical mobilities in both systems.

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7. The tautomeric structure 2-methyl-5-methylamino-4-nitro-1H-benzimidazole is not excluded by our evidence; structure (**5**) is favored because it provides additional H-bonding opportunitites to the nitro group.
8. The mutagenicities of compounds (**2**) and (**7**) in the presence of microsomal activation were not determined. However, based on results with the related compound HOIQ, it is likely that both HOMeIQx and iso-HOMeIQx would show mutagenicity comparable to that of MeIQx in the presence of S9 mix.[1c]

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