

Multifunctional Antioxidants for the Treatment of Age-Related Diseases

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Analogues of *N,N*-dimethyl-4-(pyrimidin-2-yl)piperazine-1-sulfonamide possessing a free radical scavenger group (FRS), chelating groups (CHL), or both (FRS + CHL) have been synthesized. Electro-spray ionization mass spectrometry studies indicate that select members of this series bind ions in the relative order of $\text{Cu}^{1+} = \text{Cu}^{2+} > \text{Fe}^{2+} = \text{Fe}^{3+} > \text{Zn}^{2+}$ with no binding of Ca^{2+} or Mg^{2+} observed. In vitro evaluation of these compounds in human lens epithelial, human retinal pigmented epithelial, and human hippocampal astrocyte cell lines indicates that all analogues possessing the FRS group as well as the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid protect these cells against decreased cell viability and glutathione levels induced by hydrogen peroxide. In addition, those compounds possessing CHL groups also protected these cells against hydroxyl radicals generated by the Fenton reaction. These compounds are good candidates for the preventive treatment of cataract, age-related macular degeneration (AMD), and Alzheimer's dementia (AD).

Introduction

Oxidative stress damages cells and is an accepted determinant of both lifespan and health span. Oxidative stress results from the generation of reactive oxygen species (ROS^a), primarily H_2O_2 , and is enhanced by the age-related decrease of cellular antioxidant defenses and accumulation of iron and/or copper.¹ The increased cellular presence of these redox-active metals enhance H_2O_2 -linked ROS by generating hydroxyl radicals through the Fenton reaction.^{2,3} Age-related increases in ROS are believed to trigger biochemical cascades that lead to neurodegenerations such as AD,^{4–6} retinal degenerations such as age-related macular degeneration (AMD),^{7–10} and cataract formation.^{7,11} While these diseases are pathologically different, studies suggest that they all undergo similar oxidative damage from ROS associated with redox-active metal ions. For example, ROS-associated A β in amyloid plaques, which are characteristic of AD,^{4,12–15} is also present in AMD^{16–18} and age-related cataract.^{19–21}

The oxidative pathways associated with neurodegenerations and age-related ocular diseases have been targeted as a potential therapeutic approach to the treatment of these age-related diseases.⁵ A wide variety of antioxidants have been examined to reduce ROS. These range from natural products with antioxidant properties such as aged garlic extract, curcumin, melatonin, resveratrol, *Ginkgo biloba* extract, green tea, vitamin C, L-carnosine, vitamin E, and cannabinoids to derivatives of lipoic acid, analogues of coenzyme Q (MitoQ), and “thiol-delivering” glutathione mimics such as tricyclo-decan-9-yl xanthogenate.^{22–29} Some of these agents have shown limited clinical efficacy with AMD,^{30,31} but none have

demonstrated clinical efficacy in reducing the clinical signs of neurodegeneration or cataract.

Reducing ROS through the chelation of redox-active metals is another approach that has been investigated; however, this approach has primarily been used to treat neurodegenerations. Examples are illustrated in Figure 1. Of these compounds, only the iron chelator desferrioxamine (DFO, *N'*-{5-[acetyl(hydroxy)amino]pentyl}-*N*-[5,4-[(5-aminopentyl)-(hydroxy)amino]-4-oxobutanoyl]amino]pentyl]-*N*-hydroxy-succinamide) and clioquinol (5-chloro-7-iodoquinolin-8-ol) has been investigated for both neurological^{32,33} and age-related ocular diseases.^{34–36} Desferrioxamine is not orally active and does not significantly cross the blood–brain barrier.^{37,38} Clioquinol is an orally active antibiotic that was extensively used as an antibiotic in Asia in the 1960s. In 1971 it was banned in Japan after being linked to subacute myeloptoptic neuropathy (SMON) in over 10 000 Japanese people. Its chelating ability was subsequently identified during neurotoxicity studies³⁹ where it has been demonstrated to decrease Cu uptake and counteract Cu efflux activities of the amyloid precursor protein of AD,⁴⁰ disaggregate the metal ion-induced aggregates of A β (1–40), and retard fibril growth through a Zn(II) clioquinol complex formation.⁴¹ Its efficacy has been demonstrated in vitro, in in vivo animal models, and in several small clinical trials of AD patients.^{15,42,43} Because of the promising results of clioquinol, several other 8-hydroxy-quinoline analogues have been developed: PBT2 (structure not disclosed to date),^{44,45} M-30 (5-((methyl(prop-2-ynyl)-amino)methyl)quinolin-8-ol),^{46,47} VK-28 5-((4-(2-hydroxyethyl)-piperazin-1-yl)methyl)quinolin-8-ol),⁴⁶ HLA-20 (5-((4-(prop-2-ynyl)piperazin-1-yl)methyl)quinolin-8-ol),⁴⁶ deferiasirox (4-(3,5-bis(2-hydroxyphenyl)-1*H*-1,2,4-triazol-1-yl)benzoic acid),^{46–48} deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1*H*)-one),⁴⁹ feralax (2-(3-hydroxy-2-methyl-4-oxo-3,4-dihydropyridin-1(2*H*)-yl)-*N*-((3*R*,4*R*,5*S*,6*R*)-2,4,5-trihydroxy-6-(hydroxylmethyl)-tetrahydro-2*H*-pyran-3-yl)acetamide,^{50,51} D-penicillamine ((*S*)-2-amino-3-mercapto-3-methylbutanoic acid),⁵²

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^a Abbreviations: AD, Alzheimer's dementia; AMD, age-related macular degeneration; BSA, bovine serum albumin; CHL, chelating groups; FRS, free radical scavenger groups; hLECs, human lens epithelium cells; HA-h, human hippocampal astrocytes; ROS, reactive oxygen; SDI, sorbitol dehydrogenase inhibitor.

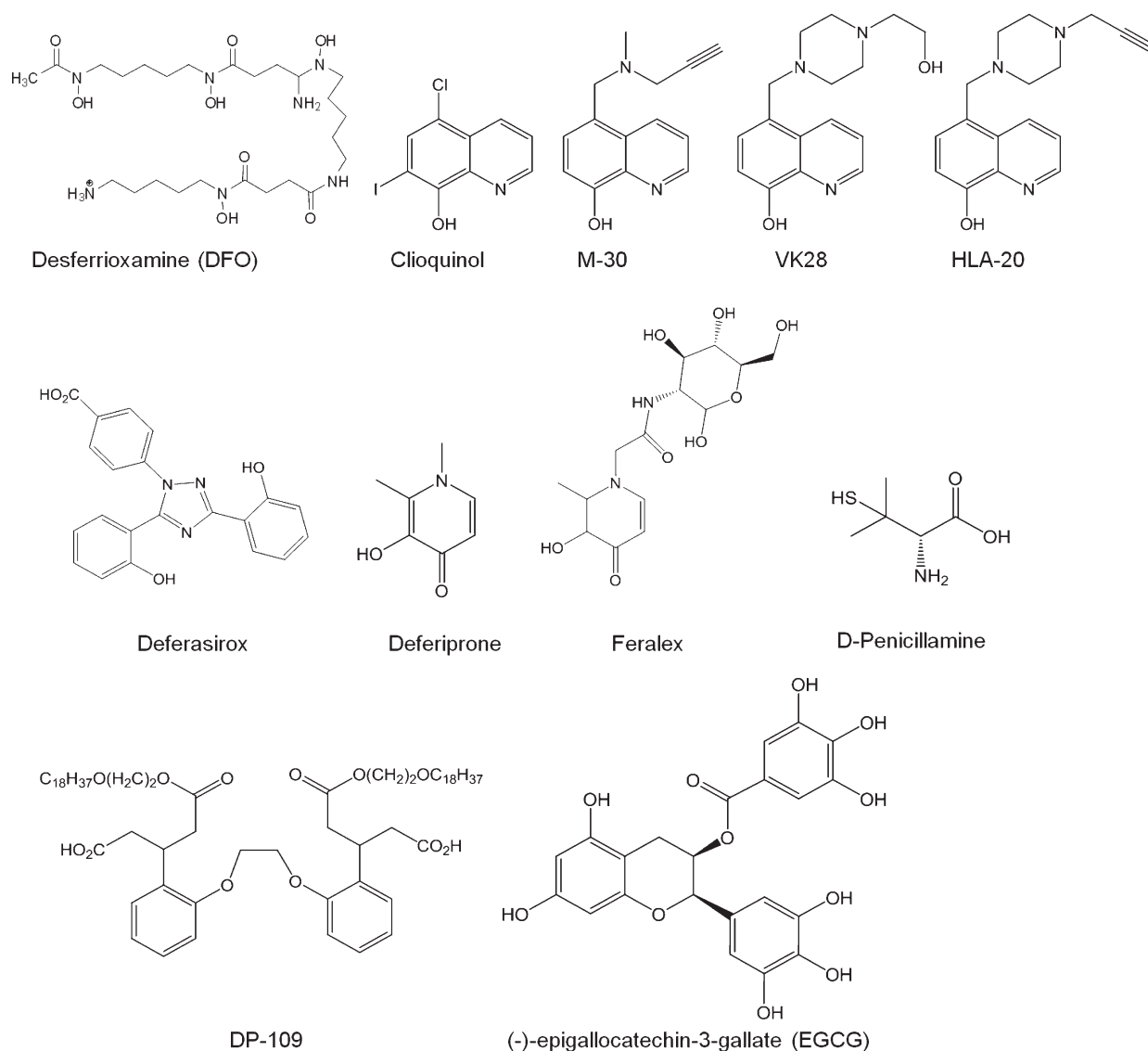


Figure 1. Structure of antioxidant and chelators evaluated for cataract, AMD, and AD.

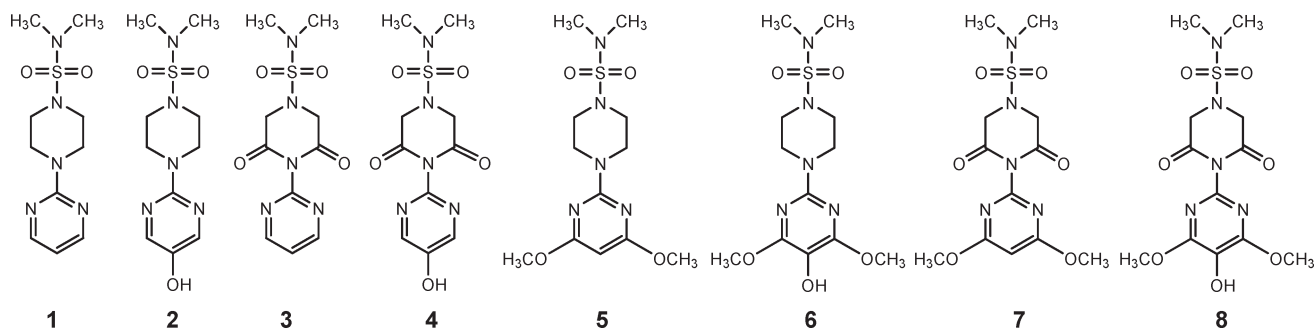


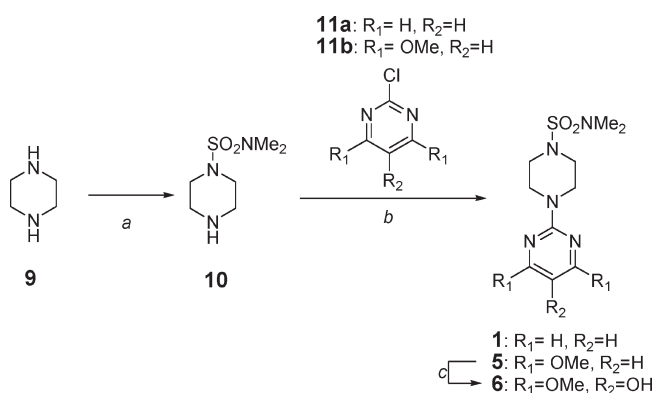
Figure 2. Analogues of *N,N'*-dimethyl-4-(pyrimidin-2-yl)piperazine-1-sulfonamide (**1**) synthesized and evaluated.

DP-109 (3,3'-(2,2'-(ethane-1,2-diylbis(oxy))-bis(2,1-phenylene))-bis(5-(2-(octadecyloxy)ethoxy)-5-oxopentanoic acid),⁵³ and (-)-epigallocatechin 3-gallate (EGCG) ((2*R*,3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5-trihydroxybenzoate).⁵⁴

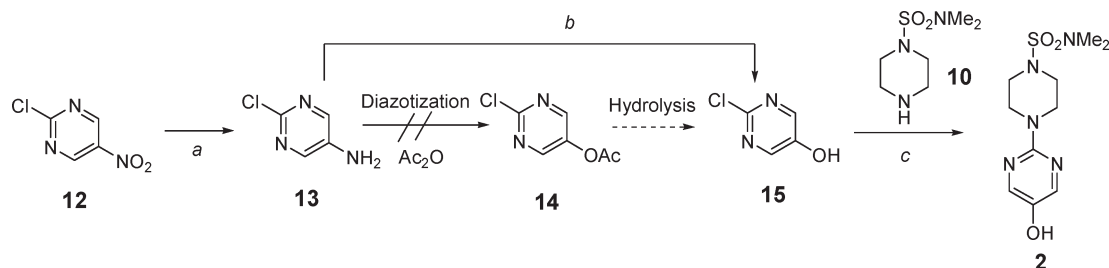
To date, many research efforts on the treatment of ROS-linked complications have focused on therapeutic targets that enhance cellular antioxidant defenses, demonstrate antioxidant activity, or regulate cellular levels of transition metal ions.^{43,55} Because multiple mechanisms are involved

in the development of ROS-linked disorders, drugs with at least two mechanisms of action targeted at ROS may offer more therapeutic benefit than those only targeting a single mechanism. Toward this end, we have synthesized a series of multifunctional analogues of *N,N'*-dimethyl-4-(pyrimidin-2-yl)piperazine-1-sulfonamide (**1**) (Figure 2) possessing a FRS group, (analogues **2**, **6**), CHL groups (CHL, analogues **3**, **7**), or both (analogues **4**, **8**).⁵⁶ The ring structure of the parent compound **1** was derived from studies investigating the effect

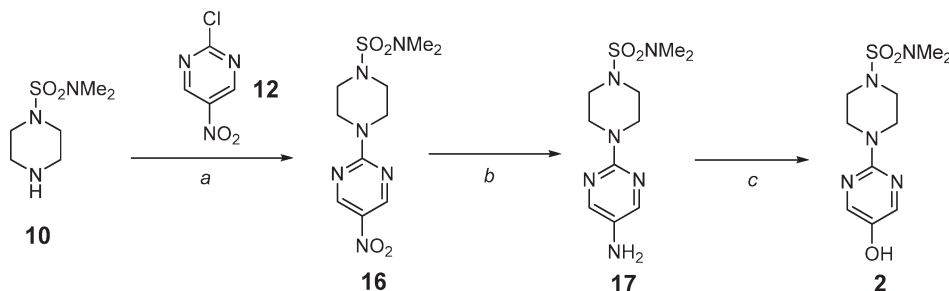
of sorbitol dehydrogenase inhibitors (SDI) on sugar cataract formation.⁵⁷ FRS activity was introduced to **1** by addition of an -OH group in the 5-position of the pyrimidine ring. This was based on a report that 5-pyrimidinols are more effective antioxidants than their corresponding phenols, with 2-*N,N*-dimethyl-4,6-dimethyl-5-hydroxypyrimidine being 5-fold more reactive toward alkyl radicals and essentially equally reactive to peroxy radicals compared to α -tocopherol.⁵⁸ Methoxy groups, rather than methyl groups, were added to the pyrimidine ring because methoxy groups stabilize the radical scavenger slightly better than the methyl groups and are not as readily subjected to metabolic oxidation as the methyl groups.^{59–61} The ability to chelate was introduced by adding carbonyl groups directly adjacent to the amino group connecting the piperazine ring to the pyrimidine ring. This was based on a report that 2-*N*-succinamide-1,3-pyrimidine easily forms complexes with transition metals such as Fe^{3+} and Cu^{2+} .⁶²

Scheme 1^a

^a Reagents and conditions: (a) $\text{ClSO}_2\text{NMe}_2$, K_2CO_3 , EtOH, room temperature; (b) TEA, THF, reflux; (c) *n*-BuLi, THF, O_2 , -78°C to room temperature.

Scheme 2^a

^a Reagents and conditions: (a) Fe, AcOH, reflux; (b) 2 M H_2SO_4 , reflux; (c) TEA, THF, reflux.

Scheme 3^a

^a Reagents and conditions: (a) TEA, THF, room temperature; (b) Fe, NH_4Cl , EtOH- H_2O , reflux; (c) 2 M H_2SO_4 , reflux.

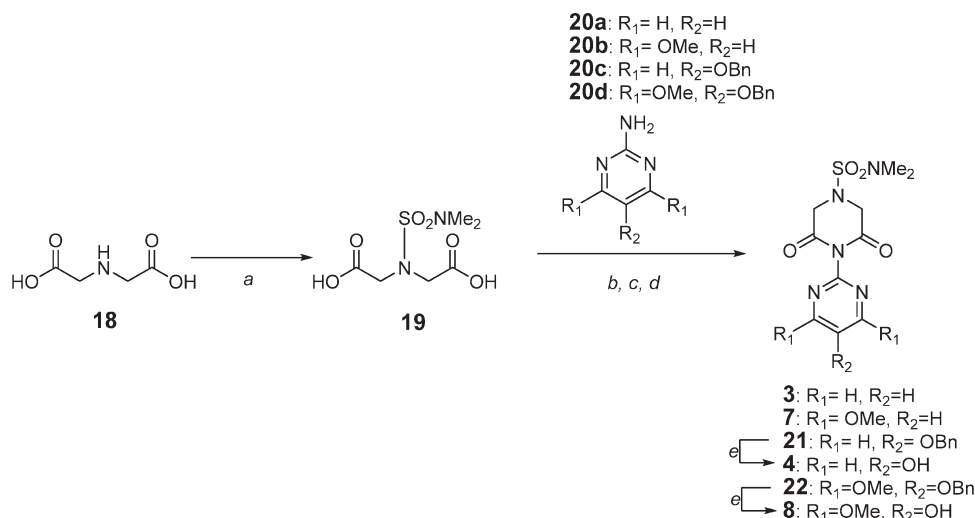
Chemistry

Compounds **1**, **5**, and **6** were synthesized as outlined in Scheme 1. *N,N*-Dimethylpiperazine-1-sulfonamide **10** was obtained from commercially available piperazine **9** according to the literature.⁶³ Nucleophilic substitution of 2-chloropyrimidines **11a** and **11b** with *N,N*-dimethylpiperazine-1-sulfonamide **10** gave **1** and **5**, respectively. Compound **6** was obtained through directed hydroxylation of **5**. The aromatic anion of **5**, which was generated in the presence of *n*-BuLi, was treated with oxygen gas to give **6**.⁶⁴

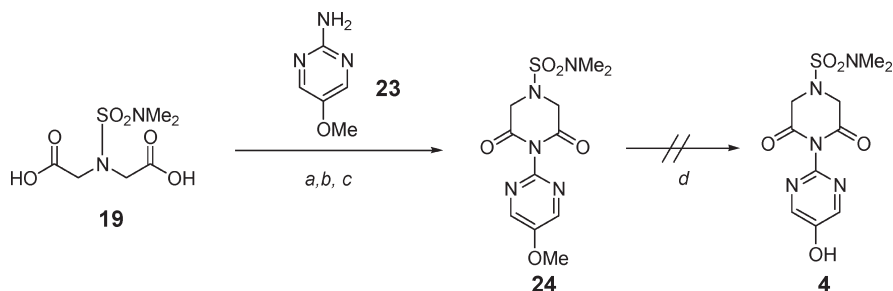
The 5-hydroxypyrimidine analogue **2** was also initially obtained by nucleophilic substitution of 2-chloro-5-hydroxypyrimidine (**15**) with *N,N*-dimethylpiperazine-1-sulfonamide (**10**) (Scheme 2). Compound **15** was obtained from commercially available 2-chloro-5-nitropyrimidine (**12**) by first reducing the nitro group to 2-chloro-5-aminopyrimidine (**13**) by refluxing with acetic acid in the presence of iron. Subsequent diazotization of the amine **13** failed; however, 2-chloro-5-hydroxypyrimidine **15** could be obtained in poor yield (33%) by refluxing **13** with 2 M sulfuric acid.⁶⁵

Because of the poor yield in obtaining **15**, an alternative approach to compound **2** was employed. As outlined in Scheme 3, an initial nucleophilic reaction of *N,N*-dimethylpiperazine-1-sulfonamide **10** with 2-chloro-5-nitropyrimidine **12** gave the nitro product **16** which was reduced to the amine **17** by refluxing with NH_4Cl and iron. Refluxing amine **17** with 2 M sulfuric acid gave **2** in 56% yield.

The piperazine-2,6-dione containing analogues were synthesized by condensation of 2,2'-(*N,N*-dimethylsulfamoylazanediyldiacetic acid (**19**), obtained from commercially available iminodiacetic acid (2,2'-azanediylidiacetic acid) (**18**), with primary aromatic amines **20a–d** (Scheme 4). The piperazine-2,6-diones **3**, **7**, **21**, and **22**, respectively, were obtained by activation of the 2,2'-(*N,N*-dimethylsulfamoyliminodiacetic acid (**19**) with acetic anhydride (step b), addition of **20a–d** (step c), and subsequent dehydration (step d).⁶⁶ The benzyl

Scheme 4^a

^a Reagents and conditions: (a) ClSO₂NMe₂, NaOH, Na₂CO₃, acetone–H₂O, 0 °C to room temperature; (b) Ac₂O, 85 °C; (c) acetone, toluene, 80 °C; (d) Ac₂O, 80 °C; (e) Pd–C, H₂, EtOAc, room temperature.

Scheme 5^a

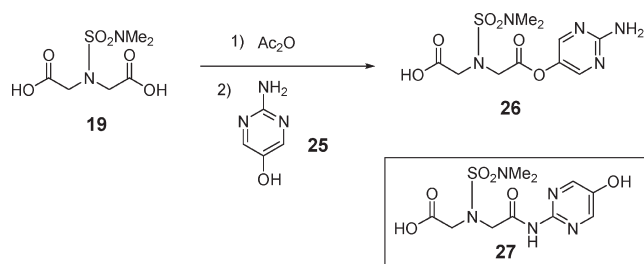
^a Reagents and conditions: (a) Ac₂O, 85 °C; (b) acetone, toluene, 80 °C; (c) Ac₂O, 80 °C; (d) BBr₃, benzene, room temperature; or TMSI, CHCl₃; or MeCN.

protecting groups of **21** and **22** were removed by hydrogenolysis to give final **4** and **8**, respectively. Compounds **20a** and **20b** were commercially available.

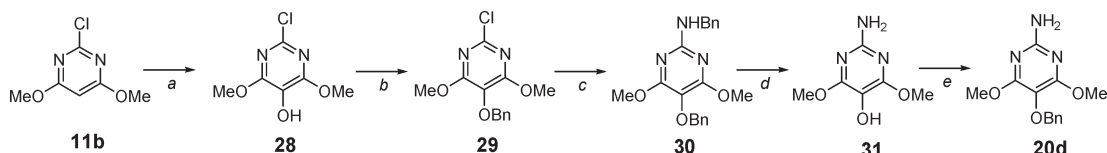
To obtain compound **4**, the synthesis outlined in Scheme 5 was initially employed. The methoxy **24** was obtained in 60% yield in a three-step pot reaction from **19** and **23** according to published procedures.⁶⁷ However, attempts at selectively cleaving the methoxy group of **24** failed. In the presence of BBr₃, a number of cleavage products were obtained, presumably because of the instability of 2,6-dioxopiperazine in the presence of the strong Lewis acid. Alternatively, no reaction was observed with mild treatment of **24** with TMSI for 12 h. Condensation of **19** with 2-amino-5-hydroxypyrimidine, **25**, the cleaved methoxy product of **23**, also did not result in the anticipated amide **27** (Scheme 6). Instead, ester **26** was formed because the 5-hydroxyl group of 2-amino-5-hydroxypyrimidine, **25**, is more reactive; however, protection of the hydroxyl group with an easily removable benzyl group gave amine **20c** which when condensed yielded compound **21**. Subsequent hydrogenolysis of **21** gave the desired compound **4**.

2-Amino-5-(benzyloxy)-4,6-dimethoxypyrimidine (**20d**) was obtained from commercially available 2-chloro-4,6-dimethoxypyrimidine **11b** (Scheme 7). Oxidation of the aromatic anion of **11b** generated in the presence of dioxigen gave the hydroxyl **28** in 44% yield. This yield was increased to 94% when dioxigen was replaced with lithium *tert*-butyl peroxide which was generated in situ from *tert*-butylperoxy alcohol

Scheme 6



and *n*-butyllithium.⁶⁸ Following benzylation of the hydroxyl group, the 2-chlorine atom was replaced with an amino group. Attempted substitution of the chlorine with an ammonia–methanol solution or ammonia hydroxide was unsuccessful. Refluxing chloride **29** in toluene with benzylamine resulted in the formation of the 2-benzylamine **30** in 33% yield and a 64% yield (according to the procedure for preparation of compound **30**) with 25% recovery of chloride **29**. Replacing toluene with dioxane increased the yield of **30** to 55% with 25% recovery of **29**. Attempted selective hydrogenolysis of **30** for 2 h at room temperature and 1 atm resulted in 2-benzylamino-4,6-dimethoxy-5-hydroxypyrimidine in 90% yield and 2-amino-4,6-dimethoxy-5-hydroxypyrimidine **31** in 10% yield. Both benzyl groups were removed to give **31** in 98% yield when hydrogenolysis was increased to 12 h. The benzyl group was

Scheme 7^a

^a Reagents and conditions: (a) LDA, *t*-BuOOLi, THF, -78°C ; (b) BnBr, K_2CO_3 , MeCN, room temperature; (c) BnNH₂, K_2CO_3 , dioxane, reflux; (d) Pd-C, H₂, MeOH, room temperature; (e) BnBr, K_2CO_3 , MeOH, room temperature.

Table 1. Percent Parent Peaks Remaining at 10:1 Metal Ion Solution/Compound Obtained by ESI-MS

compd	% parent peaks in ion solutions						
	Fe ²⁺	Fe ³⁺	Cu ¹⁺	Cu ²⁺	Zn ²⁺	Ca ²⁺	Mg ²⁺
1	100.0	100.0	100.0	100.0	100.0	100.0	100.0
2	100.0	100.0	100.0	100.0	100.0	100.0	100.0
3	8.1	10.0	3.6	4.4	15.3	100.0	100.0
4	6.9	8.2	4.2	3.0	12.7	100.0	100.0
5	100.0	100.0	100.0	100.0	100.0	100.0	100.0
6	100.0	100.0	100.0	100.0	100.0	100.0	100.0
7	7.3	7.6	4.1	3.4	13.8	100.0	100.0
8	7.5	8.2	4.0	3.6	14.6	100.0	100.0

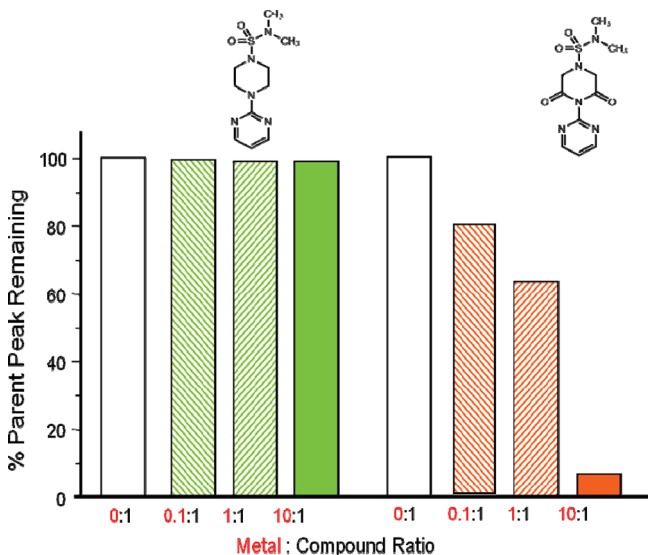


Figure 3. ESI-MS analysis of parent peak of *N,N'*-dimethyl-4-(pyrimidin-2-yl)piperazine-1-sulfonamide (**1**) and *N,N'*-dimethyl-3,5-dioxo-4-(pyrimidin-2-yl)piperazine-1-sulfonamide (**3**) in the presence of increasing amounts of Fe²⁺ solutions.

then reintroduced to the 5-hydroxy group to give **20d** under conditions similar to those employed for the synthesis of **20c**.

Chelation Studies. The relative amounts of Fe²⁺, Fe³⁺, Cu¹⁺, Cu²⁺, Zn²⁺, Ca²⁺, Mg²⁺ ions bound by compounds **1–8** were determined by electrospray ionization mass spectrometry (ESI-MS) by mixing each ion solution with compound in molar ratios of 10:1, 1:1, and 1:10 and then directly infusing each solution under constant ionization energy. Table 1 summarizes the relative percent parent peak observed with a 10:1 metal ion/compound solution for each compound. Under these conditions, a relative decrease in compound parent peak height only occurs with increased ion binding. This is illustrated in Figure 3 where the parent peak heights of compounds **1** and **3** are compared. Only compound **3** which contains the necessary piperazine-2,6-dione groups required for ion binding shows a decrease in peak height with increasing concentration of Fe²⁺. Ion

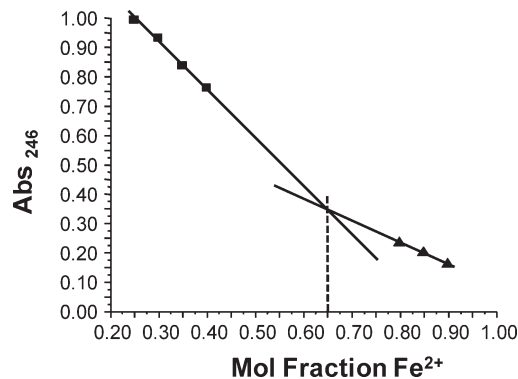


Figure 4. Job's plot of compound **4** in acetate solution (1 mM, pH 6.5) at 23°C . The final concentration of compound **4** and Fe²⁺ ion was maintained constant at 0.1 mM.

Table 2. Stoichiometry of Metal Ion Binding to Compounds Obtained from Job Plots^a

compd	stoichiometry in ion solutions						
	Fe ²⁺	Fe ³⁺	Cu ¹⁺	Cu ²⁺	Zn ²⁺	Mg ²⁺	Ca ²⁺
1	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b
2	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b
3	2:1.06	2.101	1.212	1:2.03	1:1.94	0 ^b	0 ^b
4	2:1.08	2.096	1.194	1:1.99	1:2.08	0 ^b	0 ^b
5	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b
6	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b
7	2:1.08	2.099	1.194	1:1.99	1:1.86	0 ^b	0 ^b
8	2:1.10	2.099	1.190	1:2.08	1:1.99	0 ^b	0 ^b

^a The total concentration of compound to metal ion was maintained at 0.1 mM. ^b Nonintersecting lines in the Job plot.

binding was only observed with compounds **3**, **4**, **7**, and **8** all of which possess the required piperazine-2,6-dione groups. These compounds bound ions with a relative binding order of Cu¹⁺ = Cu²⁺ > Fe²⁺ = Fe³⁺ > Zn²⁺. No binding was observed with either Ca²⁺ or Mg²⁺.

To obtain the stoichiometry of the chelator–ion complexes, Job plots were prepared for compounds **1–8** with Fe²⁺, Fe³⁺, Cu¹⁺, Cu²⁺, Zn²⁺, Ca²⁺, and Mg²⁺. The maximum absorbance for each compound as a function of the mole fraction of the metal ion was determined and plotted to give linear dependences at high and low molar fractions. The stoichiometry of the formed complexes was determined by the intersections of these lines. The intersection of these lines corresponded to the stoichiometry of the complex formed (Figure 4). Intersecting lines were not observed with compounds **1**, **2**, **5**, or **6** or any compounds incubated with Ca²⁺ and Mg²⁺; therefore, no stoichiometry could be obtained for these compounds or ions. This was consistent with the ESI-MS data. Binding stoichiometry for compounds **3**, **4**, **6**, and **7**, summarized in Table 2, was determined to be 1 metal chelator/2 metal ions for those ions with a tetrahedral/square planar geometry (Cu¹⁺, Cu²⁺, Zn²⁺) or to be 2 metal

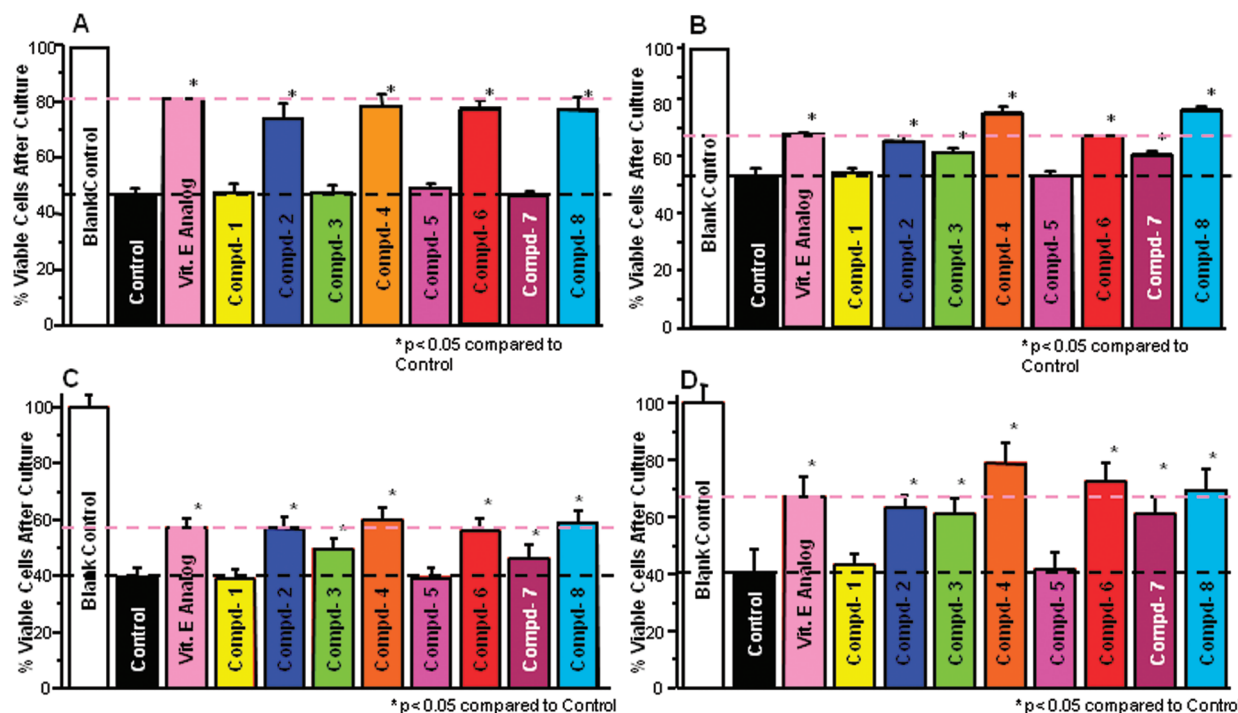


Figure 5. In vitro MTS viability assay of HLECs subjected to ROS. Part A illustrates exposure of HLECs exposed for 2 h with 1 mM H_2O_2 with/without the presence of the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or compounds 1–8. Part B illustrates 2 h exposure of HLECs with 1 mM Fenton's reagent with/without the presence of the vitamin E analogue or compounds 1–8. Parts C and D illustrate similar 2 h exposure of hRPE and HA-h cells, respectively, with 1 mM Fenton's reagent with/without the presence of the vitamin E analogue or compounds 1–8. All studies were normalized against MTS cell staining obtained without ROS (blank control), and the results represent the mean $\pm$ SD of three separate experiments. Significant differences ($p > 0.05$), calculated by ANOVA, were compared to control staining obtained with ROS in the absence of either the vitamin E analogue or compounds 1–8.

chelators/1 metal ion for ions having an octahedral geometry (Fe^{2+} , Fe^{3+}). This stoichiometry was consistent with the data of a similar chelating moiety in 2-(*N*-succinimidyl) pyrimidine.⁶²

In Vitro Evaluation of Antioxidant Activity. Compounds 1–8 were evaluated for their ability to protect SRA-1 human lens epithelium cells (hLECs),⁶⁹ commercially available ARPE-19 human retinal pigmented epithelial cells (RPEs),⁷⁰ and HA-h human hippocampal astrocytes (HA-hs). These three cell types are instrumental in the development of cataract, AMD, and AD, respectively.

In the first set of studies, the ability of compounds 1–8 to protect cells against ROS-induced cell death was evaluated using a MTS Cell Viability assay. The results were compared to the protective activity of the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. All cells were exposed for 2 h of either 1.0 mM H_2O_2 or Fenton reagents (1.0 mM H_2O_2 and 1.0 mM Fe^{2+}) with/without 1 mM compounds 1–8 or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. As summarized in Figure 5A, the presence of H_2O_2 reduced the viability of the hLECs to less than 50%. A similar loss of cell viability was observed for compounds 1, 3, 5, and 7, analogues not possessing the 2-amino-5-hydroxy-1,3-pyrimidine group required for FRS activity. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid significantly protected cell viability against ROS, and similar protection was also observed with compounds 2, 4, 6, and 8; all compounds possess the 2-amino-5-hydroxy-1,3-pyrimidine FRS group. When ROS was generated by the Fenton reaction (Figure 5B), in addition to the FRS containing compounds 2, 4, 6, and 8, some protection was now also demonstrated with compounds 3 and 7 which contain the 2,8-dioxopiperazine CHL group. Moreover, the

multifunctional compounds possessing both FRS and CHL groups (compounds 4 and 8) demonstrated better protection than 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. Similar results were observed for the RPE and HA-h cells. The protection afforded by compounds 1–8 against Fenton's reagent is summarized in Figure 5C and Figure 5D, respectively.

In these three cell types, the rapid intracellular reduction of cellular glutathione (GSH) is a sensitive marker of oxidative stress. As illustrated in Figure 6A, the presence of 1 mM H_2O_2 rapidly resulted in a reduction of GSH levels in hLECs and a similar reduction was also observed with compounds 1, 3, 5, and 7, analogues not possessing the FRS group. However, GSH levels were maintained when similar cells were treated with either the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or compounds 2, 4, 6, and 8 which possess the FRS group. All compounds possess the 2-amino-5-hydroxy-1,3-pyrimidine FRS group. With Fenton generated ROS (Figure 6B), protection was also afforded by the CHL containing compounds 3 and 7. In addition, the multifunctional compounds possessing both FRS and CHL groups (compounds 4 and 8) demonstrated slightly better protection than 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. Again, similar results were observed for the RPE and HA-h cells. The protection afforded by compounds 1–8 against Fenton's reagent is summarized in Figure 6C and Figure 6D, respectively.

Experimental Section

NMR spectra were obtained with a Varian 500 MHz spectrometer. EI-MS utilized an Agilent 5973N MSD, and ESI-MS was conducted with a Finnigan MAT LCQ. UV–visible spectra were

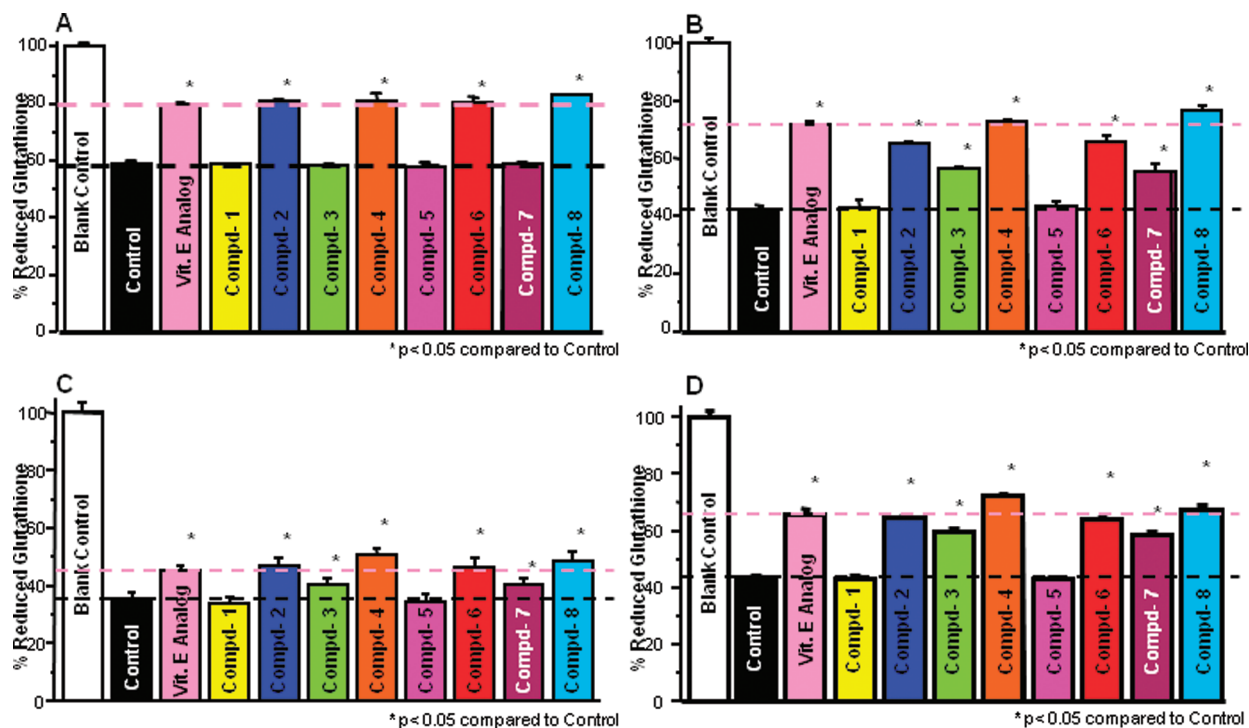


Figure 6. GSH levels in in vitro cells subjected to ROS. Part A illustrates exposure of HLECs exposed for 2 h with 1 mM H₂O₂ with/without the presence of the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or compounds 1–8. Part B illustrates 2 h exposure of HLECs with 1 mM Fenton's reagent with/without the presence of the vitamin E analogue or compounds 1–8. Parts C and D illustrate similar 2 h exposure of hRPE and HA-h cells, respectively, with 1 mM Fenton's reagent with/without the presence of the vitamin E analogue or compounds 1–8. GSH levels were expressed as nmol of GSH/mg of protein, with values normalized to GSH levels in cells not exposed to H₂O₂ (blank control, 100%). The results represent the mean $\pm$ SD of three separate experiments. Significant differences ($p > 0.05$), calculated by ANOVA, were compared to control staining obtained with ROS in the absence of either the vitamin E analogue or compounds 1–8.

measured on a Molecular Devices SpectraMax Plus³⁸⁴ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Melting points were uncorrected. Column chromatography (CC) utilized Merck silica gel (230–400 mesh). Final compound purities were assessed as $\geq 99\%$ and intermediate compound purities were assessed as $\geq 96\%$ by reverse phase HPLC using a 250 mm $\times$ 4.6 mm C18 Luna column (5 μ 100 Å) with a mobile phase of 75:25 methanol/water at a flow rate of 0.9 mL/min and detection at 220, 254, and 280 nm. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ.

***N,N'*-Dimethylsulfamoylpiperazine (10).** 1 was obtained according to the literature.⁶³

General Procedure for the Preparation of *N,N'*-Dimethyl-4-(pyrimidin-2-yl)piperazine-1-sulfonamide (1) and 4-(4,6-dimethoxy-pyrimidin-2-yl)-*N,N'*-Dimethylpiperazine-1-sulfonamide (5). Et₃N (0.73 mL, 5.2 mmol) was added to 1.0 g of *N,N'*-dimethylsulfamoylpiperazine 10 (5.2 mmol) dissolved in 20 mL of THF. 2-Chloro-4,6-dimethoxypyrimidine 11a (0.9 g, 5.2 mmol) dissolved in 5 mL of THF was then added, and the mixture was refluxed with stirring for 40 h. After the mixture was cooled, THF was removed under vacuum and the remaining yellow solid was dissolved in 300 mL of CHCl₃, washed with 0.5 N HCl, water, and brine, dried over Na₂SO₄, and filtered. Removal of solvent and CC with 1:1 CHCl₃/hexane gave 1.4 g (81%) of white compound 1, mp 157–158 °C. ¹H NMR (CDCl₃) δ 8.34 (s, 1H), 8.33 (s, 1H), 6.55 (t, J = 4.88 Hz, 1H), 3.92 (appt, J = 5.13 Hz, 4H), 3.31 (appt, J = 5.13 Hz, 4H), 2.86 (s, 6H); ¹³C NMR (CDCl₃) 161.4, 157.3, 110.5, 46.1, 43.4, 38.2; ESI-MS (m/z) 294 ([M + Na]⁺). Anal. Calcd for C₁₀H₁₇N₅O₂S: C, 44.26; H, 6.31; N, 25.81; S, 11.82. Found: C, 44.12; H, 6.42; N, 25.58; S, 12.03. The yield of compound 5, mp 103–105 °C, was 81%. ¹H NMR (CDCl₃) δ 5.41 (s, 1H), 3.86 (s, 6H), 3.89–3.86 (m, 4H), 3.28 (appt, J = 4.88 Hz, 4H), 2.86 (s, 6H); ¹³C NMR (CDCl₃) 172.0, 160.6, 78.5, 53.5, 46.2, 43.5, 38.2; EI-MS (m/z) 331 (M⁺). Anal.

Calcd for C₁₂H₂₁N₅O₄S: C, 43.49; H, 6.39; N, 21.13; S, 9.68. Found: C, 43.60; H, 6.47; N, 21.11; S, 9.85.

4-(5-Hydroxy-4,6-dimethoxypyrimidin-2-yl)-*N,N'*-dimethylpiperazine-1-sulfonamide (6). To a solution of compound 5 (1.66 g, 5 mmol) in 40 mL of THF at –78 °C and under an Ar atmosphere was added 0.3 mL of *n*-BuLi (7 mmol). After the mixture was stirred for 3 h at –60 °C, the Ar atmosphere was replaced with oxygen. The reaction mixture was then gradually warmed to room temperature (RT), and stirring was continued for an additional 6 h. After adjusting the pH to 6–7 with dilute HCl, THF was removed by evaporation and the remaining aqueous layer was extracted with CHCl₃. The CHCl₃ layers were washed with brine, dried over Na₂SO₄, and filtered. Solvent evaporation gave a yellow solid, which was purified by CC with 1:1:2 CHCl₃/EtOAc/hexane to yield 530 mg (30%) of compound 6, mp 113–114 °C. The structure was confirmed by ¹H NMR (CDCl₃) δ 4.21 (s, 1H), 3.95 (s, 6H), 3.76 (s, 4H), 3.29 (s, 4H), 2.86 (s, 6H); EI-MS (m/z) 347 (M⁺). Anal. Calcd for C₁₂H₂₁N₅O₅S: C, 41.49; H, 6.09; N, 21.16; S, 9.23. Found: C, 43.61; H, 6.13; N, 19.96; S, 9.18.

***N,N'*-Dimethyl-4-(5-nitropyrimidin-2-yl)piperazine-1-sulfonamide (16).** Triethylamine (7.2 mL, 51.8 mmol) was added to a solution of 10 (10 g, 51.8 mmol) dissolved in 400 mL of THF. 2-Chloro-5-nitropyrimidine 12 (7.7 g, 48.3 mmol) dissolved in 20 mL of THF was added to the stirred mixture. After the mixture was stirred for 24 h at RT, THF was removed under vacuum and 600 mL of CHCl₃ was added to the residue. The CHCl₃ layer was washed with 0.5 N HCl, water, and brine, dried over Na₂SO₄, and filtered. Solvent evaporation gave 14.1 g (92%) of straw-yellow solid 16. ¹H NMR (CDCl₃) δ 9.08 (s, 2H), 4.09 (appt, J = 5.13 Hz, 4H), 3.35 (appt, J = 5.13 Hz, 4H), 2.87 (s, 6H).

4-(5-Aminopyrimidin-2-yl)-*N,N'*-dimethylpiperazine-1-sulfonamide (17). NH₄Cl (1.43 g, 26.76 mmol) was added to a suspension of 16 (14.1 g, 44.6 mmol) in a mixture of EtOH (175 mL) and

H₂O (48 mL), followed by iron powder (7.5 g, 133.9 mmol). After refluxing 1 h, the reaction mixture was filtered and concentrated to give a brown solid which was dissolved in 800 mL of CHCl₃, washed with NaHCO₃ and brine, dried over Na₂SO₄, and filtered. Solvent evaporation gave a yellow solid which after CC with 100:1 CHCl₃/MeOH gave 11.5 g (90%) of solid yellow **17**. ¹H NMR (CDCl₃) δ 7.98 (s, 2H), 3.76 (t, *J* = 4.88 Hz, 4H), 3.30 (t, *J* = 4.88 Hz, 4H), 2.86 (s, 6H); EI-MS (*m/z*) 286 (*M*⁺).

4-(5-Hydroxypyrimidin-2-yl)-*N,N*-dimethylpiperazine-1-sulfonamide (2). A suspension of **17** (5.76 g, 20.2 mmol) in 100 mL of 2 M H₂SO₄ was refluxed for 1.5 h until the suspension became clear. After the mixture was cooled to RT, the solution pH was adjusted to 6–7 with 10 N NaOH, and the cloudy mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over Na₂SO₄, and filtered. Solvent evaporation gave a yellow solid, which was purified by CC using 1:1 EtOAc/hexane to yield 3.23 g (56%) of white solid compound **2**, mp 134–135 °C. ¹H NMR (CDCl₃) δ 8.08 (s, 2H), 3.78 (appt, *J* = 5.13 Hz, 4H), 3.29 (appt, *J* = 5.13 Hz, 4H), 2.86 (s, 6H); ¹³C NMR (CDCl₃) 156.9, 145.8, 143.5, 46.0, 44.3, 38.1; EI-MS (*m/z*) 287 (*M*⁺). Anal. Calcd for C₁₀H₁₇N₅O₃S: C, 41.80; H, 5.96; N, 24.37; S, 11.16. Found: C, 41.60; H, 5.98; N, 24.16; S, 11.02.

5-Hydroxy-2-aminopyrimidine (25). Under argon at RT, BBr₃ (2.6 mL, 50 mmol) was added dropwise to a solution of **23** (1.25 g, 10 mmol) in 60 mL of benzene, and the mixture was refluxed for 6 h. After the mixture stood overnight at RT, the solvent was evaporated and the residue was carefully treated with 10 mL of ice cold H₂O. The pH of the solution was adjusted to 6–7 with 6 N NaOH and then extracted with EtOAc. The EtOAc layers were washed with brine, dried over Na₂SO₄, and filtered. Solvent evaporation gave a yellow solid, which after CC using 20:1 CHCl₃/MeOH gave 840 mg (75%) of white solid 5-hydroxy-2-aminopyrimidine **25**. ¹H NMR (DMSO-*d*₆) δ 8.90 (s, 1H), 7.86 (s, 2H), 5.91 (s, 2H); EI-MS (*m/z*) 111 (*M*⁺).

5-Benzoyloxy-2-aminopyrimidine (20c). K₂CO₃ (276 mg, 2 mmol) was added to 222 mg of **25** (2 mmol) in 5 mL of methanol, followed by BnBr (0.24 mL, 2 mmol). After the mixture was stirred for 14 h at RT, the reaction was stopped by addition of water. Methanol was evaporated. The remaining aqueous layer was extracted with CHCl₃. The combined CHCl₃ layers were washed with brine, dried over Na₂SO₄, and filtered. Removal of the solvent followed by CC using 67:1 CHCl₃/MeOH yielded 300 mg (75%) of white solid **20c**. ¹H NMR (CDCl₃) δ 8.08 (s, 2H), 7.40–7.32 (m, 5H), 5.03 (s, 2H), 4.77 (br s, 2H).

2-Chloro-5-hydroxy-4,6-dimethoxypyrimidine (28). To a stirred suspension of 10.44 g 2-chloro-4,6-dimethoxypyrimidine **11b**, (60 mmol) in 200 mL of THF under argon at –78 °C was added dropwise a solution of LDA (2 M in THF/heptane/ethyl benzene, 60 mL, 120 mmol). To a separate round-bottomed flask at –78 °C under argon containing *tert*-butyl hydroperoxide (5.5 M in decane, 22.7 mL, 125 mmol) in 150 mL of THF was added a solution of *n*-BuLi (1.6 M in hexane, 78 mL, 125 mmol). Then, after being stirred for 1 h, the hydroperoxide anion solution was added via a double-ended needle to the aromatic anion. The temperature was gradually raised to 0 °C, with stirring continuing an additional 3 h. The reaction was quenched with 6 N HCl until a pH of 7 was obtained. After THF evaporation the remaining aqueous layer was extracted with CHCl₃. The CHCl₃ layers were washed with brine, dried over Na₂SO₄, and filtered. Removal of solvent and subsequent purification by CC using 100:1 CHCl₃/MeOH gave 10.67 g (93.4%) of white solid **28**. ¹H NMR (CDCl₃) δ 4.93 (s, 1H), 4.05 (s, 6H); ¹³C NMR (CDCl₃) 158.1, 146.7, 123.0, 55.1.

2-Chloro-5-(benzyloxy)-4,6-dimethoxypyrimidine (29). K₂CO₃ (10.0 g, 70 mmol) was added to **28** (13.24 g, 69.5 mmol) dissolved in 200 mL of MeCN followed by BnBr (8.3 mL, 69.5 mmol). After the mixture was stirred for 14 h at RT, the reaction was stopped by addition of water. MeCN was evaporated, the remaining aqueous layer was extracted with CHCl₃. The combined CHCl₃ layers were washed with brine, dried over Na₂SO₄, and filtered. Removal of

the solvent followed by CC using 25:1 hexane/EtOAc gave 16.57 g (85%) of benzyloxy **29**. ¹H NMR (CDCl₃) δ 7.41–7.31 (m, 5H), 5.00 (s, 2H), 3.98 (s, 6H); ¹³C NMR (CDCl₃) 163.6, 150.5, 136.4, 128.5, 128.4, 128.3, 124.4, 74.8, 54.0.

***N*-Benzyl-5-(benzyloxy)-4,6-dimethoxypyrimidin-2-amine (30)**. A mixture of **29** (3.18 g, 11.33 mmol), BnNH₂ (2.2 mL, 20 mmol), and K₂CO₃ (1.66 g, 12 mmol) in dioxane (60 mL) was refluxed for 4 days. The mixture was filtered and the filtrate concentrated in vacuo to give a yellow oil, which after CC with 25:1 to 10:1 hexane/EtOAc gave 2.54 g (64%) of white solid **30**. ¹H NMR (CDCl₃) δ 7.45–7.24 (m, 10H), 5.10 (br s, 1H), 4.85 (s, 2H), 4.55 (d, *J* = 5.5 Hz, 2H), 3.86 (s, 6H); ¹³C NMR (CDCl₃) 163.5, 156.0, 139.7, 137.5, 128.5, 128.4, 128.1, 127.8, 127.6, 127.1, 117.7, 75.2, 53.7, 45.9.

2-Amino-4,6-dimethoxypyrimidin-5-ol (31). Compound **30** (2.54 g, 7.24 mmol) in 40 mL of MeOH was hydrogenated for 12 h at RT in the presence of 635 mg of 10% Pd/C catalyst. After filtration and solvent evaporation, a white solid was obtained, which after CC using 50:1 CHCl₃/MeOH yielded 1.21 g (98%) of **31**. ¹H NMR (CDCl₃) δ 4.50 (s, 2H), 4.30 (s, 1H), 3.93 (s, 6H).

5-(Benzoyloxy)-4,6-dimethoxypyrimidin-2-amine (20d). To 4.16 g of **31** (24.3 mmol) in MeOH (240 mL) was added K₂CO₃ (3.5 g, 25 mmol), followed by BnBr (3.0 mL, 25 mmol). After the mixture was stirred for 12 h at RT, water was added to the reaction mixture. MeOH was evaporated, and the water layer was extracted with CHCl₃. The combined CHCl₃ layers were washed with brine, dried over Na₂SO₄, and filtered. Removal of CHCl₃ gave a yellow solid, which after purification by CC with 10:1 to 2:1 hexane/EtOAc yielded 5.14 g (81%) of 2-amine-4,6-dimethoxy-5-benzyloxy-pyrimidine **20d**. ¹H NMR (CDCl₃) δ 7.44–7.27 (m, 5H), 4.86 (s, 2H), 4.60 (s, 2H), 3.87 (s, 6H).

2,2'-(*N,N*-Dimethylsulfamoylazanediy)diacetic Acid (19). An amount of 26.62 g of the iminodiacetic acid **18** (200 mmol) was added to 16.0 g of NaOH (400 mmol) in 50 mL of H₂O at 0 °C, followed by 21.2 g of Na₂CO₃ (200 mmol) in 100 mL of H₂O. Then dimethylsulfamoyl chloride (200 mmol) dissolved in 50 mL of acetone was slowly added with stirring and the stirring was continued at RT. After 48 h, the reaction mixture was extracted with EtOAc. The pH of the aqueous layer was adjusted to 2 with 12 N HCl and extracted again with EtOAc. The organic layer was dried over Na₂SO₄ and filtered. Solvent evaporation gave 28.75 g (60%) of **19** as a white powder which was recrystallization from EtOAc. The structure was confirmed by ¹H NMR (DMSO-*d*₆) δ 12.81 (br s, 2H), 3.99 (s, 4H), 2.71 (s, 6H); ¹³C NMR (DMSO-*d*₆) 170.9, 49.7, 37.8.

General Procedure for Preparation of *N,N*-Dimethyl-3,5-dioxo-4-(pyrimidin-2-yl)piperazine-1-sulfonamide (3), 4-(4,6-Dimethoxypyrimidin-2-yl)-*N,N*-dimethyl-3,5-dioxopiperazine-1-sulfonamide (7), 4-(5-(Benzoyloxy)pyrimidin-2-yl)-*N,N*-dimethyl-3,5-dioxopiperazine-1-sulfonamide (21), and 4-(5-(Benzoyloxy)-4,6-dimethoxypyrimidin-2-yl)-*N,N*-dimethyl-3,5-dioxopiperazine-1-sulfonamide (22). A suspension of 1.44 g of diacid **19** (6 mmol) in 8.0 mL of Ac₂O was heated to 85 °C for 5 min until the solution became clear. After excess Ac₂O was removed under vacuum at 60 °C, the resulting anhydride was obtained as a yellow oil. The resulting anhydride, in 10 mL of toluene, was then reacted with 475 mg of 2-aminopyrimidine **20a** (5 mmol) dissolved in 7 mL of acetone by heating the mixture to 80 °C for 6 h. Solvent evaporation gave the amide as a yellow oil. Heating amide for 4 h at 80 °C with 8 mL of Ac₂O resulted into the final piperidine-2,6-ring product as a red, clear solution. Removal of the remaining Ac₂O under vacuum at 60 °C followed by CC with 3:1:1 hexane/EtOAc/CHCl₃ gave 760 mg (50.8%) of compound **3**, mp 179–181 °C. ¹H NMR (CDCl₃) δ 8.90 (d, *J* = 4.88 Hz, 2H), 7.45 (t, *J* = 4.88 Hz, 1H), 4.31 (s, 4H), 2.96 (s, 6H); ¹³C NMR (CDCl₃) 167.0, 159.5, 154.8, 121.4, 49.5, 38.3; ESI-MS (*m/z*) 322 ([*M* + Na]⁺). Anal. Calcd for C₁₀H₁₃N₅O₄S: C, 40.13; H, 4.38; N, 23.40; S, 10.71. Found: C, 40.40; H, 4.54; N, 23.41; S, 10.66.

The white solid of compound **7**, mp 200–202 °C, was obtained in 57.9% yield. ¹H NMR (CDCl₃) δ 6.10 (s, 1H), 4.30 (s, 4H), 3.92

(s, 6H), 2.967 (s, 6H); ^{13}C NMR (CDCl_3) 172.8, 166.7, 152.8, 90.5, 54.7, 49.4, 38.2; ESI-MS (m/z) 382 ($[\text{M} + \text{Na}]^+$). Anal. Calcd for $\text{C}_{12}\text{H}_{17}\text{N}_5\text{O}_6\text{S}$: C, 40.11; H, 4.77; N, 19.49; S, 8.92. Found: C, 40.31; H, 4.84; N, 19.28; S, 9.10.

Crude compound **21** was purified by CC with 100:1 $\text{CHCl}_3/\text{MeOH}$, and white solid of **21** was obtained in 69% yield. ^1H NMR (CDCl_3) δ 8.56 (s, 2H), 7.44–7.26 (m, 5H), 5.20 (s, 2H), 4.29 (s, 4H), 2.95 (s, 6H).

Crude compound **22** was purified by CC using 100:1 $\text{CHCl}_3/\text{MeOH}$, and pale-yellow solid of **22** was obtained in 41% yield. ^1H NMR (CDCl_3) δ 7.46–7.32 (m, 5H), 5.05 (s, 2H), 4.29 (s, 4H), 3.95 (s, 6H), 2.96 (s, 6H).

General Procedure for Preparation of 4-(5-Hydroxypyrimidin-2-yl)-N,N-dimethyl-3,5-dioxopiperazine-1-sulfonamide (4) and 4-(5-Hydroxy-4,6-dimethoxypyrimidin-2-yl)-N,N-dimethyl-3,5-dioxopiperazine-1-sulfonamide (8). Compound **21** (2.0 g, 4.9 mmol) dissolved in 100 mL of EtOAc was hydrogenated with 500 mg of 10% Pd/C catalyst at RT for 12 h. After filtration and solvent evaporation, compound **4** recrystallized from EtOAc to give 1.83 g (80%) of a white powder, mp 225–226 °C. ^1H NMR ($\text{DMSO}-d_6$) δ 11.0 (s, 1H), 8.45 (s, 2H), 4.39 (s, 4H), 2.85 (s, 6H); ^{13}C NMR (CDCl_3) 168.9, 152.7, 146.9, 146.1, 50.0, 38.5; ESI-MS (m/z) 338 ($[\text{M} + \text{Na}]^+$). Anal. Calcd for $\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_5\text{S}$: C, 38.09; H, 4.16; N, 22.21; S, 10.17. Found: C, 38.18; H, 4.35; N, 21.98; S, 9.93.

Crude compound **8** was purified by CC using 80:1 $\text{CHCl}_3/\text{MeOH}$ to yield 1.15 g (91%) of white solid **8**, mp 222–224 °C. ^1H NMR (CDCl_3) δ 9.67 (s, 1H), 4.44 (s, 4H), 3.88 (s, 6H), 2.51 (s, 6H); ^{13}C NMR (CDCl_3) 168.1, 159.4, 140.9, 124.7, 54.7, 49.3, 37.9; ESI-MS (m/z) 398 ($[\text{M} + \text{Na}]^+$). Anal. Calcd for $\text{C}_{12}\text{H}_{17}\text{N}_5\text{O}_7\text{S}$: C, 38.40; H, 4.56; N, 18.66; S, 8.54. Found: C, 38.60; H, 4.66; N, 18.49; S, 8.58.

Chelation Studies. The relative amounts of ions bound by compounds **1–8** were determined by electrospray ionization mass spectrometry (ESI-MS) according to the method of Baron and Hering.⁷¹ Stock solutions (1 mM) were prepared with doubly distilled water from ammonium iron(II) sulfate hexahydrate, iron(III) nitrate nonahydrate, copper(I) chloride, copper(II) chloride dihydrate, zinc chloride, magnesium chloride, and calcium chloride. Each ion solution was mixed with compounds **1–8**, respectively, in ratios of 10:1, 1:1, and 1:10 metal ion/compound so that a final compound concentration of 50 μM was maintained. Forty-five minutes after mixing, each solution was directly infused into the MS under constant ionization energy.

The stoichiometry of the complexes of compounds **1–8** with Fe^{2+} , Fe^{3+} , Cu^{1+} , Cu^{2+} , Zn^{2+} , Ca^{2+} , and Mg^{2+} was determined using the method of continuous variation (Job plots).⁷² Because of their low solubility, compounds **1–8** were first dissolved in acetonitrile (HPLC grade). These concentrated solutions were then diluted to 0.1 mM stock solutions with 1 mM acetate (pH 6.5). In a typical experiment (at 23 °C), several solutions of the compound and metal ion of interest were prepared at a constant total concentration of compound and ion but with a different mole fraction of one component. After an equilibration period of 45 min, the change in UV absorbance was monitored from 210 to 310 nm. The maximum absorbance for each compound was then recorded as a function of the mole fraction of the metal ion. Two linear dependences were obtained, at low and high molar fractions, which intersect at a mole fraction value that corresponds to the stoichiometry of the complex.

Cell Culture Studies. Human lens epithelial cells (SRA-1), obtained as a gift from Dr. Reddy,⁶⁹ were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 4 mM L-glutamine, 1.5 g/L NaHCO_3 , and 1% penicillin/streptomycin solution. Human retinal pigmented epithelial cells (ARPE-19), obtained from the American Type Culture Collection (Manassas, VA), were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12) medium with 2.5 mM L-glutamine and

15 mM HEPES buffer (Gibco, Carlsbad, CA) containing 10% heat-inactivated FBS and 1% penicillin/streptavidin solution. Human hippocampal astrocytes (HA-h), obtained from the ScienCell Research Laboratories (Carlsbad, CA), were cultured on poly-L-lysine coated containers (2 $\mu\text{g}/\text{cm}^2$) with astrocyte medium (ScienCell, Carlsbad, CA) composed of 500 mL of basal medium, 5 mL of astrocyte growth supplement, and 5 mL of penicillin/streptomycin solution. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air until subconfluent (2–4 days). Cells were passaged when 80–90% confluent by treatment with trypsin–EDTA and then plated at a density of 1×10^4 cells onto 96-well plates or 1×10^6 cells onto 150 mm dishes. For compound evaluation, FBS-free media (blank and control) or FBS-free media containing either 100 μM compounds **1–8** or 100 μM water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were added to each individual well/dish. After 1 h, an additional aliquot of FBS-free media was added to the blank well/dish, and a similar volume of FBS-free media containing 1 mM H_2O_2 was added to all remaining wells/dishes. For the Fenton studies, addition of H_2O_2 was immediately followed by addition of 1 mM ammonium iron(II) sulfate hexahydrate. After 2 h of exposure, all media were removed and the cells were washed at room temperature with PBS. All studies were conducted in triplicate.

Cell viability studies were conducted in 96-well plates using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI). Briefly, after 2 h of exposure to either H_2O_2 or Fenton reagents, media were removed from all wells and each well was washed with PBS. After removal of the PBS wash, 100 μL of blank media was added to each well followed by addition of 20 μL of the MTS solution. The cells were further incubated at 37 °C for 1.5 h and then spectrophotometrically evaluated at 490 nm in a plate reader. The results were normalized to blank control (100%) of cells not treated with H_2O_2 .

GSH measurements were conducted on cells cultured in either 100 or 150 mm dishes. After 2 h of exposure to either H_2O_2 or Fenton reagents, the media were removed and the cells were washed with PBS. The cells were removed from each dish by scraping and homogenized in glass homogenizers. Following centrifugation at 4 °C, protein levels in the supernatant were measured according to Bradford.⁷³ The cell homogenates were then deproteinized with equal volumes of 20% trichloroacetic acid (TCA), and GSH levels were measured at 412 nm according to the DTNB method.⁷⁴ GSH levels were expressed as nmol of GSH/mg of protein, with values subsequently normalized to GSH levels in cells not exposed to H_2O_2 (blank control, 100%).

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