Chroman/Catechol Hybrids: Synthesis and Evaluation of Their Activity against Oxidative Stress Induced Cellular Damage

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Three series of chromans substituted at positions 2 or 5 by catechol derivatives were synthesized, and their activity against oxidative stress induced cellular damage was studied. Specifically, the ability of the new molecules to protect cultured cells from H_2O_2-induced DNA damage was evaluated using single cell gel electrophoresis (comet assay), while the neuroprotective activity of the new compounds against oxidative stress induced programmed cell death was studied using glutamate-challanged hippocampal HT22 cells. The majority of the new compounds are stronger neuroprotectants than quercetin. 5-Substituted chroman analogues such as the caffeic acid amides **12** and **16** and the dihydrostilbene analogue **24** were the most potent against both H_2O_2- and glutamate-induced damage in Jurkat T cells and HT22 cells, respectively.

Introduction

Low amounts of reactive oxygen species (ROS) are continuously generated in the cells of aerobic organisms, and they are likely to play important physiological roles, especially in signal transduction processes.[1,2] However, elevated steady-state levels of these species (a situation usually called "oxidative stress") has been implicated in the initiation or progression of various pathological conditions including neurological diseases, particularly neurodegenerative diseases, as well as cardiovascular diseases and cancer.[3−6]

Oxidative damage and disease progression may be retarded by administering exogenous protective compounds, which can act in several different ways such as chain breaking antioxidants or free radical scavengers, inhibitors of ROS formation, transition metal chelators, etc. The protective efficacy of antioxidants depends on the type of ROS that is generated, the place of generation (body barriers such as blood−brain barrier reduce the permeability of most antioxidants), and the severity of the insult.

The central nervous system (CNS) is particularly vulnerable to damage by ROS due to several factors, including low levels of the natural antioxidant glutathione in neurons, membranes rich in polyunsaturated fatty acids, the presence of excitatory amino acids such as glutamate, and increased requirement for oxygen because of the high metabolic activities of the brain.[7a−b] Moreover, there is evidence that transition metals such as iron are mediators of ROS production.[7c] Since iron is the most abundant transition metal in the brain, it is considered a potent potential toxin. Histological and quantitative changes in iron have been reported in most neurodegenerative diseases.[7d] Iron catalyzes the formation of hydroxyl radicals (•OH) by Fenton-type reaction.[8] The exact intracellular location of iron is likely to be of outmost importance for the ultimate effect, because •OH, due to their extreme reactivity, interact exclusively in the vicinity of the bound metal.[9] Formation of •OH close to DNA results in its damage, including base modifications and single and double strand breakage. However, there are indications that location of iron at positions other than near the DNA may contribute indirectly to DNA damage and ensuing apoptosis.[10]

Compounds bearing 3,4-dihydroxyphenyl (catechol) moieties possess a wide spectrum of biological activities, which is related to their capacity to transfer electrons, to chelate ferrous ions, and to scavenge ROS.[11] Catechol and caffeic acid derivatives were found to be potent lipoxygenase inhibitors,[12] HIV integrase inhibitors,[13] neuroprotectants,[14] or cholesterol-lowering agents.[15] The natural 3,4-dihydroxy stilbene analogue asriginin exhibits strong cardioprotective activity in ischemic/reperfused rat hearts.[16] Moreover synthetic *cis*-3,4-dihydroxy stilbene analogues showed strong antiproliferative and apoptotic activity in HL60 leukemia cells.[17] Flavonoids bearing a catechol moiety in ring B such as quercetin, luteolin, and catechins showed protective effects against coronary heart disease and oxidative stress induced neuronal cell death.[18−21] Hydroxy groups in different positions of flavonoids and caffeic acid derivatives may allow scavenging of ROS but at the same time decrease lipophilicity and therefore the ability of polyphenols to penetrate and remain in the cells. For instance, quercetin aglycone is taken up by Caco-2 intestinal cells far more effectively than its more hydrophilic glucosides. However, quercetin metabolism and ensuing rapid efflux of the aglycone conjugates causes intracellular aglycone levels to decrease rapidly.[22] Thus, some of these compounds, while effective in vitro, cannot afford cytoprotection because they are inefficiently taken up by the cells or are rapidly metabolized to conjugates effectively transported out of the cells.

Vitamin E is widely studied not only as an antioxidant and oxidative stress induced programmed cell death (oxytosis) inhibitor but also for its nonantioxidant activities, such as gene regulation, inhibition of protein kinase C, 5-lipoxygenase, and phospholipase A2, and activation of protein phosphatase 2A and diacylglycerol kinase.[23] However, vitamin E showed limited efficacy in emergency reperfusion or DNA damage despite its high activity against oxytosis,[23b] which might be attributed to

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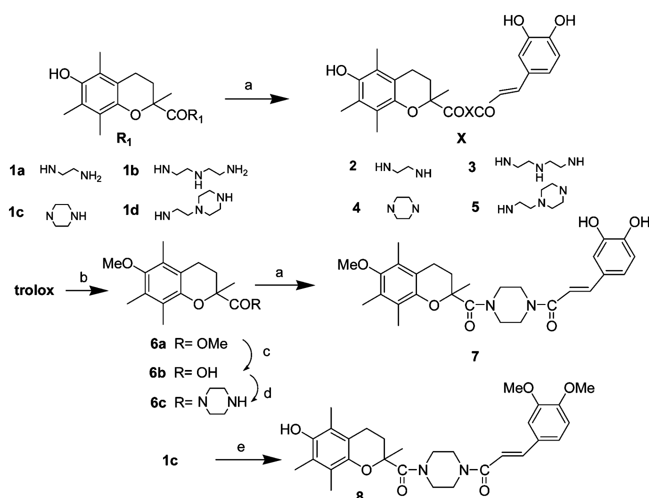
Scheme 1[a]



[a] Reagents and conditions: (a) BOP, caffeic acid, Et_3N; (b) $(MeO)_2SO_2$, K_2CO_3, (c) NaOH 1 N; (d) CDI, piperazine; (e) BOP, *trans*-3,4-dimethoxycinnamic acid, Et_3N.

its highly lipophilic properties. However, amides or amines of the synthetic vitamin E analogue trolox are more effective than vitamin E as neuroprotectants and against reperfusion-induced oxidative damage.[24−26]

As an ongoing effort[27,28] toward highly effective antioxidants, we synthesized three series of hybrids containing the chroman moiety of vitamin E and a catechol group, and we explored their ability to protect cells exposed to different types of oxidative stress.

The first series of the new analogues contain caffeic acid and trolox connected through diamine or triamine spacers. The second series are amides of caffeic acid with 2- or 5-aminomethylchroman and 5-aminochroman derivatives. The last series of compounds involves chroman analogues substituted at position 5 by catechol moieties.

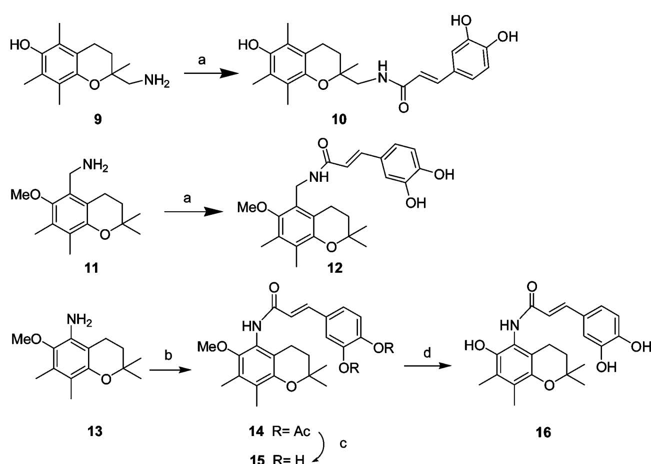
The ability of the new molecules to protect cultured cells from H_2O_2-induced DNA damage was evaluated using the highly sensitive methodology of single cell gel electrophoresis or comet assay.[29] Cellular DNA is especially sensitive to the action of H_2O_2, and we have shown that this DNA damage is mediated mainly by iron, which catalyzes the formation of the extremely reactive $^{\bullet}$OH.[10,29]

The neuroprotective activity of the new compounds was studied using HT22 cells, a neuronal cell line derived from murine hippocampus. Exposure of these cells to high concentrations (1−5 mM) of glutamate blocks cystine uptake, which leads to depletion of intracellular cysteine and loss of glutathione (GSH), an early increase in ROS, and, eventually, production of ROS by mitochondria and programmed cell death.[30a] Glutamate-induced cell death of HT22 cells is thought to mimic cytotoxicity due to oxidative stress as implicated in several neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.[30b]

Chemistry

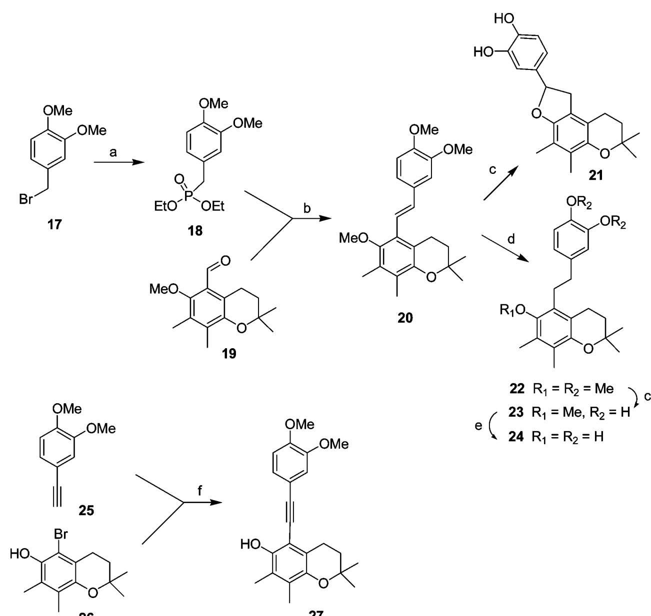
Amide analogues of caffeic acid **2**−**5**, **7**, **8**, **10**, and **12** (Schemes 1 and 2) were synthesized from the corresponding aminoamides **1** or amines **9** and **11**, prepared according to our previously described methodology,[27,28] and caffeic acid using BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate) as condensing agent. The methoxychroman derivative **7** was obtained by methylation of trolox using dimethyl sulfate and potassium carbonate, followed by

Scheme 2[a]



[a] Reagents and conditions: (a) BOP, caffeic acid, Et_3N; (b) *trans*-3,4-diacetoxycinnamoyl chloride, Et_3N; (c) MeONa, MeOH; (d) $BF_3{\cdot}SMe_2$ CH_2Cl_2.

Scheme 3[a]



[a] Reagents and conditions: (a) triethyl phosphite, TBAI; (b) *t*-BuOK, DMF; (c) $BF_3{\cdot}SMe_2$ CH_2Cl_2, 2 h; (d) Pd/C 10%, H_2, 50 psi; (e) $BF_3{\cdot}SMe_2$ CH_2Cl_2, 24 h; (f) $PdCl_2(PPh_3)_2$, Et_3N, CuI.

hydrolysis of the resulting ester **6a** and coupling with piperazine in the presence of CDI (compound **6b**) and finally with caffeic acid using BOP. The analogue **8** in which the catechol group is protected was synthesized from the corresponding piperazine amide and coupling with *trans*-3,4-dimethoxycinnamic acid using BOP.

Amide **16** was synthesized from the appropriate chroman amine and *trans*-3,4-diacetoxycinnamoyl chloride in Et_3N and THF. Deprotection of acetyl groups of compound **14** was achieved using MeONa in MeOH to give compound **15**, while for the deprotection of the methoxy group, $BF_3{\cdot}SMe_2$ was used[31] (Scheme 2).

The synthesis of the compounds of the third series is depicted in Scheme 3. 3,4-Dimethoxy benzyl alcohol was converted to the corresponding bromide using PBr_3 and then to phosphonate ester using triethyl phosphite. Horner−Emmons reaction of **18** with aldehyde **19** in the presence of *t*-BuOK in DMF[32a] gave one geometrical isomer of the stilbene analogue **20**. These

Table 1. Lipophilicity (ClogP) Data and Antioxidant Potency of the Chroman/Catechol Hybrids as Assessed Chemically as Well as Biologically Using Jurkat and HT22 Cells

compound	ClogP[a]	TEAC	IC_{50} (μM)[b] Jurkat cells	EC_{50} (μM)[c] HT-22 cells
2	3.6	6.50 ± 0.15	75 ± 8	7.36 ± 0.24
3	3.5	6.33 ± 0.18	50 ± 6	>10
4	4.1	6.55 ± 0.24	50 ± 6	1.48 ± 0.18
5	4.1	6.24 ± 0.35	20 ± 3	6.35 ± 0.04
7	4.8	6.12 ± 0.21	50 ± 8	2.11 ± 0.39
8	5.0	1.26 ± 0.04	*d*	0.65 ± 0.13
10	4.3	6.53 ± 0.18	25 ± 2	1.14 ± 0.35
12	4.8	3.80 ± 0.23	1 ± 0.1	2.15 ± 0.59
15	4.4	6.53 ± 0.12	20 ± 1	>10
16	4.5	4.28 ± 0.22	5 ± 2	1.23 ± 0.26
20	7.0			>10
21	5.3	5.90 ± 0.34	10 ± 2	1.00 ± 0.21
22	6.7	0.05 ± 0.01	*d*	4.59 ± 0.45
23	5.8	1.60 ± 0.08	10 ± 2	1.17 ± 0.15
24	5.2	3.74 ± 0.12	5 ± 2	0.93 ± 0.19
27	6.3			>10
quercetin	1.3	4.30 ± 0.16	5 ± 1	2.98 ± 0.31
caffeic acid	1	1.2	*d*	>10000
trolox	3.1	1	*d*	

[a] Calculated with the program ChemDraw Ultra 8.0.1. [b] IC_{50} values are test compound concentration promoting a 50% reduction of the H_2O_2-induced DNA single-strand breakage. [c] EC_{50} values are test compound concentrations required to achieve viability of the glutamate-exposed cells equal to 50% of that of the nonexposed cells (see legend to Figure 1). Values are mean ± SEM of at least three independent experiments. [d] Inactive.

reaction conditions usually afford the trans isomer as the major product. In the NMR spectrum, olephinic protons appear as a singlet at 7.05 ppm, which is in accordance with previously observed chemical shift for the trans isomer.[32b] Thus, we assume that **20** should be trans.

Reaction of **20** with $BF_3{\cdot}SMe_2$ afforded the furan analogue **21** instead of the deprotected stilbene analogue. This is not surprising since $BF_3{\cdot}SMe_2$ is a Lewis acid and could catalyze this cyclization reaction. The absence of optical rotation means that **21** is racemic. The same furan analogue was obtained by using 9-iodo-9-BBN for the cleavage of methoxy groups. Attempts to deprotect the methoxy groups using a large excess of EtSNa in DMF gave a complex mixture of products.

Hydrogenation of **20** using Pd/C gave the trimethoxy dihydrostilbene analogue **22**. Deprotection of the catechol group was achieved using $BF_3{\cdot}SMe_2$ for 2 h (compound **23**). Under the same reaction conditions but for 24 h, the trihydroxy analogue **24** was obtained.

Alkyne analogue **27** was obtained from Sonogashira reaction of bromochroman **26** and alkyne **25** in the presence of $(PPh_3)_2PdCl_2$, CuI, and triethylamine.[33] Alkyne was prepared from 3,4-dimethoxybenzaldehyde and dimethyl-1-diazo-2-oxopropylphosphonate.[34] Attempts to deprotect methoxy groups using $BF_3{\cdot}SMe_2$ result in an unstable product.

Results and Discussion

Our goal was to examine the influence on antioxidant activity (assessed chemically and biologically) of catechol groups at position 2 or 5 of the chroman nucleus. In addition, we explored the differences in activity when the hydroxy groups of catechol or chroman moieties are free or protected. The results are reported in Table 1.

Values of trolox equivalent activity concentration (TEAC) for the new compounds were determined according to Rice-Evans and Miller.[35] The TEAC value of an antioxidant was calculated experimentally using 0.3 mM trolox solution and normalizing data to 1.0 mM trolox (Table). The trihydroxy analogues (compounds **2**−**5**) of the first series are very strong antioxidants with TEAC values of 6.24−6.55 μM, while the TEAC value of the protected analogue **8** is 1.26 ± 0.04 μM. The most active compounds from the second series are **10** and **15** with TEAC values of 6.53 μM. Among the compounds of the third series, benzofuran analogue **21** is the strongest antioxidant with TEAC = 5.90 ± 0.34 μM.

The oxidative insult by hydrogen peroxide has been widely used to assess cytoprotection. The effects of continuously generated H_2O_2 (by the action of added glucose oxidase) on DNA damage was assessed by single cell gel electrophoresis or "comet assay" using Jurkat T-cells.

The IC_{50} values of the analogues bearing di- or triamine spacers are between 20 and 75 μM. The most active is compound **5** with IC_{50} = 20 ± 3 μM. while compounds **3**, **4**, and **7** have similar activity (IC_{50} = 50 μM). Protection of the chroman hydroxy group did not diminish the activity of the piperazine analogue, while protection of the catechol group abolishes the cytoprotective activity.

Among the amides of the second series, the most active is compound **12** with IC_{50} = 1.0 ± 0.1 μM. Compound **16** is also very active against DNA damage with IC_{50} = 5.0 ± 2.0 μM, while its methoxy analogue **15** has IC_{50} = 20 ± 1 μM. The 2-substituted chroman **10** has IC_{50} = 25 ± 2 μM.

Both benzofuran analogue **21** and the trihydroxy analogue **24** from the third series are strong inhibitors of DNA damage with IC_{50} = 10 ± 2 μM and IC_{50} = 5 ± 2 μM respectively. The trimethoxy analogue **22** is inactive.

The above results may be taken to suggest that the presence of the catechol group is necessary for the activity of our compounds against DNA damage. The 5-substituted chroman analogues exhibited the highest cytoprotective activity. All these analogues probably act by a mechanism that inhibits the formation of hydroxyl radical (i.e., iron chelation). The results of our recent study[36a] strongly support the idea of intracellular binding of redox active iron as the basis for the protective capacity of flavonoids. In addition, previous literature data[11,36b] suggest that the presence of the catechol moieties in polyphenolic compounds is responsible for the protection offered against oxidative stress induced DNA damage. Moreover, we have observed that many strong antioxidants, commercially available or compounds synthesized in our laboratory, lacking catechol moieties, failed to protect cells from H_2O_2-induced DNA damage. Thus, we can deduce that the protective effects of our catechol derivatives are probably mediated by an iron-binding mechanism.

The mouse hippocampal cell line HT22 has been used to elucidate sequential cellular events during the programmed cell death cascade triggered by glutamate-induced oxidative stress.[23c,30a] Although HT22 cells lack ionotropic glutamate receptors that could mediate excitotoxicity, they are killed by oxytosis within 24 h following exposure to 1−5 mM glutamate.[23c] Several natural (e.g., flavonoids) and synthetic (e.g., tyrphostins) polyphenols have tested positive against glutamate oxytosis of HT22 cells[19,37a] as assessed using the MTT to colored formazan conversion assay.[19,23b,37a] Importantly, however, their HT22 protective effect was harnessed at concentrations ≥1 μM. The majority of the new analogues are stronger neuroprotectants than quercetin (Figure 1). In addition, they are not toxic to the cells up to the higher concentrations tested. Compounds **2** and **5** from the first series exhibit similar albeit rather weak neuroprotective activity with EC_{50} = 7.36 ± 0.24 and 6.35 ± 0.04 μM, respectively. Piperazine analogues **4** and **7** are better neuroprotectants than those containing ethylenediamine moieties, and

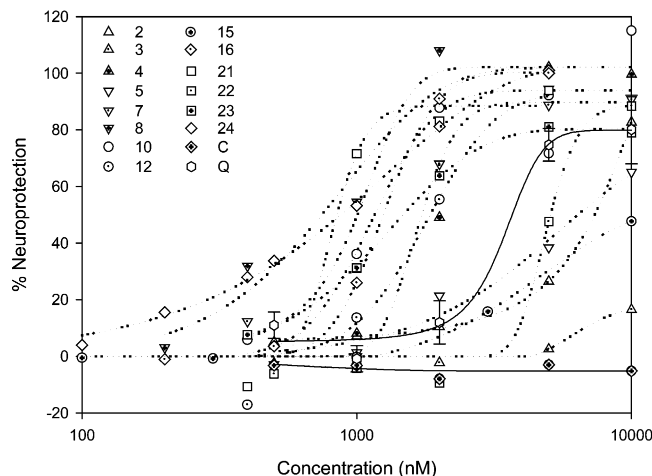


Figure 1. Inhibition of glutamate-induced oxytosis of HT22 cells by chroman/catechol hybrids. HT22 cells were exposed for 24 h to 5 mM glutamate in the absence or presence of increasing concentrations of caffeic acid (C), quercetin (Q), and the indicated hybrids, and changes in cell viability were assessed using MTT conversion to colored formazan as a measure of the number of living cells. Effects of the hybrids on the viability of HT22 cells that were not exposed to glutamate were similarly assessed. Direct interference of the hybrids with the MTT conversion could be excluded (see Experimental Section). Neuroprotection refers to the number of viable glutamate-exposed cells as a percentage of the respective number of viable nonexposed cells at each of the test compound concentrations tested. Data (mean ± SEM of 3−7 independent experiments) were curve-fitted using SigmaPlot 9.0. SEM values were similar to those depicted for Q. EC_{50} values were calculated as described in the legend of Table 1.

their activity was somewhat better than that of quercetin (EC_{50} = 2.98 ± 0.31 μM). Surprisingly, analogue **8**, bearing the protected catechol group, is the most active of the new compounds.

Trihydroxy amides **10** and **16** exhibited strong neuroprotective activity with EC_{50} = 1.14 ± 0.35 and 1.23 ± 0.26 μM, respectively. The methoxy chroman analogue **12** is slightly less active with EC_{50} = 2.15 ± 0.59 μM. Analogue **15** may be taken to be weakly active, although it is also significantly toxic to the cells at concentrations higher than 1 μM.

The furan analogue **21**, the methoxy analogue **23**, and the trihydroxy analogue **24** of the third series possess strong neuroprotective activity with EC_{50} = 1.00 ± 0.21, 1.17 ± 0.15, and 0.93 ± 0.19 μM, respectively. Protection of all the hydroxy groups diminishes (compound **22**) or abolishes (compound **20**) neuroprotection. The presence of a hydroxy group on the diphenylacetylene analogue **27** does not improve the neuroprotective activity.

Although compounds **2** and **3** are very strong antioxidants, they show decreased activity against DNA damage and weak neuroprotective activity. Piperazine analogues show high cytoprotective activity. Protection of the chroman hydroxy group of compound **4** (compound **7**) does not significantly compromise cytoprotective activity. However, protection of the catechol group of compound **4** abolishes its activity against DNA damage but increases its neuroprotective activity. Thus, the presence of the catechol group is requisite for activity against H_2O_2-induced cellular damage, probably because of its iron chelating properties, while for neuroprotective activity of this series of compounds, the chroman hydroxy group appears to be most effective when combined with a protected catechol group. These data may be taken to indicate that increased compound lipophilicity may facilitate uptake of **8** and allow for chroman accumulation in HT22 cells to levels higher than can be achieved with **4**. 5-Substituted chromans of the second and third series, such as the catechol derivative **12** and the trihydroxy analogues **16** and **24**, are strong antioxidants against ROS produced chemically and highly potent against H_2O_2- and glutamate-induced damage in Jurkat T cells and HT22 cells, respectively.

Although in some cases there is a correlation between cytoprotective activity and lipophilicity, this is not applicable for all these compounds. This is expected, however, considering that the antioxidant potential of a compound at the cellular level does not depend only on its capacity to scavenge ROS but also on the cellular substrate. The level and activity of scavenging entities and their distribution between different cellular compartments is highly dependent on cell-type specific metabolism of the antioxidants as well. For instance, Murota et al.[22] have reported that uptake of quercetin by Caco-2 intestinal cells is accompanied by extensive conjugation during or after adsorption and is followed by rapid efflux of the conjugates, that Caco-2 uptake of the more hydrophilic quercetin glycosides lagged substantially behind that of quercetin aglycone, and that conjugation and efflux of the glycosides was far less effective by comparison. Thus, it appears that, while free hydroxy groups contribute to antioxidant activity, they are subject to conjugation, which in turn provides for rapid efflux of the conjugates. In this light, an attempt to stage a structure−activity relationship (SAR) discussion based only on log *P* values would appear unjustified. However, we could deduce an optimum ClogP range of 4.1−6.0 for the chromans exhibiting improved neuroprotective capacity. Higher or lower values result in decreased activity (Table 1). Moreover, it appears that a combination of ClogP values 4.5−5.8 and substitution by catechol moieties at position 5 of benzopyran ring results in highly active compounds against oxidative stress induced cellular damage.

Although the activity against DNA damage can be largely attributed to the iron chelating properties of the chroman/catechol hybrids, their neuroprotective activity may be due not only to their antioxidant properties but perhaps also to their capacity to maintain intracellular GSH at a high enough level or to interfere with other cell signaling cascades implicated in oxytosis.[38] For instance, Herrera et al.[38a] reported recently that the sesquiterpene lactone parthenolide may increase GSH levels in hippocampal HT22 cells by activating the antioxidant/electrophilic response element (ARE) in the promoter of the gene coding of glutamyl cystine synthase gene, the rate-limiting enzyme for GSH synthesis. Torres et al.[38b] have shown that epicatechin−cysteine conjugates can rescue HT22 cells from glutamate oxytosis by maintaining glutathione levels rather than by scavenging ROS. Sagara et al.[37a] have reported that tyrphostins protect HT22 cells from glutamate oxytosis by three distinct mechanisms, depletion of ROS, inhibition of ROS production by mitochondria, and increasing cellular glutathione levels. Ishige et al.[19] have reported that flavonoids protect HT22 cells from glutamate by increasing intracellular GSH, as well as scavenging ROS and preventing the influx of Ca^{2+} in the presence of high levels of ROS. Whether chroman/catechol prevention of HT22 oxytosis involves, in addition to ROS depletion, maintenance of GSH levels, inhibition of ROS production, or interference with other cell signaling cascades implicated in oxytosis is not known, however.

Conclusion

We synthesized new chroman/catechol hybrids possessing cytoproprotective activity against oxidative stress. The 5-substituted chromans **12**, **16**, and **24** are very potent against H_2O_2- and glutamate-induced cellular damage. Compounds **21** and **23**

are less potent against DNA damage than the above three analogues but more active than the 2-substituted chromans, and they exhibit strong neuroprotective activity. While the activity of the chromans against DNA damage may be largely attributed to their iron-chelating properties, their activity against glutamate-induced oxytosis of HT-22 cells is currently a matter of conjecture.

Experimental Section

General Procedure for the Preparation of Analogues 2−5. To a solution of caffeic acid (49 mg, 0.27 mmol) and 0.14 mL of triethylamine in 2 mL anhydrous DMF were added at 0 °C the appropriate aminoamide **1** (0.27 mmol) and a solution of BOP (121 mg, 0.27 mmol) in 2 mL anhyd CH_2Cl_2. The mixture was stirred at 0 °C for 1 h and at ambient temperature for 24 h. Ethyl acetate was then added, and the mixture was washed with 1 N HCl, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl. The organic layer was dried and evaporated in vacuo.

1-[(3,4-Dihydro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-yl)carbonyl]-4-[3-(3,4-dihydroxyphenyl)-2-propenoyl]piperazine (4). Column chromatography (CH_2Cl_2/MeOH 90/10). Yield 95%, yellow solid, mp 100−103 °C. Anal. ($C_{27}H_{32}N_2O_6$) C, H, N.

***N*-[(3,4-Dihydro-6-methoxy-2,2,7,8-tetramethyl-2*H*-1-benzopyran-5-yl)methyl]-3-(3,4-dihydroxyphenyl)-2-propenamide (12).** Compound **12** was prepared from chroman-5-methylamine **11**[28] (87 mg, 0.48 mmol) and caffeic acid (120 mg, 0.48 mmol) using BOP (212 mg, 0.48 mmol). Purification by column chromatography (CH_2Cl_2/MeOH 90/10) afforded 113 mg (58%) of white solid, mp. 230−233 °C. Anal. ($C_{24}H_{29}NO_5$) C, H, N.

***N*-(3,4-Dihydro-6-hydroxy-2,2,7,8-tetramethyl-2*H*-1-benzopyran-5-yl)-3-(3,4-dihydroxyphenyl)-2-propenamide (16).** Compound **16** was prepared from **15** as described in the literature.[31] Column chromatography using CH_2Cl_2/MeOH 95/5 as eluent afforded 59% yield of a yellow viscous oil. Anal. ($C_{22}H_{25}NO_5$) C, H, N.

(*E*)-3,4-Dihydro-5-[2-(3,4-dimethoxyphenyl)ethenyl]-6-methoxy-2,2,7,8-tetramethyl-2*H*-1-benzopyran (20). To a solution of phosphonate **18** (694 mg, 2.41 mmol) in 4 mL of anhyd DMF was added at 0 °C potassium *tert*-butoxide (270 mg, 2.41 mmol), and the mixture was stirred at 0 °C for 1 h. Aldehyde **19** (400 mg, 1.61 mmol) in 2 mL of anhyd DMF was then added, and the mixture was stirred at ambient temperature for 1 h, at 110 °C for 2 h, and at ambient temperature for 24 h. The reaction mixture was then cooled at 0 °C, and H_2O and a solution of HCl (1 N) were added until the pH was acidic, and the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was washed with saturated aqueous NaCl, dried, and evaporated. The residue was purified by column chromatography (petroleum ether/AcOEt 90/10) to give 220 mg (36%) of **20** as white waxy solid. ^{1}H NMR δ: 7.07−6.97 (m, 2H) 7.05 (s, 2H, CH═CH), 6.87 (d, J = 7.9 Hz, 1H, ArH), 3.96 (s, 3H, Ar-OCH_3), 3.91 (s, 3H, Ar-OCH_3), 3.63 (s, 3H, -OCH_3), 2.85 (t, J = 6.5 Hz, 2H), 2.23 (s, 3H, Ar-CH_3), 2.15 (s, 3H, Ar-CH_3), 1.78 (t, J = 6.5 Hz, 2H), 1.33 (s, 6H, 2CH_3). ^{13}C NMR δ: 149.5, 149.1, 148.7, 148.1, 132.9, 131.5, 128.4, 126.6, 124.9, 121.3, 119.3, 116.7, 111.3, 108.8, 72.9, 60.1, 55.9, 33.1, 26.9, 21.9, 12.5, 12.2. Anal. ($C_{24}H_{30}O_4$) C, H, N.

1,7,8,9-Tetrahydro-2-(3,4-dihydroxyphenyl)-4,5,7,7-tetramethyl-2*H*-furan[3,2-*f*]benzopyran (21). To a solution of stilbene **20** (30 mg, 0.08 mmol) in 8 mL of anhyd CH_2Cl_2 was added 0.62 mL (5.85 mmol) of boron trifluoride dimethyl sulfide at 0 °C, and the mixture was stirred at ambient temperature for 24 h and then treated as described in the literature[31] to give 26 mg (100%) of **21** as light brown oil. ^{1}H NMR δ: 6.91 (s, 1H, Ar*H*), 6.81 (s, 2H, Ar*H*), 5.57 (pseudo triplet, J = 7.9 Hz and J = 9.2 Hz, 1H, -OC*H*CH_2-), 3.55−3.35 (m, 1H, -OCHC*H*H-), 3.05−2.90 (dd, J = 9.2 Hz and J = 15.3 Hz, 1H, OCHCH*H*-), 2.59−2.46 (m, 2*H*, CH_2-chroman), 2.13 (s, 3H, Ar-C*H*$_3$), 2.09 (s, 3H, Ar-C*H*$_3$), 1.76 (t, J = 6.5 Hz, 2H, C*H*$_2$), 1.30 (s, 6H, 2C*H*$_3$). ^{13}C NMR δ: 150.6, 145.8, 143.6, 143.4, 135.5, 123.9, 120.6, 118.7, 116.6, 114.6, 113.2, 113.1, 83.2, 73.2, 37.9, 32.6, 29.7, 26.9, 20.7, 12.3, 11.6. Anal. ($C_{21}H_{24}O_4$) C, H, N.

3,4-Dihydro-5-(3,4-dimethoxyphenyl)-6-methoxy-2,2,7,8-tetramethyl-2*H*-1-benzopyran (22). A solution of compound **20** (110 mg, 0.29 mmol) in 8 mL of AcOEt was added to 11 mg of Pd/C 10%, and the mixture was hydrogenated at 50 psi and at ambient temperature for 5 h. The mixture was then filtrated through Celite and washed with AcOEt, and the solvent was evaporated. Yield 110 mg (100%), yellow oil. ^{1}H NMR δ: 6.81 (bs, 2H, Ar*H*), 6.72 (s, 1H, Ar*H*), 3.87 (s, 6H, -Ar-OC*H*$_3$), 3.72 (s, 3H, -OCH_3-chroman), 2.87−2.84 (m, 2H, -C*H*$_2$-CH_2-), 2.79−2.76 (m, 2H, -CH_2-C*H*$_2$-), 2.62 (t, J = 6.5 Hz, 2H, C*H*$_2$), 2.23 (s, 3H, Ar-C*H*$_3$), 2.12 (s, 3H, Ar-C*H*$_3$), 1.75 (t, J = 6.5 Hz, 2H, C*H*$_2$), 1.28 (s, 6H, 2CH_3). ^{13}C NMR δ: 149.6, 148.8, 148.1, 147.2, 135.3, 129.7, 128.1, 123.7, 120.2, 116.9, 111.9, 111.3, 72.8, 61.1, 55.9, 55.7, 36.1, 32.9, 29.2, 26.9, 20.3, 12.8, 11.9. Anal. ($C_{24}H_{32}O_4$) C, H, N.

3,4-Dihydro-5-(3,4-dihydroxyphenyl)-6-methoxy-2,2,7,8-tetramethyl-2*H*-1-benzopyran (23). To a solution of **22** (40 mg, 0.11 mmol) in 8 mL of anhyd CH_2Cl_2 was added boron trifluoride dimethyl sulfide (0.16 mL, 1.56 mmol) at 0 °C, and the mixture was stirred at ambient temperature for 2 h.[28] Purification by column chromatography using CH_2Cl_2/MeOH 95/5 afforded **23** as colorless oil. Yield 17 mg (46%). Anal. ($C_{22}H_{28}O_4$) C, H, N.

3,4-Dihydro-5-(3,4-dihydroxyphenyl)-2,2,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (24). To a solution of **22** (60 mg, 0.17 mmol) in 10 mL of anhyd CH_2Cl_2 was added boron trifluoride dimethyl sulfide (0.27 mL, 2.52 mmol) at 0 °C, and the mixture was stirred at ambient temperature for 24 h and then treated as previously described.[28] Purification by column chromatography using CH_2Cl_2/MeOH 95/5 afforded 30 mg (53%) of **24** as colorless oil. Anal. ($C_{21}H_{26}O_4$) C, H, N.

3,4-Dihydro-5-(3,4-dimethoxyphenylethynyl)-2,2,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (27). A solution of 5-bromo-3,4-dihydro-2,2,7,8-tetramethyl-2*H*-1-benzopyran-6-ol, **26** (44 mg, 0.15 mmol), and alkyne **25** (25 mg, 0.15 mmol) in anhyd triethylamine (0.18 mL) was degassed in vacuo. $PdCl_2(PPh_3)_2$ (1.7 mg, 0.003 mmol), CuI (1 mg, 0.006 mmol), and 1.8 mL of anhyd DMF were then added, and the mixture was stirred at ambient temperature for 3 h. Saturated aqueous NH_4Cl was added to the mixture and extracted with diethyl ether. The organic layer was washed with saturated aqueous NaCl and dried over Na_2SO_4, the solvent was evaporated, and the residue was purified by column chromatography (petroleum ether/AcOEt 70/30) to give 50 mg (89%) of **27** as brown viscous oil. ^{1}H NMR δ: 7.18 (d, J = 8.5 Hz, 1H), 7.08 (s, 1H), 6.84 (d, J = 8.5 Hz, 1H), 3.90 (s, 3H), 3.88 (s, 3H), 2.82 (t, J = 6.5 Hz, 2H), 2.05 (s, 6H), 1.68 (t, J = 6.5 Hz, 2H), 1.31 (s, 6H). ^{13}C NMR δ: 150.6, 149.3, 141.6, 140.0, 135.1, 130.3, 128.1, 125.8, 114.5, 112.0, 110.9, 81.6, 70.9, 55.9, 42.3, 29.1, 24.2, 12.6. Anal. ($C_{23}H_{26}O_4$) C, H, N.

Evaluation of the Activity of the New Compounds against H_2O_2-Induced DNA Damage. Cell Culture and Treatment. The ability of individual compounds to protect cellular DNA from H_2O_2-induced damage was investigated by using Jurkat cells (a human T-lymphocytic cell line, ATCC, clone E6-1). One hundred microliters of RPMI 1640 growth medium (supplemented with 10% fetal calf serum, penicillin 100 IU/ml, streptomycin 100 μg/mL, and glutamine 300 μg/mL) containing 1.5×10^5 cells was placed into each of 96 wells of ELISA plastic plates and incubated for 1 h at 37 °C, 95% air, 5% CO_2. Cells were subsequently treated for 10 min with 60 ng of the enzyme glucose oxidase, which was able to generate 11.8 ± 1.5 μM H_2O_2 per minute in the absence of cells. Additions of the compounds at the indicated concentrations were done 30 min prior to the addition of glucose oxidase. Following the treatment, cells were collected by centrifugation (250 × g at 4 °C for 5 min) for further analysis.

Single Cell Gel Electrophoresis. The assay used was essentially the same as previously described.[29] Cells were suspended in 1% low-melting-point agarose in PBS (pH 7.4) and pipetted onto superfrosted glass microscope slides precoated with a layer of 1% of normal-melting-point agarose (warmed at 37 °C prior to use). The agarose was allowed to set at 4 °C for 10 min, and then the

slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris at pH 10, 1% Triton X-100 v/v) at 4 °C for 1 h to remove cellular proteins. Slides were then placed in single rows in a 30-cm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA, pH > 13, at 4 °C for 40 min to allow for separation of the two DNA strands (alkaline unwinding). Electrophoresis was performed in the unwinding solution at 30 V (1 V/cm), 300 A, for 30 min. The slides were then washed three times for 5 min each with 0.4 M Tris, pH 7.5, at 4 °C before staining with Hoechst 33342 (20 μg/mL).

Image Analysis and Scoring. Stained nucleoids were examined under a UV microscope with an excitation filter of 435 nm and a magnification of 400. The damage was not homogeneous, and visual scoring of the cellular DNA on each slide was based on characterization of 100 randomly selected nucleoids. The comet-like DNA formations were categorized into five classes (0, 1, 2, 3, and 4) representing an increasing extent of DNA damage seen as a "tail". Each comet was assigned a value according to its class. Accordingly, the overall score for 100 comets ranged from 0 (100% of comets in class 0) to 400 (100% of comets in class 4). In this way, the overall DNA damage of the cell population can be expressed in arbitrary units. Visual scoring expressed in this way correlates nearly linearly with other parameters such as percent of DNA in tail estimated after computer image analysis using a specific software package. Observation and analysis of the results were always carried out by the same experienced person using a specific pattern when moving along the slide.

Statistical Analysis. Student's *t*-test analysis was used to examine statistically significant differences. The differences between total minus control (background) values were estimated and tested for statistical significance. Each value represents the mean ± SD of triplicate measurements of two independent experiments.

Evaluation of the Activity of the Chroman/Catechol Hybrids against Glutamate Neurotoxicity. HT22 cells were maintained in phenol red free DMEM medium (SIGMA) supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS, GIBCO), and 1% GlutaMAX-1 at 37 °C and 5% CO_2. For the cytotoxicity assay, the cells were plated in a 96-well flat bottom plate at a density of 4000 cells/well in 100 μL of the above DMEM−Hepes−GlutaMAX medium containing 10% dextran-coated charcoal treated FBS (DCC-FBS) for 24 h prior to exposure of the cells to serial dilutions of the test compounds in fresh DMEM−Hepes−GlutaMAX−DCC-FBS in the absence or presence of 5 mM glutamate for an additional 24 h at 37 °C. Following treatment, the number of viable cells was determined using conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) to colored formazan as a measure of the number of living cells, essentially as already described by others.[19,23b,37a] Cells that did not receive glutamate served to test compound cytotoxicity at each of the concentrations tested, whereas those that received glutamate as well as test compound served to assess the respective neuroprotective activity. Positive (glutamate only) and negative (vehicle only) neurotoxicity controls served to normalize the neuroprotective activity. Direct interference of the chroman/catechol hybrids with MTT conversion to formazan was excluded for all concentrations tested using mock cultures deprived of HT22 cells. Interference of the chromans with mitochondrial conversion of MTT to formazan under normal growth conditions was excluded using the trypan blue exclusion assay to directly determine the number living cells.

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Supporting Information Available: Detailed experimental procedures for the synthesis of compounds **2**, **3**, **5−8**, **10**, **14**, **15**, **18**, and **25**, spectroscopic data for compounds **4**, **12**, **16**, **23**, and **24**, and analytical data for the final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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