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1,4-Dihydro-2,3-quinoxalinediones as Potential Flavin Metabolites and Excitatory Amino Acid Receptor Ligands. Part 1: Synthesis and Pharmacological Evaluation of the Benzylic Oxidation Series of 1,4-Dihydro-6,7-dimethyl-2,3-quinoxalinedione

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Abstract—A series of five 6,7-disubstituted 1,4-dihydro-2,3-quinoxalinediones was prepared, two of which are known microbial flavin metabolites and three of which are potential flavin metabolites. Four of the five compounds inhibited specific binding of [^{3}H]-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid ([^{3}H]AMPA), [^{3}H]kainic acid, and [^{3}H]6-cyano-1,4-dihydro-7-nitro-2,3-quinoxalinedione ([^{3}H]CNQX) in rat brain homogenate fractions, with IC_{50} values in the low micromolar range (the fifth compound competed only with [^{3}H]CNQX). Two of the compounds were moderately potent AMPA antagonists in an in vitro functional test.

Introduction

Certain synthetic 1,4-dihydro-2,3-quinoxalinediones, such as 6-cyano-1,4-dihydro-7-nitro-2,3-quinoxalinedione (CNQX, **1**; Figure 1) and the 6,7-dinitro analog (DNQX, **2**), antagonize excitatory responses elicited from many neurons in the mammalian central nervous system by glutamate and by the receptor-subtype-selective glutamate agonists AMPA and kainic acid.[1] Excitations elicited by *N*-methyl-D-aspartate (NMDA) are also antagonized to a lesser extent, apparently due to competition for the glycine coagonist binding site associated with the NMDA receptor complex.[2] A substantial number of compounds with structural similarities to the 2,3-quinoxalinediones or 3-oxoquinoxaline-2-carboxylic acids (**3**, Figure 1) have been synthesized as potential therapeutic agents;[3] such compounds appear especially promising as early intervention agents in stroke.[4]

To date, no endogenous ligand for 'quinoxalinedione receptors' has been identified. A few naturally occurring 2,3-quinoxalinediones have been reported in the literature, either in plant sources or as microbial metabolites, or precursors, of riboflavin (see below), and we are therefore addressing the question of whether flavin metabolism is somehow relevant to excitatory amino acid neurotransmission; hypothetically, there might be endogenous or naturally occurring xenobiotic, flavin-related compounds that play neuroexcitatory, neuromodulatory, or neuroprotective roles, or act as neurotoxins.

In the late 1950s and early 1960s, Stadtman and coworkers[5–8] showed that a strain of *Pseudomonas*

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riboflavinus (designated as *Pseudomonas* RF by these workers), an aerobic soil bacterium that utilizes riboflavin as its sole source of energy, degrades riboflavin via 2,3-quinoxalinedione derivatives **4** and **5** to 3,4-dimethyl-6-carboxy-α-pyrone (**6**), ribose or ribitol, oxamide, carbon dioxide, and urea (Figure 2). Kato and Makida[9] also reported **4** and **5** among the riboflavin degradation products formed by a soil bacillus. Chemically, alkaline hydrolysis of riboflavin gives urea and the 1-D-ribityl-3-quinoxalinecarboxylic acid **7** (Figure 3).[10] Alkaline hydrolysis with peroxide gives **4** or **5**, depending on the conditions.[11,12] It also was reported by Miles et al.[13] that the carboxylic acid **7** could be oxidatively converted to **4** by hydrogen peroxide in acetic acid (Figure 3); however, **7** may not be an intermediate in the microbial conversion of riboflavin to **4**. In a model for such biological oxidations, alkaline ferricyanide chemical oxidation of **4**[14] gave **5** and the quinoxaline-6-carboxylic acid **8**, among other products (Figure 4).

The 2,3-quinoxalinediones are not yellow, and have a fluorescence emission range of 200–350 nm. To date, it appears that mammalian flavin metabolism studies have concentrated on yellow-colored compounds with fluorescence emission at wavelengths above 500 nm. Furthermore, radioisotopic studies of mammalian riboflavin metabolism have only been carried out with [2-^{14}C]riboflavin; therefore, any 2,3-quinoxalinedione metabolite would be lacking a radiolabel. ([^{14}C]Urea *has* been detected in various studies—see Discussion section.) 2,3-Quinoxalinediones as a class are polar compounds, but are not very water-soluble. Their polarity causes them not to partition efficiently from aqueous phases into water-immiscible organic solvents (such as chloroform or phenol, two solvents that have commonly been used in flavin metabolism studies); neither are they readily soluble in such solvents.

A number of 2,3-quinoxalinediones and 3-oxoquinoxaline-2-carboxylic acids that are potential flavin

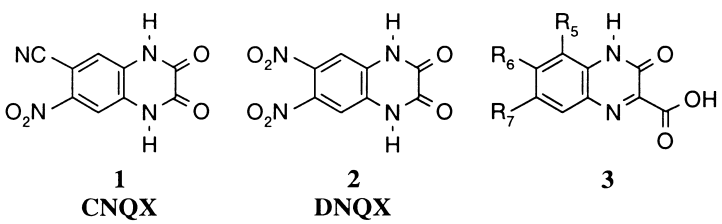


Figure 1. 2,3-Quinoxalinedione and 3-oxoquinoxaline-2-carboxylic acid excitatory amino acid receptor ligands.

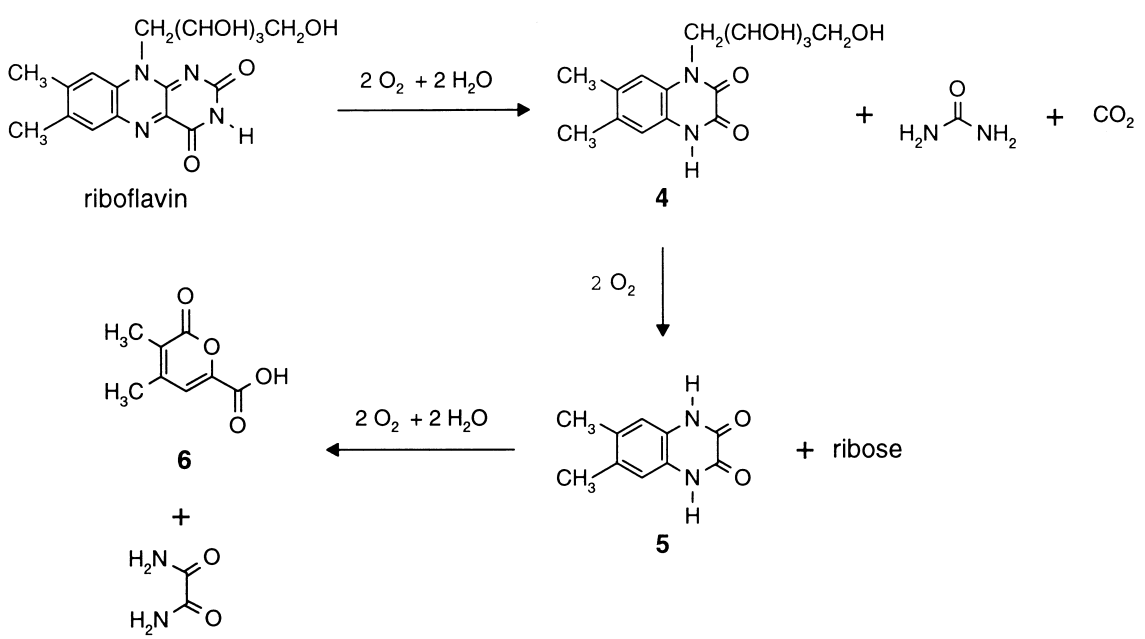


Figure 2. Metabolism of riboflavin by *Pseudomonas riboflavinus*.

metabolites have not been synthesized previously. The availability of synthetic compounds as analytical standards facilitates the development of methods for their detection and quantitation in biological samples. Herein, we report the synthesis of the compounds representing the benzylic oxidation series of **5**, along with the results of an initial pharmacological evaluation that provides one index of the likelihood that these compounds could play a neuromodulatory role in the mammalian central nervous system.

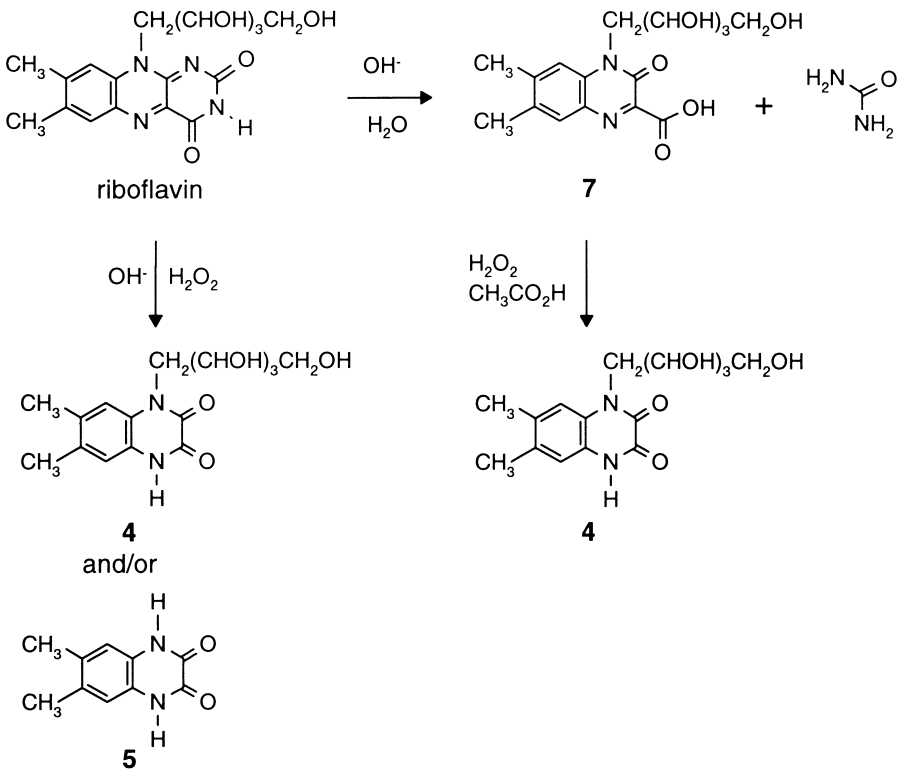


Figure 3. Chemical degradation pathways of riboflavin leading to 2,3-quinoxalinediones and 3-oxoquinoxaline-2-carboxylic acids.

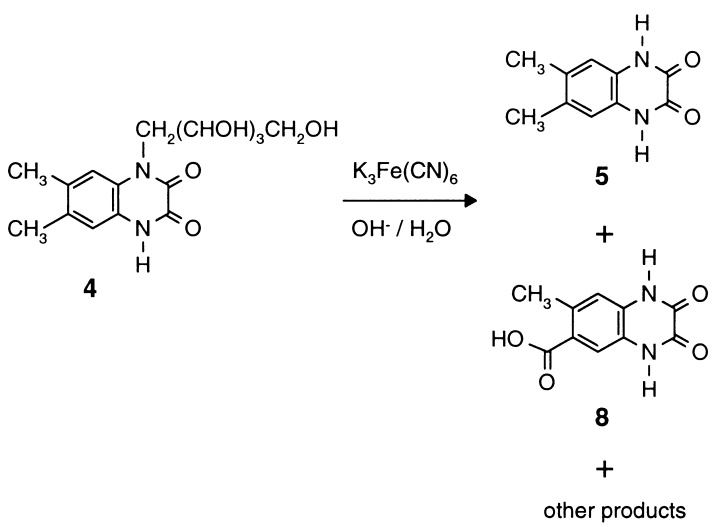


Figure 4. Alkaline ferricyanide oxidation as a model for oxidative metabolism of compound **4** by bacteria (from Ref. 17).

Results

Synthetic chemistry

The known[15] compound **5** was synthesized by refluxing 3,4-dimethyl-1,2-phenylenediamine with diethyl oxalate in tetrahydrofuran (THF) under a positive pressure of argon gas (Figure 5).[16] Compound **5** is poorly soluble in solvents other than dimethylformamide or dimethyl sulfoxide; to allow free-radical bromination, it was therefore converted to the corresponding 2,3-dimethoxyquinoxaline: the 2,3-quinoxalinedione **5** was treated with phosphorus oxychloride to provide the corresponding 2,3-dichloro derivative **9**,[17] which was further treated with sodium methoxide in anhydrous methanol to yield the dimethoxy compound **10**.[18] Free-radical bromination was carried out using *N*-bromosuccinimide in carbon tetrachloride, which gave compounds **11** and **12**, always together with unreacted starting material (**10**). The bromomethyl and 6,7-bis(bromomethyl) compounds **11** and **12** were separated from each other and from **10** by column chromatography. Compound **11** was hydrolyzed (Figure 6) in 1.2 N hydrochloric acid[19] to provide the 6-hydroxymethyl-7-methyl-2,3-quinoxalinedione **13**. Similarly, the bis(bromomethyl) derivative **12** was hydrolyzed to **14**. The bromomethyl compound **11** was also reacted with hexamethylenetetramine to prepare the aldehyde derivative **15** in a Sommelet reaction (Figure 6).[20]

A number of approaches were pursued for synthesizing the known (and deceptive by its apparent simplicity) 7-methylquinoxaline-6-carboxylic acid **8**, and the resulting body of work will be the subject of another paper. Herein we report the synthesis of this compound from 2-methyl-4,5-dinitrobenzoic acid (**16**), prepared essentially by the method of Goldstein and Tardent,[21] except that the nitrile hydrolysis was carried out with basic peroxide (Figure 7). Reduction to the diamine could be accomplished either with hydrazine and Raney nickel or tin metal in aqueous HCl. Careful optimization of the reaction conditions was required with both methods because further conversion of the diamine **17** to at least one unidentified material was problematic. The diamine free base was unstable in neutral or basic solutions, and such solutions quickly turned red and then brown. Straightaway cyclization of the free base prepared by the hydrazine/Raney nickel reduction to the 2,3-quinoxalinedione **8** was readily accomplished with diethyl oxalate in refluxing THF, however.

Pharmacological evaluations

The 6,7-dimethyl (**5**), 6-hydroxymethyl-7-methyl (**13**), 6-formyl-7-methyl (**15**), and 6,7-bis(hydroxymethyl) (**14**) 2,3-quinoxalinediones were inhibitors of [^{3}H]CNQX and [^{3}H]AMPA specific binding in rat brain homogenates, although not exceedingly potent; IC_{50} values were in the low micromolar range (Table 1). The

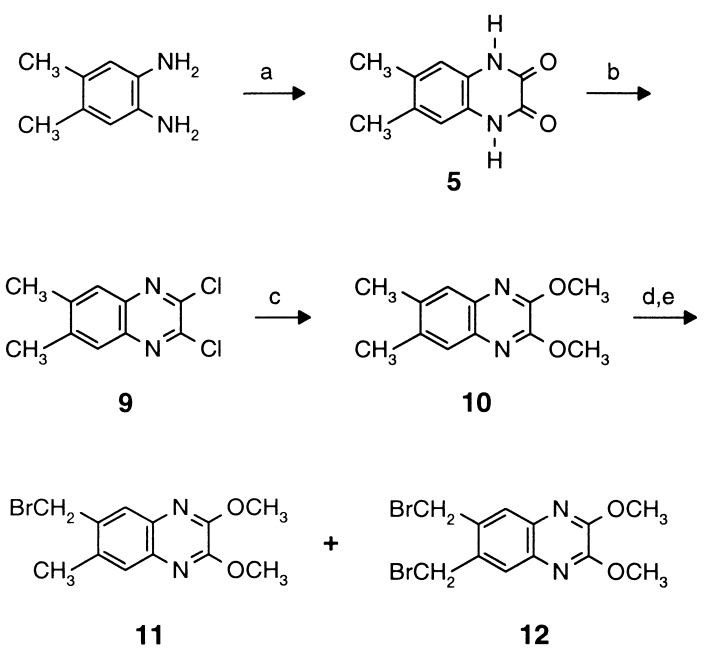


Figure 5. Synthesis of intermediates **11** and **12**. Reagents and conditions: (a) $EtOCOCO_2Et$, THF, CH_3CO_2H (cat.), Ar(g), reflux; (b) $POCl_3$, *N,N*-dimethylaniline, reflux; (c) CH_3ONa, CH_3OH, reflux; (d) NBS, BzOOBz (cat.), CCl_4, reflux; (e) silica chromatography.

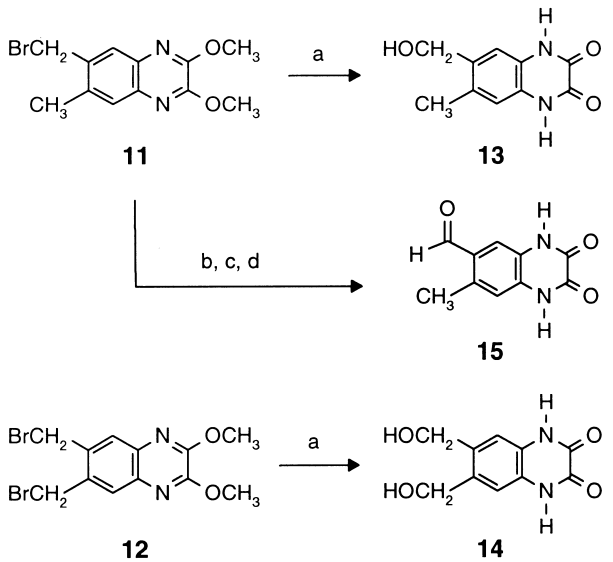


Figure 6. Synthesis of target compounds **13–15**. Reagents and conditions: (a) 1.2 N HCl, Δ; (b) $C_6H_{12}N_4$; (c) CH_3CO_2H, reflux; (d) HCl, reflux.

compounds were all about tenfold less potent as inhibitors of specific [^{3}H]kainate binding. Unlabelled CNQX (**1**) and DNQX (**2**) were tested as reference compounds, and were somewhat more potent in inhibiting the specific binding of all three radioligands. This is consistent with the well-documented observation that electron-withdrawing functional groups in the R^6 or R^7 positions confer greater receptor affinity, and in fact the most potent compound in our series is the aldehyde **15**. The diol **14** was more active than we had expected, however. Compounds **14** and **15** were modestly potent antagonists of AMPA-receptor-stimulated [^{3}H]norepinephrine release from rat hippocampal nerve endings in vitro, though again somewhat less active than the standard compound DNQX.

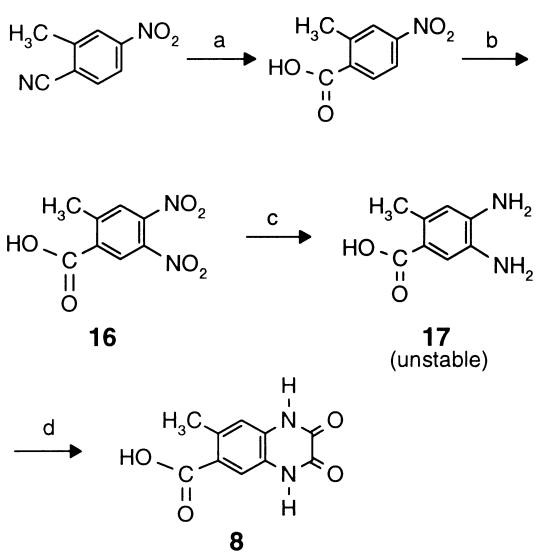


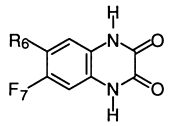
Figure 7. Synthesis of quinoxaline-6-carboxylic acid **8**. Reagents and conditions: (a) H_2O_2, 10% NaOH, reflux; (b) HNO_3, H_2SO_4 (1:1); (c) NH_2NH_2, Ra(Ni); (d) $EtOCOCO_2Et$, THF, CH_3CO_2H (cat.), Ar(g), reflux.

A nitro group is present at the R^6 or R^7 position in many of the 2,3-quinoxalinediones known to exhibit the most potent AMPA-receptor antagonism. Both DNQX and CNQX are sufficiently acidic as to be predominantly ionized at physiological pH. The nitro group would be a locus for a considerable portion of the resulting negative charge (Figure 8). Even in the absence of ionization, the electron-withdrawing nitro group is a region of negative charge buildup. We therefore anticipated that the carboxylic acid **8** might be a potent AMPA receptor ligand, because ionization of the carboxyl group is essentially total under physiological conditions. This compound proved to be inactive, however, as an inhibitor of specific [^{3}H]AMPA and [^{3}H]kainate binding, and its modest inhibition of specific [^{3}H]CNQX binding might be due to activity at the NMDA-receptor-associated glycine coagonist site.[2] The isoalloxazines riboflavin and lumiflavin (**18**) were inactive, as was the alloxazine lumichrome (**19**).

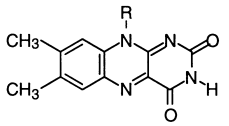
Discussion

Tillotson and Karcz[22] confirmed the presence in rat urine of at least ten [^{14}C]flavin-related compounds

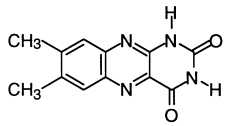
Table 1. Radioligand binding and in vitro functional studies



1, 2, 5, 8, 13-15



18, lumiflavin: R = CH_3
riboflavin: R = $CH_2(CHOH)_3CH_2OH$



19, lumichrome

Compound	R_6	R_7	IC_{50} [^{3}H]CNQX[a] (μM)	IC_{50} [^{3}H]AMPA[b] (μM)	IC_{50} [^{3}H]KAIN[c] (μM)	IC_{50} [^{3}H]NE release[d] (μM)
Riboflavin	—	—	I[e]	I	I	ND[f]
18 (lumiflavin)			I	I	I	ND
19 (lumichrome)			I	I	I	ND
5	CH_3	CH_3	6.4 ± 1.3	16 ± 2.7	> 100	1000
13	CH_2OH	CH_3	33 ± 5.6	13 ± 2.9	> 100	> 1000
15	CHO	CH_3	4 ± 1	11 ± 3.2	54 ± 9	158 ± 43
8	CO_2H	CH_3	20 ± 11	> 100	> 100	ND
14	CH_2OH	CH_2OH	9.2 ± 3.4	9.3 ± 6.4	> 100	450 ± 72
1 (CNQX[a])	CN	NO_2	0.2 ± 0.0	0.56 ± 0.24	2.3 ± 0.7	ND
2 (DNQX[g])	NO_2	NO_2	1.4 ± 0.1	0.93 ± 0.42	2.2 ± 0.4	41 ± 6

Data are expressed as the mean ± sem of at least three independent determinations.
[a]CNQX: 6-cyano-1,4-dihydro-7-nitro-2,3-quinoxalinedione.
[b]AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid.
[c]KAIN refers to kainic acid.
[d]Antagonism of [^{3}H]norepinephrine release elicited by 100 μM AMPA from hippocampal slices; see text for details.
[e]No detectable competition at 100 μM concentration.
[f]Not determined.
[g]DNQX: 6,7-dinitro-1,4-dihydro-2,3-quinoxalinedione.

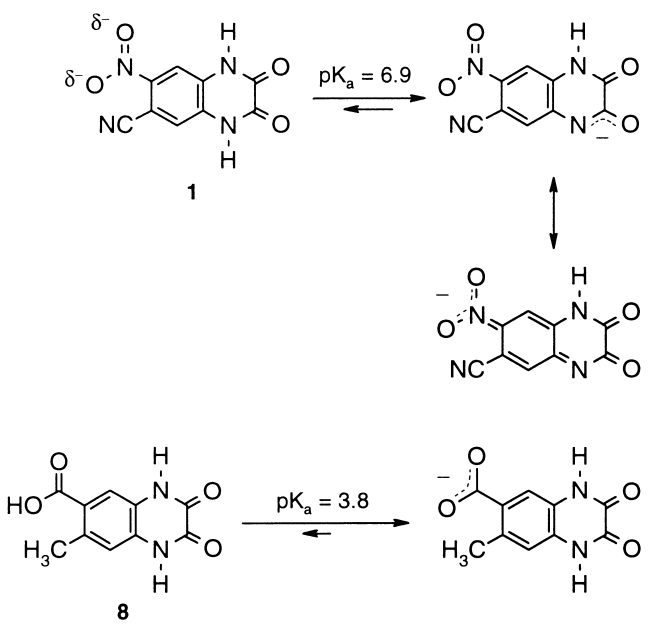


Figure 8. Comparison of the conjugate base of CNQX (**1**) with the carboxylate anion of compound **8**.

resulting from metabolism and chemical degradation of orally administered [2−^{14}C]riboflavin. These compounds have not all been identified, and only about 10–20% of the total ^{14}C was excreted in the urine; as much as 9% of this urinary ^{14}C appeared as [^{14}C]urea. [^{14}C]Urea was also identified in rat urine in [2−^{14}C]riboflavin metabolism studies by Christensen,[23] though in smaller amounts. These results confirm the biological degradation of the pyrimidinoid portion of the isoalloxazine ring of flavins, although it is still not clear whether such degradation occurs somatically or (more likely) through the action of microorganisms in the gut in mammals. Various reports suggest (by physical descriptions of spots on paper and thin-layer chromatograms) the likely presence of quinoxalinediones in human feces,[24] rabbit urine,[25] and the urines of goats, calves, and sheep.[26]

The 1-ribityl-2,3-quinoxalinedione **4** (see Figs 2–4) was reported to be present in the pea plant and several other plant species,[27,28] and could apparently serve as a riboflavin precursor. Furthermore, juvenile rats fed compound **4** exhibited considerably better growth than those fed flavin-deficient diets or diets containing **5**.[29] Although the latter could be due to a 'flavin-sparing effect',[30] these two observations suggest an alternate biosynthetic pathway for riboflavin involving quinoxalinone intermediates, essentially the reverse of the microbial degradation pathway discussed above, which may occur more widely in nature than heretofore recognized.

We have developed methods for extracting and analyzing the 2,3-quinoxalinediones **5**, **13**, **15**, and **8** in rat brain, rat liver, rat urine, rat feces, rabbit urine, and rabbit feces. The availability of synthetic analytical standards was essential to this work. Preliminary results indicate the presence of each of these compounds, in the high parts-per-billion to low parts-per-million range, in one or more of the matrices examined. To the best of our knowledge, these compounds have never been reported to be present in these matrices, or in any other samples of mammalian or animal origin, and our methods and results will be reported separately in the near future.

Conclusions

The four compounds representing the benzylic oxidation series of 1,4-dihydro-6,7-dimethyl-2,3-quinoxalinedione have been synthesized. Three of the compounds (but not the carboxylic acid **8**) compete with radioligands for binding to AMPA/kainate receptors, with IC_{50} values in the low micromolar range. Any possible neurophysiological significance remains to be demonstrated.

Experimental

Synthetic chemistry general procedures

Melting points were determined using a Thomas–Hoover capillary melting point apparatus and are reported without an emergent stem correction. Infrared spectra of samples (as KBr pellets unless otherwise noted) were obtained on a Nicolet Analytical 5MX FT or Nicolet Impact 400D FT spectrometer. NMR spectra were obtained with a JEOL FX-90 (90 MHz) spectrometer or a Brüker AM-400 (400 MHz) spectrometer. Chemical shifts were reported in parts per million (δ) relative to tetramethylsilane (unless otherwise noted). Electron-impact mass spectra were recorded on a Finnigan MAT TQS 4510 or Finnigan MAT 900 double-focusing magnetic sector spectrometer. Fast-atom bombardment mass spectra (FABMS) were obtained on a Finnigan MAT TSQ-70 equipped with an ANTEK cesium ion source and an acceleration voltage of 5 kV; or a Finnigan MAT 900 double-focusing instrument with a Finnigan cesium ion source and an acceleration voltage of 6 kV. HPLC analyses were performed on an ISCO binary gradient chromatograph equipped with two Model 2350 pumps, a Valco C6W injection valve (VICI, Inc.), and a UA-5 fixed-wavelength detector. All analyses were carried out with wavelength cutoff filters to select the 254 nm or 280 nm mercury emission lines, and using an Altex Ultrasphere™-Octyl 5 μm C8 (25 cm×4.6 mm) or ISCO 5 μm Spherisorb® C18 (25 cm×4.6 mm) column. Mobile phases were isocratic (compositions noted in each case below) or gradient combinations of the following solvents: Solvent A, 5% CH_3CN/0.1% CF_3COOH/H_2O; Solvent B, 75% CH_3CN/0.1% CF_3COOH/H_2O. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN, Oneida Research Services, Whitesboro, NY, or Atlantic Microlabs, Inc., Norcross, GA. The abbreviations used are tetrahydofuran, THF; *N,N*-dimethyl formamide, DMF; dimethylsulfoxide, DMSO. All reagents and solvents were used as obtained without further purification, unless otherwise noted. All solvents for recrystallization were analytical grade or better.

Synthetic methods

1,4-Dihydro-6,7-dimethyl-2,3-quinoxalinedione (5). 4,5-Dimethyl-1,2-phenylenediamine (3.54 g, 26.0 mmol) was dissolved in 52 mL of THF; diethyl oxalate (24.2 g, 165 mmol) and six drops of glacial acetic acid were then added. The flask was flushed with argon gas and maintained under a positive pressure of argon for three days of reflux, during which time the product crystallized out of the boiling solution. Upon cooling the reaction mixture, diethyl ether (200 mL) was added, and the suspension was shaken vigorously and filtered to provide a

cream-colored solid. This solid was resuspended in diethyl ether (200 mL), shaken vigorously, and filtered again. This procedure was repeated twice more, and the collected product was dried in air to a constant weight to yield 4.76 g (96%) of **5** as a tan powder. Recrystallization from a large volume of methanol/ethanol gave white plates, mp >340 °C [lit.[15a] >250 °C; lit.[15b] 340 °C (dec); lit.[15c] >325 °C (acetic acid); lit.[15d] 340 °C (dec)]; ^{1}H NMR (DMSO-d_6) δ 11.8 (s, 2H, NH), 6.89 (s, 2H, ArH), 2.18 (s, 6H, CH_3); these shift values are consistent with those reported by Cai et al.;[15a] IR (KBr, cm^{-1}) 3151 (NH), 1630 (C=O).

6,7-Dimethyl-2,3-dichloroquinoxaline (9). A solution of compound **5** (2.38 g, 0.0125 mol, dried overnight in an oven) in phosphorus oxychloride (10 mL, 0.11 mol) and *N,N*-dimethylaniline (2.6 g, 0.021 mol) was refluxed for 8 h under a dry argon atmosphere. The reaction mixture was then added very slowly with stirring into crushed ice (300 g). The resulting precipitate was collected by suction filtration, washed with water, and dried to provide an off-white amorphous solid (2.6 g, 94%), mp 177–179 °C; mp 180–181 °C after recrystallization from petroleum ether [lit.[31] mp 183 °C; lit.[32] mp 182 °C; lit.[15b] mp 270 °C (petroleum ether; this value seems likely to be erroneously reported)]; ^{1}H NMR ($CDCl_3$) δ 7.8 (s, 2H, ArH), 2.5 (s, 6H, CH_3); IR (KBr, cm^{-1}) {1618, 1540, 1478} (ring stretches), 1250s, 1195m, 1132s, 987s.

2,3-Dimethoxy-6,7-dimethylquinoxaline (10). Compound **9** (1.63 g, 7.2 mmol) was added to a mixture of sodium methoxide (0.90 g, 16.7 mmol) in dry methanol (10 mL), and the mixture was refluxed under a positive pressure of dry argon gas for 5 h. The reaction was cooled to room temperature, filtered, and the filter cake was dried overnight to obtain a yellow product. This product was suspended in a minimal amount of ether, the suspension was stirred for 30 min, and the product was collected by suction filtration to yield a light-yellow amorphous solid (1.48 g, 94%), mp 123–125 °C. Further purification could be achieved either by (1) direct recrystallization from ethanol or (2) partitioning between ethyl acetate and water, evaporating the (dried) ethyl acetate in vacuo, and recrystallizing the residue from ethanol; mp 139–140 °C (ethanol), [lit[18]. 142–144 °C (ethanol)]; ^{1}H NMR ($CDCl_3$) δ 7.67 (s, 2H, H5, H8; lit[18]. δ 7.53 in DMSO-d_6), 4.15 (s, 6H, OCH_3), 2.42 (s, 6H, CH_3); IR (KBr, cm^{-1}) {1640, 1576, 1560, 1479, 1390} (ring stretches), {1321, 1251} (C-O).

6-Bromomethyl-7-methyl-2,3-dimethoxyquinoxaline (11) and 6,7-bis(bromomethyl)-2,3-dimethoxyquinoxaline (**12**). Compound **10** (0.50 g, 2.3 mmol) was dissolved in carbon tetrachloride (10 mL). *N*-Bromosuccinimide (0.41 g, 2.3 mmol) and benzoyl peroxide (0.079 g, 0.33 mmol) were added, and the solution was refluxed under a dry argon atmosphere for 14 h. After cooling, the resulting suspension was passed through glass wool to remove the crystallized succinimide, and the filtrate was concentrated in vacuo to a small volume. This solution was chromatographed on a silica gel column (45 cm×5.1 cm), eluting with benzene. The fractions containing compound **11** were combined, as were those for compound **12**, and the benzene was distilled off on a rotary evaporator to provide the compounds **11** (0.37 g, 54%) and **12** (0.14 g, 16%). Recrystallization of each compound from ethyl acetate/hexane gave analytical samples: **11**, mp 148.5–149.5 °C; **12**, mp 154–155.5 °C.

Analytical data for compound **11**: ^{1}H NMR ($CDCl_3$) δ 7.73 (s, 1H, H5), 7.57 (s, 1H, H8), 4.64 (s, 2H, CH_2), 4.13 (s, 6H, OCH_3), 2.55 (s, 3H, CH_3); IR (KBr, cm^{-1}) 2987 (CH), {1580, 1518, 1483, 1385} (ring stretches), {1331, 1261} (C-O); MS *m/e* 298 (M^+, ^{81}Br), 296 (M^+, ^{79}Br), 217 (base, – Br). Anal. calcd for $C_{12}H_{13}BrN_2O_2$: C, 48.50; H, 4.41; N, 9.43; found: C, 48.52; H, 4.40; N, 9.35.

Analytical data for compound **12**: ^{1}H NMR ($CDCl_3$) δ 7.8 (s, 2H, H5), 4.83 (s, 4H, CH_2), 4.15 (s, 6H, OCH_3); IR (KBr, cm^{-1}) 2986 (CH), {1585, 1518, 1483, 1452, 1381} (ring stretches), {1336, 1260} (C-O); MS *m/e* 378 (M^+, $^{81}Br_2$), 376 (M^+, $^{79}Br^{81}Br$), 374 (M^+, $^{79}Br_2$), 297 (base, $-^{79}$Br), 295 (base, $-^{81}$Br). Anal. calcd for $C_{12}H_{12}Br_2N_2O_2$: C, 38.33; H, 3.22; N, 7.45; found: C, 38.45; H, 3.23; N, 7.46.

1,4-Dihydro-6-hydroxymethyl-7-methyl-2,3-quinoxalinedione (13). Compound **11** (0.50 g, 1.7 mmol) was refluxed in 1.2 N HCl (30 mL) overnight, and then allowed to cool. The precipitated solid was collected by vacuum filtration. Chloroform (15 mL) was added to this solid and the suspension was stirred at room temperature for 10 min. The solid was again collected by filtration, dried in a stream of air, and recrystallized from 95% ethanol to yield the dihydrate (0.20 g, 49%), mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 11.83 (bs, 2H, NH), 7.18 (s, 1H, ArH), 6.87 (s, 1H, ArH), 5.14 (s, 1H, OH), 4.45 (s, 2H, CH_2), 2.18 (s, 3H, CH_3); IR (KBr, cm^{-1}) 3440 (NH), 1685 (C=O), 1656 (ring stretch); MS (FAB) *m/e* 206, 207 (M+1). A separate analytical sample of the monohydrate was prepared by recrystallization from 95% ethanol. Anal. calcd for $C_{10}H_{10}N_2O_3{\cdot}H_2O$: C, 53.57; H, 5.39; N, 12.49; found: C, 53.61, 53.52; H, 5.42, 5.45; N, 12.55, 12.49.

1,4-Dihydro-6,7-bis(hydroxymethyl)-2,3-quinoxalinedione (14). In a similar fashion as for **11**, compound **12** (0.50 g, 1.3 mmol) was refluxed in 1.2 N HCl (30 mL) for 10 h. On cooling, the solid precipitate was collected by filtration, resuspended in chloroform (15 mL), and stirred at room temperature for 10 min. The product was again

collected by filtration and dried in a stream of air to give a shiny, light-tan-colored solid (0.22 g, 69%), mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 11.89 (s, 2H, NH), 7.19 (s, 2H, Ar-H), 4.48 (s, 4H, CH_2); IR (KBr, cm^{-1}) 1682 (C=O), 1390 (ring stretch), 1105 (C-O); MS (FAB) *m/e* 223 (M+1). Anal. calcd for $C_{10}H_{10}N_2O_4 \cdot 0.9 H_2O$: C, 50.38; H, 4.99; N, 11.75; found: C, 50.57; H, 4.97; N, 11.75. (Water of hydration was confirmed with a micro loss-on-drying analysis).

6-Formyl-7-methyl-2,3-quinoxalinedione (15). Compound **11** (1.50 g, 5.05 mmol) was dissolved in chloroform (8.4 mL), and a solution of hexamethylenetetramine (2.40 g, 17.9 mmol) in chloroform (4.2 mL) was added with vigorous stirring. A white solid separated almost immediately. This suspension was refluxed for 30 min, cooled, and the precipitate collected by filtration and washed with light petroleum (5 mL) to give a white solid. This solid was then combined with 50% aqueous acetic acid (20 mL), and the mixture was refluxed for 2 h. Concd aq HCl (4.2 mL) was then added, and the solution was refluxed for 5 min. The reaction mixture was cooled and kept in a freezer overnight, and the resulting solid was filtered off and recrystallized from ethanol to provide light-tan crystals (0.80 g, 76%), mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 12.16 (s, 1H, N(1)H), 12.02 (s, 1H, N(3)H), 10.13 (s, 1H, CHO), 7.52 (s, 1H, H5), 6.97 (s, 1H, H8), 2.57 (s, 3H, CH_3); IR (KBr, cm^{-1}) 3590 (NH), 1716 (aldehyde C=O), 1685 (quinoxalinedione C=O); MS (FAB) *m/e* 204. Anal. calcd for $C_{10}H_8N_2O_3 \cdot 0.25 H_2O$: C, 57.55; H, 4.11; N, 13.42; found: C, 57.66; H, 4.26; N, 13.08.

2-Methyl-4-nitrobenzoic acid. A mixture of 6.7 g (41 mmol) of 2-methyl-4-nitrobenzonitrile,[21,33] 10% aq sodium hydroxide (81 mL), and 10% hydrogen peroxide (42 mL) was brought to reflux. After 2.5 h, the water circulation in the condenser was halted for 5–10 min to remove dissolved ammonia. The water flow was restored, reflux was continued for 1 h, and the reaction mixture was then allowed to cool. Concd HCl was added until the product precipitated completely; it was then collected by suction filtration and washed with a little cold water. Recrystallization from hot water yielded 6.17 g (83%) of light-yellow needles, mp 149–152 °C (lit.[33] 151 °C (water), lit.[34] 152 °C (water(?)), lit.[35] 153–154 °C; lit.[36] 153–154 °C); ^{1}H NMR (DMSO-d_6) δ 8.21–7.97 (m, 3H, ArH), 2.64 (s, 3H, $ArCH_3$); IR (KBr, cm^{-1}): 1698 (C=O), 1526 (NO_2), 1354 (NO_2), 1275 (C-O).

4,5-Dinitro-2-methylbenzoic acid (16). The procedure was that of Goldstein and Tardent.[21] From 1.81 g (1.00 mmol) of 2-methyl-4-nitrobenzoic acid, 2.02 g (89%) of **16** was obtained (after recrystallization from hot water) as pale-yellow needles, mp 184–189 °C (lit.[21] 186 °C (water)); ^{1}H NMR (DMSO-d_6) δ 8.51 (s, 1H, ArH), 8.23 (s, 1H, ArH), 2.68 (s, 3H, $ArCH_3$); IR (KBr, cm^{-1}): 1712 (C=O), 1547 (NO_2), 1368 (NO_2), 1275 (C-O).

4,5-Diamino-2-methylbenzoic acid (17). A 250 mL round-bottomed flask was charged with 4,5-dinitro-2-methylbenzoic acid (**16**; 1.25 g, 5.52 mmol), hydrazine hydrate (100%, 3.2 mL), and ethanol (65 mL). A catalytic amount of Raney nickel was added portionwise with stirring while the reaction mixture was heated under reflux.[37] The reaction was monitored by HPLC (30% CH_3CN/0.1% CF_3COOH/H_2O, 1.2 mL/min; t_R = 15.6 min and 2.2 min for the starting material and product, respectively), and continued just to the point of completion, about 2–3 h. The reaction mixture was filtered, and the filtrate concentrated under reduced pressure to a semisolid residue, which was recrystallized from ethanol to give **17** (688 mg, 74.8%). The pure compound is presumably colorless, but the isolated pinkish solid quickly turns red and eventually brown upon standing, particularly when exposed to light; ^{1}H NMR (DMSO-d_6) δ 7.18 (s, 1H, ArH), 6.32 (s, 1H, ArH), ~4.25 (bs, NH), 2.33 (s, 3H, $ArCH_3$). Elemental analysis was obtained for the dihydrochloride salt (see below).

4,5-Diamino-2-methylbenzoic acid dihydrochloride. A 15 mL round-bottomed flask was charged with 4,5-dinitro-2-methylbenzoic acid (**16**; 417 mg, 1.84 mmol) and powdered tin (712 mg, 6.0 mmol), and equipped with a reflux condenser. Concd HCl (4.5 mL) was added slowly; the reaction is quite vigorous. The progress was monitored by HPLC (flow rate 1.2 mL/min, eluting 3 min with Solvent A, then a linear gradient to Solvent B over 9 min, then Solvent B for 4 min; starting material: t_R = 14 min, product: t_R = 10 min), and the reaction was continued just to the point of completion, about 25–40 min. The resulting mixture was diluted with water (200 mL) to yield a clear solution, and hydrogen sulfide gas was passed into the liquid until all the tin was precipated as sulfide (to monitor this process, a small sample was filtered from time to time and treated with hydrogen sulfide to check for additional precipitate formation). The precipitate was allowed to settle overnight, the clear liquid was decanted, and the remaining residue was gravity-filtered through paper. The combined filtrate and decantated liquid was evaporated in vacuo until crystals appeared, and then cooled in ice. The crystals were collected by suction filtration, washed with a little concd HCl, and dried in air to yield 321 mg (72.9%) of dihydrochloride, dp 218 °C; ^{1}H NMR (DMSO-d_6) δ 7.78 (s, 1H, ArH), 6.64 (s, 1H, ArH), ~6.5 (v br s, NH_3^+ and H_2O), 2.41 (s, 3H, $ArCH_3$). Anal. calcd for $C_8H_{10}N_2O_2 \cdot 2HCl \cdot 0.75H_2O$: C, 38.34; H, 4.63; N, 11.18; Cl, 28.29; found: C, 38.75, 38.69; H, 4.89, 4.92; N, 11.26, 11.29; Cl, 28.25.

1,4-Dihydro-7-methyl-2,3-dioxoquinoxaline-6-car-boxylic acid (8). 4,5-Diamino-2-methylbenzoic acid (**17**, 436 mg, 2.63 mmol) was dissolved in THF (5.2 mL). Diethyl oxalate (2.3 mL) was added, followed by two drops of glacial acetic acid, and the mixture was refluxed under a positive pressure of argon gas for three days. Upon cooling the reaction mixture, diethyl ether (30 mL) was added to make a suspension, which was shaken vigorously and then filtered to provide a cream-colored solid. This solid was resuspended in diethyl ether (30 mL) and filtered off again, and the collected product was dried in air overnight to give 535 mg (91%) of **8** as an off-white powder, mp $>250\,^{\circ}C$; ^1H NMR (DMSO-d_6) δ 11.92 (bs, 2H, NH), 7.68 (s, 1H, ArH), 6.95 (s, 1H, ArH), 2.48 (s, 3H, CH_3). Recrystallization from 95% ethanol gave small, light-tan rhombic crystals of the monoethanolate; ^1H NMR (DMSO-d_6) δ 12.74 (bs, 1H, COOH), 12.04 (bs, 1H, NH), 11.92 (bs, 1H, NH), 7.68 (s, 1H, ArH), 6.95 (s, 1H, ArH), 4.36 (bs, 1H, $CH_3CH_2O\mathit{H}$), 3.37 (m, 2H, $CH_3C\mathit{H}_2OH$), 2.48 (s, 3H, $ArCH_3$), 1.05 (t, 3H, $J = 7.0$ Hz, $C\mathit{H}_3CH_2OH$); ^{13}C NMR (DMSO-d_6) 168.2, 156.0, 155.4, 135.2, 129.0, 124.7, 124.0, 118.2, 117.8, 56.6 (CH_3CH_2OH), 21.7 ($ArCH_3$), 19.7 (CH_3CH_2OH). Anal. calcd for $C_{10}H_8N_2O_4{\cdot}CH_3CH_2OH$: C, 54.13; H, 5.30; N, 10.52; found: C, 54.46; H, 5.50; N, 10.62.

Pharmacological testing

AMPA binding assay. The interaction of compounds with AMPA receptors was assessed by inhibition of specific [^{3}H]AMPA binding in washed membrane preparations of rat brain following the procedure originally published by Honore et al.[38] After the freeze/thaw cycle and two additional washings, the preparation was incubated with 0.04% Triton X-100 at 37 °C for 30 min to remove an inhibitor protein. After another wash, membranes were incubated with [^{3}H]AMPA (1.4 nM final concentration) and solutions of test compounds at appropriate concentrations in 50 mM tris-HCl buffer containing 2.5 mM $CaCl_2$ and 100 mM KSCN, pH 7.2. The mixture was incubated on ice for 60 min with periodic shaking, following which bound and free ligand were separated by rapid filtration through Whatman GF/C filter paper using a Brandel M-12R cell harvester (Gaithersburg, MD). The resulting filter disks, containing membrane-bound radioligand, were dissolved in Scintiverse-E® cocktail for scintillation counting. Nonspecific binding was determined in the presence of 1 mM glutamate. For determination of potency, a range of test compound concentrations (0.01–100 μM) was tested. IC_{50} values were calculated using nonlinear regression analysis (InPlot® software, GraphPad, Inc.).

Kainate binding assay. Interaction of compounds with kainate receptors was assessed by inhibition of specific [^{3}H]kainate binding sites. Tissue preparation was exactly the same as for the AMPA studies to the point of the freeze/thaw cycle. After thawing, the membranes were washed twice with 50 mM tris-HCl buffer containing 2.5 mM $CaCl_2$, pH 7.1. Binding was then performed by incubating membranes with [^{3}H]kainate (1 nM final concentration), and solutions of test compounds at appropriate concentrations, in a 50 mM Tris-citrate buffer, pH 7.4. The mixture was incubated for 60 min on ice before separating bound and free ligand by the method described above.

CNQX binding assay. Inhibition of [^{3}H]CNQX specific binding was assessed according to the procedure of Honoré et al.[39]

Neurotransmitter release assay. This assay is based on the fact that AMPA receptor activation induces norepinephrine release from hippocampal nerve endings.[40] The following procedure has been adapted from the method of Desai et al.[41] Briefly, mouse hippocampi were dissected out and chopped into 0.3 mm×0.3 mm slices. Slices were incubated with 0.2 μM [^{3}H]norepinephrine in Krebs buffer for 30 min, and then transferred to superfusion chambers and washed for 60 min with warmed, oxygenated Krebs buffer at 0.3 mL/min. Following this, ten 5 min fractions were collected. After the 3rd collection (i.e., fraction 3), buffer containing 50 μM cyclothiazide was introduced. The presence of cyclothiazide diminishes AMPA receptor desensitization, thus allowing for a more readily quantifiable AMPA response. After the collection of fraction 4, buffer containing drug, cyclothiazide, and 100 μM AMPA was introduced for 5 min, followed by normal buffer until the end. For initial screening purposes, tissue slices were exposed to 1 mM test compound alone (test for possible agonist activity) run in parallel with tissue slices exposed to test compound plus 100 μM AMPA (test for antagonist). Each condition was run in triplicate. At least two experiments were conducted to generate an $n = 6$ for each treatment. For dose-response data, a minimum of four concentrations of each test compound were run against 100 μM AMPA (for antagonists) or alone (for agonists). Determination of IC_{50} values was accomplished utilizing median effect plot analysis (Chou and Talalay, 1983).[42]

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