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Isoxazole substituted chromans against oxidative stress-induced neuronal damage

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ABSTRACT

We have previously reported that catechol-bearing regioisomers of 5-isoxazolyl-6-hydroxy-chroman display higher in vitro neuroprotective activity, compared to hybrids with other nitrogen heterocycles, but their activity is hampered by cytotoxicity at higher concentrations. In an effort to discover non-cytotoxic isoxazole substituted chromans of high neuroprotective activity, 20 new 3- and 5-substituted (chroman-5-yl)-isoxazoles and (chroman-2-yl)-isoxazoles were synthesized using the copper(I)-catalysed cycloaddition reaction between in situ generated nitrile oxides and terminal acetylenes. An additional aim was to further explore the effect of the isoxazole ring substituents on the neuroprotective activity. The activity of these compounds against oxidative stress-induced death (oxytosis) of neuronal HT22 cells was evaluated and interesting SARs for this group of analogues were derived. The vast majority of new chroman analogues displayed high in vitro neuroprotective activity displaying EC_{50} values below 1 μM and lacked cytotoxicity. The position of substituents on the isoxazole ring influences the activity of the regioisomers, with the 3-aryl-5-(chroman-5-yl)-isoxazoles, **17** and **18** and bis-chroman **20** displaying higher neuroprotective activity (EC_{50} ~0.3 μM) compared to other (chroman-5-yl) and (chroman-2-yl)-isoxazoles.
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1. Introduction

The 1-benzopyran ring system constitutes the basic skeleton of a variety of natural compounds with interesting biological activities.[1,2] In addition, several biologically active synthetic 3,4-dihydro-2*H*-1-benzopyrans (chromans)[3,4] have been reported, suggesting that derivatives of this benzopyran motif may be capable of interacting with a variety of cellular targets. Our group has long been involved with the design and synthesis of cardioprotective[5] and neuroprotective chroman derivatives.[6] We have recently reported the synthesis of 1,2-dithiolane/chroman hybrids[6b] and chroman/catechol[6c] hybrids bearing heterocyclic rings such as isoxazole, 1,2,4- and 1,3,4-oxadiazole, 1,2,3-triazole and tetrazole and the study of the influence of these heterocycles on the activity of the compounds at the cellular level. Interestingly, we found that the two isoxazole analogues we synthesized displayed higher neuroprotective activity than chromans bearing other nitrogen heterocycles.[6c] Specifically, the regioisomers of 5-isoxazolyl-6-hydroxy-chroman bearing a catechol moiety displayed strong activity against oxytosis of HT22 hippocampal neurons (EC_{50} = 0.3–0.6 μM), but they were cytotoxic at concentrations higher than 1 μM.

A number of analogues based on the isoxazole pharmacophore have been reported to exhibit a wide range of biological activities including, antithrombotic,[7] antiviral,[8] antiapoptotic[9] and antiparasitic[10] properties. In this light it appears that the isoxazole scaffold can be incorporated in the design for a wide range of bioactive agents. We therefore set out to synthesize new analogues to explore the effect of various isoxazole ring substituents on the ability of chroman derivatives to protect glutamate-challenged HT22 hippocampal neurons from oxytosis. The specific aim of this study was to discover new 2- or 5-isoxazolyl-chromans of even higher neuroprotective activity and much lower cytotoxicity compared to the catechol-bearing regioisomers of 5-isoxazolyl-chroman previously reported.[6c] To this end we designed and synthesized 20 new analogues in which isoxazole moieties bear aliphatic or aromatic substituents. The copper(I)-catalysed cycloaddition reaction between in situ generated nitrile oxides and terminal acetylenes was applied in the synthesis of new compounds.

2. Chemistry

Isoxazoles were obtained by 1,3-dipolar cycloaddition of nitrile oxides to terminal acetylenes. The appropriate aldoximes were transformed to the corresponding nitrile oxides using

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chloramine-T trihydrate, which acts as both a halogenating agent and a base.[11] In the presence of a catalytic amount of copper(I), obtained from comproportionation of Cu metal and $CuSO_4{\cdot}5H_2O$, the in situ generated nitrile oxides reacted with the acetylene intermediates to afford the corresponding isoxazoles. Scheme 1 shows the synthesis of 5-substituted chromans: cycloaddition reaction of chroman oxime I[6c] with aliphatic or aromatic alkynes and subsequent deprotection using $BF_3{\cdot}SMe_2$ gave isoxazole derivatives **5**–**9**. The ^{1}H NMR spectrum of compounds **5**–**7** and **9** showed a singlet at ~8.4 ppm indicating a hydrogen bond between the 6-OH and the nitrogen of the isoxazole. For the synthesis of the regioisomers **14**–**18**, 5-ethynyl-benzopyran **II**[6b] reacted with the appropriate oximes to afford the isoxazole analogues **10**–**13** which were deprotected using $BF_3{\cdot}SMe_2$. Bis-chroman **19** was prepared from benzopyran oxime **I** and 5-ethynyl-benzopyran **II** and after deprotection, gave **20**.

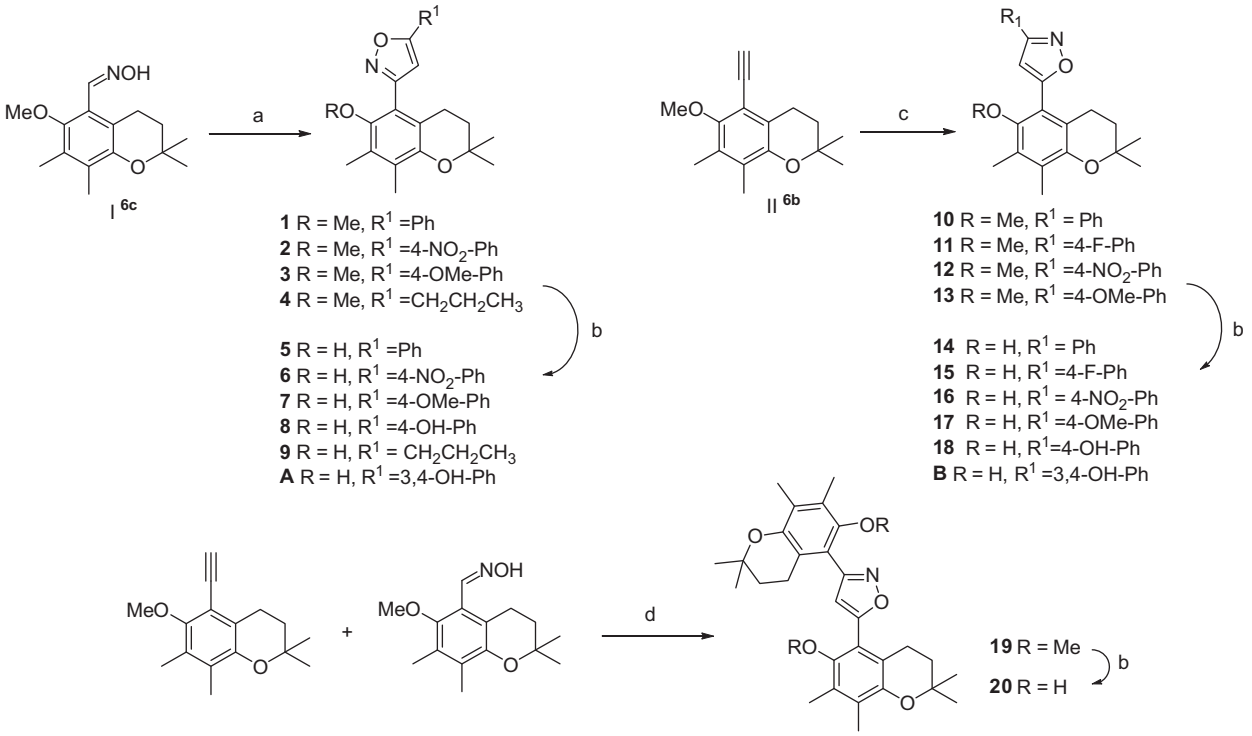
The synthesis of 2-substituted chromans is depicted in Scheme 2. Chroman-2-methanol **IV**,[12] obtained from the reduction with $LiAlH_4$ of 6-methoxy-trolox methyl ester **III**,[6a] was used as starting material. Oxidation using pyridinium chlorochromate (PCC), followed by reaction with hydroxylamine gave oxime **21**, which reacted with the appropriate alkyne to produce isoxazoles **22**–**24** which upon treatment with $BF_3{\cdot}SMe_2$ gave compounds **25**–**27**. Reaction of aldehyde **V** with dimethyl-(diazomethyl)phosphonate (generated in situ from dimethyl-1-diazo-2-oxopropylphosphonate (Bestmann–Ohira reagent[13]) by treatment with K_2CO_3, MeOH) afforded alkyne **28** which in turn gave isoxazoles **29** and **30** and, upon deprotection, analogues **31**, **32**. Cycloaddition of oxime **21** to ethynyl-benzopyran **II** gave **33** which, after deprotection, afforded the bis-chroman **34**. Alkylation of chroman-2-methanol with propargyl bromide produced **35** which reacted with aromatic oximes to give compounds **36**, **37** and, after deprotection of methoxy groups, compounds **38**–**40**.

The yields of cycloaddition depend on electronic and steric properties of oximes and alkynes. Aryl alkynes or oximes bearing electron donating substituents (compounds **3**, **13**, **37**) led to higher yields. Steric hindrance caused by 2-substituted chromans (compounds **22**–**24**, **29**, **30**) and the bis-chromans (**19**, **33**) resulted in lower yields.

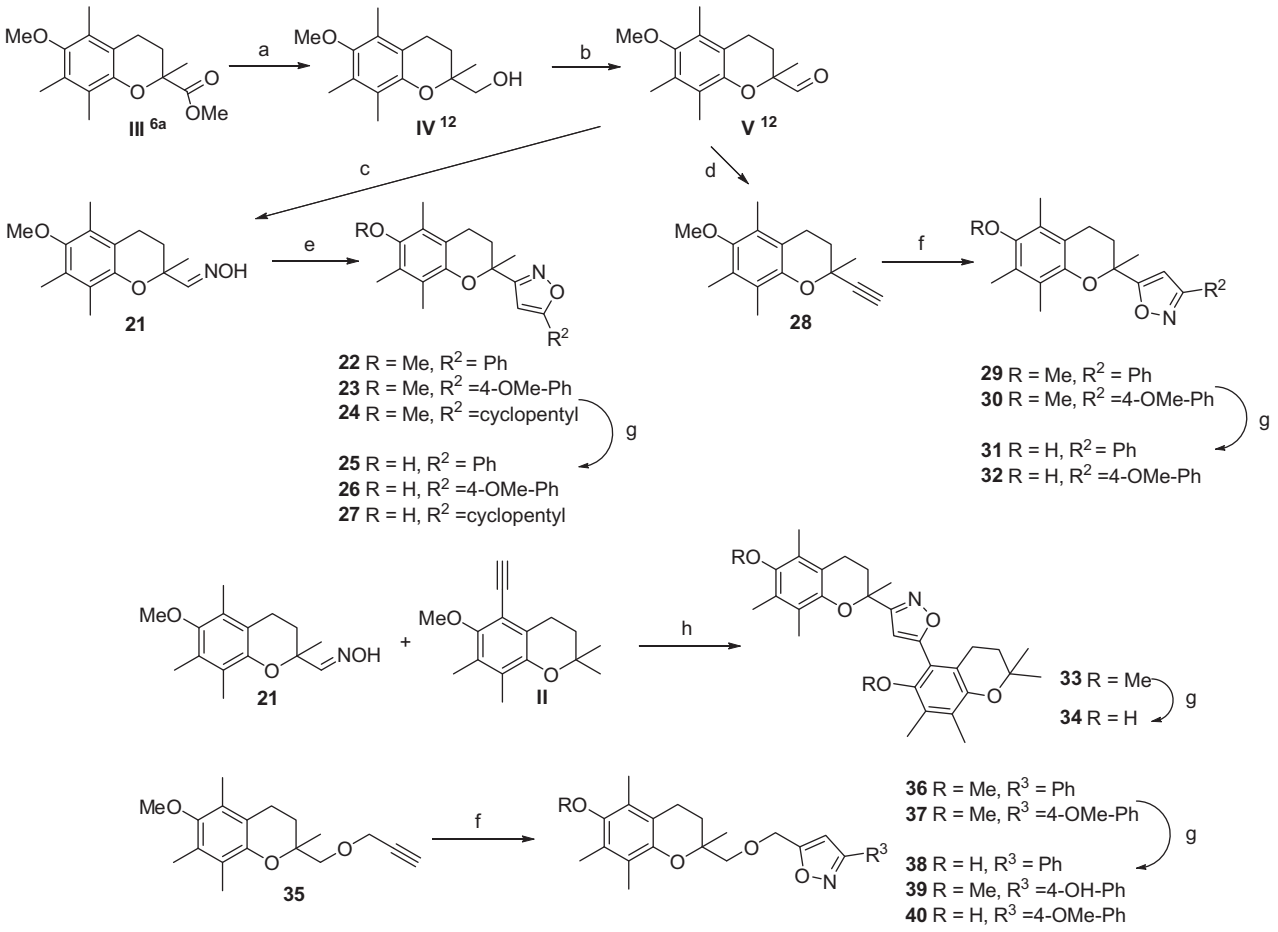
In addition, we endeavoured to synthesize the 2-subtituted chromans on solid support. Thus, trolox ethyl ester was attached to bromo Wang resin.[14] The loading to the resin did not exceed 50%. The yield of the loading was even lower using Wang[15] or Merrifield resins. Although reduction to the chroman-2-methanol using $LiAlH_4$[16] was quantitative, subsequent alkylation[17] with propargyl bromide to the corresponding propargyl ether proceeded with low yield, suggesting that the synthesis in solution was the preferred procedure for the synthesis of these chromans.

3. Results and discussion

Oxidative stress is implicated in neurodegenerative diseases such as Alzheimer and Parkinson diseases and is mediated via a series of disturbances to the redox homeostasis of the cells. The mouse hippocampal cell line HT22 has been used to elucidate sequential cellular events during oxidative stress-induced cell death (oxytosis) caused by glutamate-induced depletion of intracellular glutathione.[18–21] Glutamate-challenged HT22 cells suffer oxytosis within 24 h as a result of accumulation of reactive oxygen species (ROS). Administration of antioxidants has been shown to prevent oxytosis, and the HT22 cells have been extensively used for the identification of novel neuroprotective agents that were subsequently tested positive in reducing stroke-induced brain damage.[22,23] Figures 1 and 2 show representative assessments of the neuroprotective activity of 5- and 2-substituted chromans, respectively. The efficacy of neuroprotection, defined as the percentage of glutamate-challenged HT22 cells rescued from oxytosis



Scheme 1. Reagents and conditions: (a) alkyne, $TsN(Cl)Na{\cdot}3H_2O$, $CuSO_4{\cdot}5H_2O$, Cu^0, *t*-BuOH/H_2O, rt, 12 h, (b) $BF_3{\cdot}SMe_2$ (20 equiv), CH_2Cl_2, at 0 °C for 1 h and an additional 1 h at rt (deprotection of **3** gave a mixture of analogues **7** and **8**) (c) oxime, $TsN(Cl)Na{\cdot}3H_2O$, $CuSO_4{\cdot}5H_2O$, Cu^0, *t*-BuOH/H_2O, rt, 12 h, (d) $TsN(Cl)Na{\cdot}3H_2O$, $CuSO_4{\cdot}5H_2O$, Cu^0, *t*-BuOH/H_2O, rt, 12 h.



Scheme 2. Reagents and conditions: (a) $LiAlH_4$, THF, 70 °C, (b) PCC, anhyd CH_2Cl_2, (c) $NH_2OH{\cdot}HCl$, anhyd pyr, (d) Bestmann–Ohira reagent, K_2CO_3, MeOH, (e) alkyne, $TsN(Cl)Na{\cdot}3H_2O$, $CuSO_4{\cdot}5H_2O$, Cu^0, *t*-BuOH/H_2O, rt, 12 h, (f) oxime, $TsN(Cl)Na{\cdot}3H_2O$, $CuSO_4{\cdot}5H_2O$, Cu^0, *t*-BuOH/H_2O, rt, 12 h, (g) $BF_3{\cdot}SMe_2$, CH_2Cl_2, at 0 °C for 1 h and an additional 1 h at rt (deprotection of **36** gave a mixture of analogues **39** and **40**) (h) $TsN(Cl)Na{\cdot}3H_2O$, $CuSO_4{\cdot}5H_2O$, Cu^0, *t*-BuOH/H_2O, DMF, rt, 12 h.

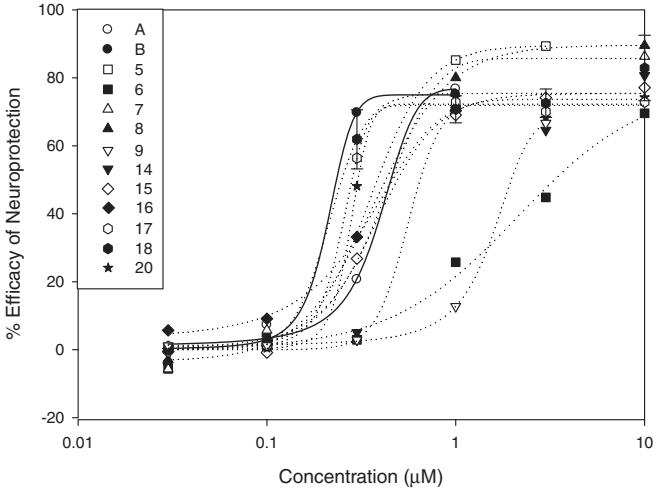


Figure 1. Protection of HT22 cells from oxytosis by 5-substituted chroman analogues. Cells were challenged with 5 mM glutamate in the presence of vehicle or increasing concentrations of analogues for 24 h and relative numbers of living cells were assessed as described in Section 5. Values are mean ± SEM of three independent experiments carried out in triplicate with an intra-assay variation similar to that shown for analogue **18**. A, B: Catechol moiety-bearing regioisomers of 5-isoxazolyl-6-hydroxy-chroman previously reported to display cytotoxicity as well as strong activity against oxytosis of HT22 cells.[6c]

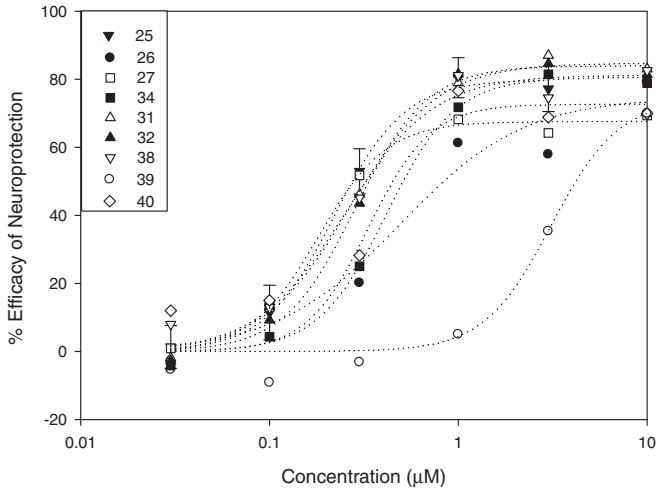


Figure 2. Protection of HT22 cells from oxytosis by 2-substituted chroman analogues. Cells were challenged with 5 mM glutamate in the presence of vehicle or increasing concentrations of analogues for 24 h and relative numbers of living cells were assessed as described in Experimental part. Values are mean ± SEM of three independent experiments carried out in triplicate with an intra-assay variation similar to that shown for analogue **25**.

in the presence of 10 μM test compound compared to vehicle, and the potency of neuroprotection, defined as the test compound concentration for 50% efficacy (EC_{50}), are listed in Tables 1 and 2 along with test compound cytotoxicity. The latter is defined as% loss of cell viability among not challenged cells in the presence of

10 μM test compound compared to vehicle. The activity and cytotoxicity of new compounds is compared to that of 5-isoxazolyl-6-hydroxy-chroman regioisomers bearing a catechol moiety previously reported (compounds A and B, Table 1).[6c]

The data of Table 1 show that the potency of 5-substituted chromans depends on the type of regioisomer as well as the substituent. With respect to the nature of the substituent in 5-chromanyl-isoxazoles, the hydroxyphenyl analogue **18** (EC_{50} = 0.33 μM), the methoxyphenyl derivative **17** (EC_{50} = 0.32 μM) and the bis-chroman derivative **20** (EC_{50} = 0.30 μM) display similar potency (p >0.05; t-test) with their catechol counterpart **B** (EC_{50} = 0.25 μM) but are not cytotoxic. Similarly, among the 3-chromanyl isoxazoles, the hydroxyphenyl analogue **8** (EC_{50} = 0.49 μM) and the methoxyphenyl derivative **7** (EC_{50} = 0.70 μM) display similar potency (p >0.05; t-test) with their catechol counterpart **A** (EC_{50} = 0.60 μM) and are also not cytotoxic. The 5-chromanyl-isoxazole **17** is more potent (p = 0.028; t-test) that the respective 3-chromanyl isoxazoles **7** by a factor of about 2, possibly reflecting a negative role of the hydrogen bond between the 6-hydroxy group and the isoxazole nitrogen in 3-chromanyl-isoxazoles. However, the potencies of **8** and **18** are not significantly different (p >0.05; t-test). Similarly, 5-chromanyl-3-phenyl-isoxazole analogue **14** (EC_{50} = 0.73 μM) displays similar potency (p >0.05; t-test) with its 3-chromanyl isomer **5** (EC_{50} = 0.61 μM), indicating that presence of a strong electron donating substituent on the phenyl group or a second antioxidant moiety are requisite for 5-chromanyl analogues of comparatively higher potency. Nonetheless, while 5-chromanyl-3-nitrophenyl isoxazole **16** (EC_{50} = 0.51 μM) displays similar potency with **17** and **18**, its 3-chromanyl isomer **6** (EC_{50} = 4.68 μM) is less potent (p <0.000; t-test) than **7** and **8** by a factor of 6.7 and 9.6, respectively, indicating that the nature as well as the position of the substituent has a strong influence on the in vitro neuroprotective activity of 5-substituted chromans.

On the other hand, 5-phenyl-isoxazole **25** (EC_{50} = 0.53 μM) displays similar potency (p >0.05; t-test) with its 3-phenyl-isoxazole isomer **31** (EC_{50} = 0.52 μM), the 3-phenyl-isoxazyl-methyloxy analogue **38** (EC_{50} = 0.54 μM) and 5-chromanyl analogue **5**. Thus, chromans substituted at position 2 by 5-aryl-isoxazole moieties are by and large as potent as their 3-aryl-isoxazole isomers (Table 2) and nearly as potent as chromans substituted at position 5 by 5-aryl-isoxazole moieties (Table 1). However, methoxyphenyl analogue **26** (EC_{50} = 1.07 μM) is again significantly less potent (p <0.002; t-test) than its isomer **32** (EC_{50} = 0.42 μM). In addition, the bis-chroman derivative **26** is significantly less potent (p <0.025; t-test) than its regioisomer **34**. Protection of the 6-hydroxy group strongly influences the activity. Thus, compound **39** (EC_{50} = 7.11 μM) is significantly less potent (p <0.000) than **40** (EC_{50} = 0.69 μM).

On the whole it appears that the bioactivities of the new analogues cannot be analysed by considering only hydrogen bonding and/or electronic properties of the substituents. Other factors not directly related to oxidative stress pathways but differentially affected by regioisomers, such as intracellular and/or intraorganellar bioavailability[21,24] or interaction with signalling entities,[25] may indirectly influence cell fate. Interestingly, it has been reported that the potential of antioxidants to protect glutamate-challenged HT22 cells from oxytosis depends on their accessibility to specific mitochondrial sites of ROS production under oxidative stress conditions as well as their intracellular bioavailability, which is also known to vary considerably among structurally related compounds.[21,24] In addition, depending on the considered structure, delocalization of the phenoxy radical, for example, between regioisomers **20** and **34**, may occur differently and this may also interfere with the antioxidant properties. Between regioisomers **20** and **34** delocalization of phenoxy radical seems to be more efficient in compound **20**. Clearly, the activity of our compounds at the cellular level depends on the nature of the heterocycle substituents. However, whether and how these substituents may differentially impact on the various pathways of ROS production and/or oxytosis modulation is presently unclear.

4. Conclusion

The majority of the new chroman analogues displayed high in vitro neuroprotective activity, with EC_{50} values below 1 μM, and lacked cytotoxicity. With respect to 5-substituted chromans, the nature of the substituents on the isoxazole ring influences the activity of the regioisomers, with 3-aryl-5-chromanyl-isoxazoles displaying higher potency than their 5-aryl-3-chromanyl-isoxazole isomers. With respect to 2-substituted chromans, 3-aryl-5-chromanyl-isoxazoles and 5-aryl-3-chromanyl-isoxazoles display largely similar potency and both are nearly equipotent with 5-subsituted chromans bearing 5-aryl-isoxazole moieties. In 5-subsituted chromans bearing 3-aryl-isoxazole substituents, the presence of an electron donating substituent or a second antioxidant moiety (analogues **17**, **18** and **20**) seems requisite for high potency. The 3,5-bis(chroman-5-yl)isoxazole analogue **20,** in particular, is more potent than its 5-(chroman-5-yl)-3-(chroman-2-yl)isoxazole congener **34** by a factor of 2.4.

5. Experimental section

5.1. Chemistry

Melting points were determined on a Buchi 510 apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian spectrometers operating at 300 MHz or 600 MHz and ^{13}C spectra were recorded at 75 MHz using $CDCl_3$ as solvent. Silica gel plates Macherey-Nagel Sil G-25 UV_{254} were used for thin layer chromatography. Chromatographic purification was performed with silica gel (200–400 mesh). The purity of the tested compounds was determined by HPLC (Thermo Scientific HPLC Spectra System) column Nucleosil 100-5 150 × 4.6 mm C18 5μ, Macherey-Nagel. Mass spectra were obtained on TSQ 7000 Finigan instrument or HPLC–MS^n Fleet-Thermo, in the ESI mode. HRMS spectra were recorded, in the ESI mode, on UPLC-MS^n Orbitrap Velos-Thermo.

5.1.1. General procedure for the synthesis of isoxazoles

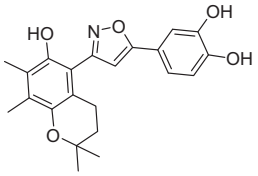
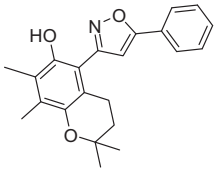
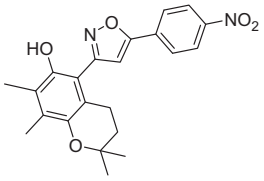
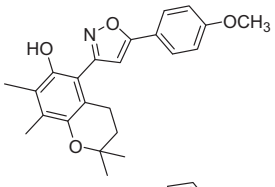
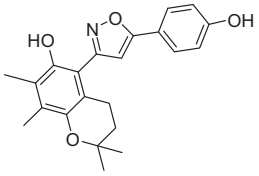
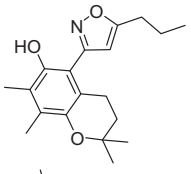
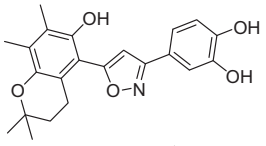
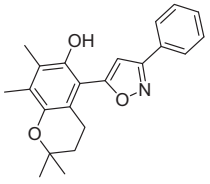
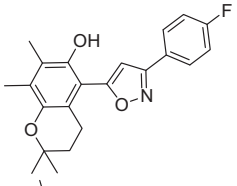
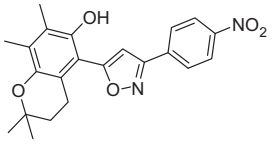
To the appropriate oxime (0.1 mmol) dissolved in 1 mL of t-BuOH/H_2O (1/1), chloramine-T trihydrate (0.1 mmol) was added in small portions over 5 min. Then, $CuSO_4{\cdot}5H_2O$ (0.01 mmol), copper turnings (catalytic amount ~0.5 mg) and the requisite alkyne (0.1 mmol) were added and the mixture was stirred overnight. The reaction mixture was poured onto ice/water and after addition of dilute NH_4OH (to remove all copper salts), it was extracted with AcOEt. The organic layer was washed with satd aqueous NaCl, dried and evaporated to dryness.

5.1.1.1. 5-(3,4-Dihydro-6-methoxy-2,2,7,8-tetramethyl-2*H*-1-benzopyran-5-yl)-3-phenyl-isoxazole (10). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield 38%, yellowish solid, mp 75–77 °C. ^{1}H NMR δ: 7.89 (m, 2H), 7.48 (m, 3H), 6.76 (s, 1H), 3.52 (s, 3H, –OCH_3), 2.72 (t, 2H, J = 6.8 Hz, –CH_2), 2.23 (s, 3H, Ar–CH_3), 2.18 (s, 3H, Ar–CH_3), 1.73 (t, 2H, J = 6.8 Hz, –CH_2), 1.34 (s, 6H, CH_3). ^{13}C NMR δ: 168.2, 162.4, 149.6, 148.3, 129.9, 129.3, 128.9, 126.8, 118.2, 118.1, 102.7, 73.6, 61.5, 32.5, 26.9, 21.4, 12.5, 12.4. MS m/z: 386.24 $(M+Na)^+$

5.1.2. General procedure for deprotection of methoxy groups

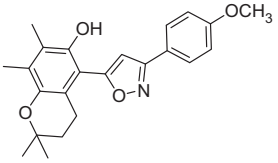
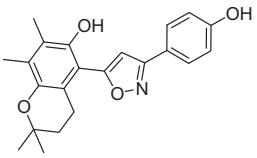
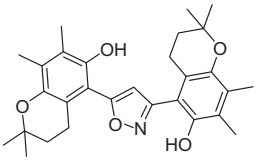
A solution of protected compound (1 equiv) in anhyd CH_2Cl_2 (C = 0.026 M), was cooled at 0 °C and $BF_3{\cdot}SMe_2$ (20 equiv) was added. The mixture was stirred at 0 °C for 1 h and an additional

Table 1
Efficacy and potency of 5-substituted chroman analogues to protect glutamate-challenged HT22 cells from oxytosis

Compound		Efficacy[a] (%)	Potency[b] (μM)	Cytotoxicity[c] (%)
A		70 ± 2[†]	0.60 ± 0.19	52 ± 3
5		79 ± 4[*]	0.61 ± 0.08	36 ± 7
6		79 ± 5	4.68 ± 0.27	ns
7		77 ± 4	0.70 ± 0.15	ns
8		81 ± 5	0.49 ± 0.11	ns
9		77 ± 1[*]	1.76 ± 0.10	33 ± 13
B		73 ± 5[†]	0.25 ± 0.04	82 ± 1
14		76 ± 5	0.73 ± 0.07	ns
15		77 ± 3	0.52 ± 0.08	ns
16		76 ± 3[*]	0.51 ± 0.17	26 ± 7

(*continued on next page*)

Table 1 (*continued*)

Compound		Efficacy[a] (%)	Potency[b] (μM)	Cytotoxicity[c] (%)
17		75 ± 3	0.32 ± 0.03	ns
18		74 ± 4	0.33 ± 0.12	ns
20		79 ± 4	0.30 ± 0.07	ns

[a] Defined as % protection of viability of glutamate-challenged HT22 cells from oxytosis at 10 μM test compound unless defined otherwise. Cell viability was assessed using the MTT assay (Section 5.2.1). Efficacy (%) of neuroprotection was calculated as described in Section 5. Values are mean ± SEM of at least three independent experiments such as those shown in Figures 1 and 2.

[b] Defined as the test compound concentration, that is, able to protect viability of glutamate-challenged HT22 cells to a level equal to 50% of that of non-challenged cells. Values are mean ± SEM of at least three independent experiments similar to those shown in Figures 1 and 2.

[c] Defined as % loss of cell viability in the presence of 10 μM test compound compared to vehicle in the absence of glutamate as assessed using the Trypan blue exclusion assay (Section 5.2.2). Values are mean ± SEM of at least three independent experiments carried out in triplicate. ns = non-significant (p >0,05; t-test).

[*] Assessed at 3 μM due to cytotoxicity at test compound concentrations ⩾10 μM.

[†] Assessed at 1 μM due to cytotoxicity at test compound concentrations ⩾3 μM.

1 h at rt. The excess of $BF_3{\cdot}SMe_2$ was removed under argon, water was added and the mixture was extracted with AcOEt. The organic layer was washed with satd aqueous NaCl, dried and concentrated in vacuo.

5.1.2.1. 3,4-Dihydro-2,2,7,8-tetramethyl-5-(5-phenylisoxazol-3-yl)-2*H*-1-benzopyran-6-ol (5). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield 49%, yellow solid, mp 155–157 °C. HPLC: 8:2 MeOH–H_2O, 0.05% TFA, flow rate: 1.5 mL/min, t_R: 6.97 min, detection at 254 nm, purity 97.9%. ^{1}H NMR δ: 8.50 (s, 1H), 7.87–7.84 (m, 2H), 7.51–7.48 (m, 3H), 6.79 (s, 1H), 2.84 (t, 2H, J = 6.7 Hz), 2.24 (s, 3H), 2.18 (s, 3H), 1.77 (t, 2H, J = 6.7 Hz), 1.36 (s, 6H). ^{13}C NMR δ: 169.5, 161.7, 146.7, 145.2, 136.5, 130.5, 129.1, 128.9, 126.0, 123.7, 115.5, 109.9, 101.1, 72.8, 32.9, 27.0, 22.8, 12.4. HRMS: calcd for: $C_{22}H_{24}NO_3$ $(M+H)^+$ 350.1751. Found: 350.1736.

5.1.2.2. 3,4-Dihydro-2,2,7,8-tetramethyl-5-(5-(4-nitrophenyl)-isoxazol-3-yl)-2*H*-1-benzopyran-6-ol (6). Purification by column chromatography (pet. ether–AcOEt 8.5:1.5). Yield: 59%, yellow-orange solid 203–205 °C. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 5.35 min, detection at 297 nm, purity 98.8%. ^{1}H NMR δ: 8.38 (d, 2H, J = 8.8 Hz) 8.04 (d, 2H, J = 8.8 Hz), 8.13 (s, 1H), 6.98 (s, 1H), 2.83 (t, 2H, J = 6.6 Hz), 2.24 (s, 3H), 2.19 (s, 3H), 1.77 (t, 2H J = 6.6 Hz), 1.36 (s, 6H). ^{13}C NMR δ: 166.9, 162.0, 148.6, 146.6, 145.4, 132.5, 129.4, 126.8, 126.7, 124.5, 123.9, 115.4, 109.4, 103.8, 72.9, 32.8, 29.7, 26.9, 22.9, 12.5, 12.4. HRMS: calcd for $C_{22}H_{23}N_2O_5$ $(M+H)^+$ 395.1607. Found: 395.1582.

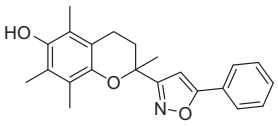
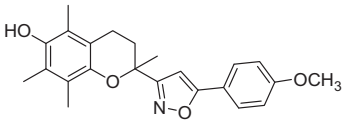
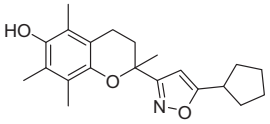
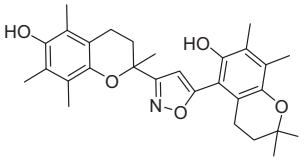
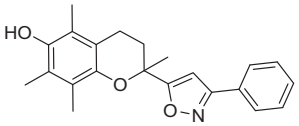
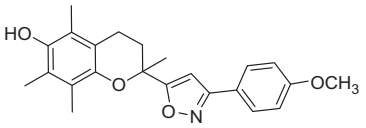
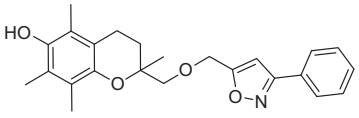
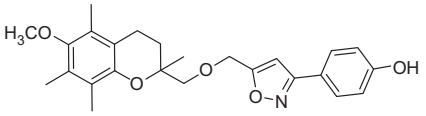
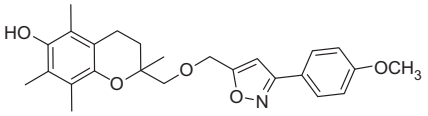
5.1.2.3. 3,4-Dihydro-2,2,7,8-tetramethyl-5-(5-(4-methoxyphenyl)-isoxazol-3-yl)-2*H*-1-benzopyran-6-ol (7). According to the general procedure, described above, a mixture of analogues **7** and **8** was obtained which was purified by column chromatography (pet. ether–AcOEt 8.5:1.5). Yield: 36%, yellowish solid, mp 160–162 °C. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 7.72 min, detection at 254 nm, purity 99.8%. ^{1}H NMR δ: 8.44 (s, 1H), 7.79 (d, 2H, J = 8.7 Hz), 7.01 (d, 2H, J = 8.7 Hz), 6.66 (s, 1H), 3.88 (s, 3H), 2.84 (t, 2H, J = 6.7 Hz), 2.24 (s, 3H), 2.18 (s, 3H), 1.76 (t, 2H, J = 6.7 Hz), 1.36 (s, 6H). ^{13}C NMR δ: 169.5, 161.7, 161.3, 146.7, 145.2, 128.8, 127.6, 123.6, 119.8, 115.5, 114.4, 110.0, 99.8, 72.8, 55.4, 32.9, 27.0, 22.8, 12.4. HRMS: calcd for $C_{23}H_{26}NO_4$ $(M+H)^+$ 380.1856. Found: 380.1847.

5.1.2.4. 3,4-Dihydro-2,2,7,8-tetramethyl-5-(5-(4-hydroxyphenyl)-isoxazol-3-yl)-2*H*-1-benzopyran-6-ol (8). Yield: 51%, yellowish solid, mp 217–219 °C. HPLC: 7:3 MeOH–H_2O, flow rate: 1 mL/min, t_R: 2.93 min, detection at 254 nm, purity 99.9%. ^{1}H NMR ($CDCl_3$, MeOD) δ: 7.66 (d, 2H, J = 8.7 Hz), 6.88 (d, 2H, J = 8.7 Hz), 6.57 (s, 1H), 2.76 (t, 2H, J = 6.6 Hz), 2.17 (s, 3H), 2.12 (s, 3H), 1.70 (t, 2H, J = 6.6 Hz), 1.30 (s, 6H). ^{13}C NMR ($CDCl_3$, MeOD) δ: 170.0, 161.5, 159.1, 146.2, 145.2, 128.6, 127.7, 127.6, 123.5, 118.8, 115.9, 115.8, 110.4, 99.4, 72.9, 32.8, 26.8, 22.6, 12.3. HRMS: calcd for $C_{22}H_{24}NO_4$ $(M+H)^+$ 366.1700. Found: 366.1691.

5.1.2.5. 3,4-Dihydro-2,2,7,8-tetramethyl-5-(5-propylisoxazol-3-yl)-2*H*-1-benzopyran-6-ol (9). Purification by column chromatography (pet. ether–AcOEt 8.5:1.5). Yield: 25%, yellowish solid, mp 160–162 °C. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 5.23 min, detection at 254 nm, purity 98.9%. ^{1}H NMR δ: 8.54 (s, 1H), 6.26 (s, 1H), 2.83–2.74 (m, 4H), 2.22 (s, 3H), 2.16 (s, 3H), 1.84–1.72 (m, 4H), 1.34 (s, 6H), 1.03 (t, 3H, J = 7.3 Hz). ^{13}C NMR δ: 173.2, 161.1, 146.7, 145.1, 128.6, 123.6, 115.5, 110.0, 102.6, 32.9, 28.6, 26.9, 22.8, 20.9, 13.7, 12.3. HRMS: calcd for $C_{19}H_{26}NO_3$ $(M+H)^+$ 316.1907. Found: 316.1895.

5.1.2.6. 3,4-Dihydro-2,2,7,8-tetramethyl-5-(3-phenylisoxazol-5-yl)-2*H*-1-benzopyran-6-ol (14). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 93%, yellow solid, mp 123–125 °C. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 4.93 min, detection at 254 nm, purity 95%. ^{1}H NMR δ: 7.89–7.87

Table 2
Efficacy and potency of 2-substituted chroman analogues to protect glutamate-challenged HT22 cells from oxytosis

Compound		Efficacy[a] (%)	EC_{50}[b] (μM)	Cytotoxicity[c] (%)
25		81 ± 3*	0.53 ± 0.13	35 ± 8
26		70 ± 5	1.07 ± 0.14	ns
27		71 ± 4	0.45 ± 0.11	ns
34		86 ± 4	0.63 ± 0.11	ns
31		82 ± 5	0.52 ± 0.09	ns
32		87 ± 8	0.42 ± 0.08	ns
38		87 ± 4	0.54 ± 0.09	ns
39		60 ± 6	7.11 ± 1.7	ns
40		74 ± 4	0.69 ± 0.04	ns

[a,b,c] As for legend to Table 1.
* Assessed at 3 μM due to cytotoxicity at 10 μM test compound.

(m, 2H), 7.51–7.48 (m, 3H), 6.71 (s, 1H), 2.73 (t, 2H, J = 6.7 Hz), 2.22 (s, 3H), 2.19 (s, 3H), 1.74 (t, 2H, J = 6.7 Hz), 1.34 (s, 6H). ^{13}C NMR δ: 167.6, 162.7, 145.8, 145.3, 130.2, 129.6, 129.0, 127.0, 123.3, 116.4, 110.2, 102.7, 73.2, 33.0, 29.7, 27.0, 22.0, 12.4, 12.2. HRMS: calcd for $C_{22}H_{24}NO_3$ $(M+H)^+$ 350.1751. Found: 350.1736.

5.1.2.7. 3,4-Dihydro-5-(3-(4-fluorophenyl)isoxazol-5-yl)-2,2,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (15). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 99%, yellow solid, mp 152–154 °C. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 4.70 min, detection at 254 nm, purity 100%. ^{1}H NMR δ: 7.87–7.83 (m, 2H), 7.17 (t, 2H, J = 8.4 Hz), 6.67 (s, 1H), 2.72 (t, 2H, J = 6.7 Hz), 2.22 (s, 3H), 2.19 (s, 3H), 1.74 (t, 2H, J = 6.7 Hz), 1.34 (s, 6H). ^{13}C NMR δ: 167.8, 165.5, 162.2, 161.7, 145.8, 145.2, 129.7, 128.8, 128.7, 125.1, 125.0, 123.3116.0, 115.9, 102.5, 73.2, 32.7, 26.8, 21.9, 12.4, 12.2. ^{19}F NMR δ (ppm): −110.3. HRMS: calcd for $C_{22}H_{23}FNO_3$ $(M+H)^+$ 368.1656. Found: 368.1642.

5.1.2.8. 3,4-Dihydro-2,2,7,8-tetramethyl-5-(3-(4-nitrophenyl)-isoxazol-5-yl)-2*H*-1-benzopyran-6-ol (16). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 70%, yellow solid, mp 172–174 °C. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 4.71 min, detection at 297 nm, purity 99.4%. ^{1}H NMR δ: 8.35 (d, 2H, J = 8.8 Hz), 8.06 (d, 2H, J = 8.8 Hz), 6.80 (s, 1H), 2.74 (t, 2H, J = 6.7 Hz), 2.22 (s, 3H), 2.19 (s, 3H), 1.75 (t, 2H, J = 6.7 Hz), 1.34 (s, 6H). ^{13}C NMR δ: 168.9, 160.8, 148.7, 145.9, 145.2, 135.0, 130.0, 127.7, 124.2, 123.2, 116.5, 110.0, 102.7, 73.2, 53.4, 32.6, 29.7, 29.6, 26.8, 21.9, 12.5, 12.2. HRMS: calcd for $C_{22}H_{23}N_2O_5$ $(M+H)^+$ 395.1607. Found: 395.1584.

5.1.2.9. 3,4-Dihydro-5-(3-(4-methoxyphenyl)isoxazol-5-yl)-2,2,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (17). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 82%, yellow solid, mp 155–157 °C. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 5.28 min, detection at 297 nm, purity 100%. ^{1}H NMR δ: 7.80

(d, 2H, *J* = 8.5 Hz), 7.00 (d, 2H, *J* = 8.5 Hz), 6.65 (s, 1H), 5.61 (s, 1H), 3.87 (s, 3H), 2.73 (t, 2H, *J* = 6.6 Hz), 2.22 (s, 3H), 2.18 (s, 3H), 1.74 (t, 2H, *J* = 6.6 Hz), 1.33 (s, 6H). ^{13}C NMR δ: 167.3, 162.3, 161.1, 145.7, 145.3, 129.5, 128.3, 124.8, 123.2, 121.3, 116.3, 114.4, 110.2, 102.4, 73.1, 55.4, 32.7, 26.8, 21.9, 12.4, 12.2. HRMS: calcd for $C_{23}H_{26}NO_4$ $(M+H)^+$ 380.1856. Found: 380.1844.

5.1.2.10. 3,4-Dihydro-5-(3-(4-hydroxyphenyl)isoxazol-5-yl)-2,2,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (18). Obtained by deprotection of **17**. Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 56%, yellow oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1 mL/min, t_R: 4.46 min, detection at 254 nm, purity 97.3%. ^{1}H NMR δ: 7.72 (d, 2H, *J* = 7.9 Hz), 6.89 (d, 2H, *J* = 7.9 Hz), 6.80 (s, 1H), 2.64 (t, 2H, *J* = 6.4 Hz), 2.18 (s, 3H), 2.14 (s, 3H), 1.73 (t, 2H, *J* = 6.4 Hz), 1.31 (s, 6H). ^{13}C NMR δ: 169.6, 163.7, 160.6, 147.0, 129.5, 129.4, 125.6, 121.7, 118.4, 116.8, 114.5, 104.1, 74.3, 33.9, 30.8, 27.1, 22.6, 12.7, 12.6. HRMS: calcd for: $C_{22}H_{24}NO_4$ $(M+H)^+$ 366.1700. Found: 366.1686.

5.1.2.11. 5,5′-(isoxazole-3,5-diyl)bis(3,4-dihydro-2,2,7,8-tetramethyl-2*H*-1-benzopyran-6-ol) (20). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 95%, yellow gummy solid. HPLC: 9:1 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 3.61 min, detection at 254 nm, purity 95.7%. ^{1}H NMR δ: 6.69 (s, 1H), 2.82 (t, 2H, *J* = 6.6 Hz), 2.71 (t, 2H, *J* = 6.7 Hz), 2.25 (s, 3H), 2.22 (s, 3H), 2.19 (s, 6H), 1.78–1.72 (m, 4H), 1.35 (s, 6H), 1.34 (s, 6H). ^{13}C NMR δ: 166.7, 161.5, 146.8, 145.9, 145.3, 145.1, 129.8, 129.1, 123.8, 123.2, 116.5, 115.5, 110.0, 109.7, 106.4, 73.2, 72.9, 32.9, 32.7, 29.7, 27.0, 26.8, 22.9, 21.9, 12.5, 12.4, 12.2. HRMS: calcd for: $C_{29}H_{36}NO_5$ $(M+H)^+$ 478.2588. Found: 478.2581.

5.1.2.12. 3,4-Dihydro-2,5,7,8-tetramethyl-2-(5-phenyl-3-isoxazolyl)-2*H*-1-benzopyran-6-ol (25). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 58%, yellow oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 4.1 min, detection at 254 nm, purity 99.0%. ^{1}H NMR δ: 7.72–7.70 (m, 2H), 7.44–7.40 (m, 3H), 6.36 (s, 1H), 2.69–2.55 (m, 3H), 2.24 (s, 3H), 2.18 (s, 3H), 2.14–2.10 (m, 1H), 2.05 (s, 3H), 1.72 (s, 3H). ^{13}C NMR δ: 169.5, 169.3, 145.3, 145.0, 130.0, 128.8, 127.4, 125.8, 122.3, 121.1, 118.6, 117.5, 98.0, 73.5, 31.6, 28.4, 20.9, 12.2, 11.9, 11.2. HRMS: calcd for: $C_{22}H_{24}NO_3$ $(M+H)^+$ 350.1751. Found: 350.1740.

5.1.2.13. 3,4-Dihydro-2,5,7,8-tetramethyl-2-[5-(4-methoxyphenyl)-3-isoxazolyl]-2*H*-1-benzopyran-6-ol (26). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 58%, yellow oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 4.17 min, detection at 254 nm, purity 99.7%. ^{1}H NMR (600 MHz) δ: 7.64 (d, 2H, *J* = 8.8 Hz), 6.93 (d, 2H, *J* = 8.8 Hz), 6.24 (s, 1H), 4.23 (s, 1H), 3.84 (s, 3H), 2.66–2.56 (m, 3H), 2.23 (s, 3H), 2.18 (s, 3H), 2.12–2.10 (m, 1H), 2.05 (s, 3H), 1.71 (s, 3H). ^{13}C NMR δ: 169.5, 169.2, 160.9, 145.3, 145.0, 127.4, 122.3, 121.1, 120.3, 118.6, 117.5, 114.2, 96.6, 55.3, 31.7, 28.4, 20.9, 12.2, 11.9, 11.2. HRMS: calcd for: $C_{23}H_{26}NO_4$ $(M+H)^+$ 380.1856. Found: 380.1844.

5.1.2.14. 3,4-Dihydro-2,5,7,8-tetramethyl-2-[5-(cyclopentyl-3-isoxazolyl]-2*H*-1-benzopyran-6-ol (27). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 64%, yellow oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1 mL/min, t_R: 6.01 min, detection at 286 nm, purity 100%. ^{1}H NMR (600 MHz) δ: 5.83 (s, 1H), 4.25 (s, 1H), 3.11–3.08 (m, 1H), 2.65–2.62 (m, 2H), 2.51–2.47 (m, 1H), 2.18 (s, 6H), 2.09–2.07 (m, 1H), 2.06 (s, 3H), 2.05–2.00 (m, 2H), 1.74–1.66 (m, 5H), 1.64 (s, 3H), 163–1.62 (m, 1H). ^{13}C NMR δ: 176.9, 168.5, 145.2, 145.0, 122.3, 121.1, 118.5, 117.5, 97.7, 73.4, 37.5, 31.8, 31.7, 31.6, 28.0, 25.2, 20.8, 12.2, 11.8, 11.2. HRMS: calcd for: $C_{21}H_{28}NO_3$ $(M+H)^+$ 342.2064. Found: 342.2051.

5.1.2.15. 3,4-Dihydro-2,5,7,8-tetramethyl-2-(3-phenyl-5-isoxazolyl)-2*H*-1-benzopyran-6-ol (31). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 33%, yellow gummy solid. HPLC: 8:2 MeOH–H_2O, flow rate: 1 mL/min, t_R: 5.68 min, detection at 254 nm, purity 96.6%. ^{1}H NMR δ: 7.75–7.72 (m, 2H), 7.42–7.35 (m, 3H), 6.24 (s, 1H), 2.71–2.62 (m, 1H), 2.61–2.48 (m, 1H), 2.47–2.33 (m, 1H), 2.24 (s, 3H), 2.19 (s, 3H), 2.10–2.09 (m, 1H), 2.04 (s, 3H), 1.75 (s, 3H). ^{13}C NMR δ: 175.9, 162.2, 145.4, 144.7, 129.9, 128.8, 126.8, 122.3, 121.3, 118.5, 117.1, 99.0, 74.2, 31.4, 27.3, 20.7, 12.2, 11.9, 11.2. HRMS: calcd for: $C_{22}H_{24}NO_3$ $(M+H)^+$ 350.1751. Found: 350.1740.

5.1.2.16. (3,4-Dihydro-2,5,7,8-tetramethyl-2-[3-(4-methoxyphenyl)-isoxazol-5-yl]-2*H*-1-benzopyran-6-ol (32). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 67% yellowish oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 3.84 min, detection at 254 nm, purity 100%. ^{1}H NMR (600 MHz) δ: 7.67 (d, 2H, *J* = 8.8 Hz), 6.92 (d, 2H, *J* = 8.8 Hz), 6.18 (s, 1H), 4.27 (s, 1H), 3.83 (s, 3H), 2.68–2.65 (m, 1H), 2.55–2.52 (m, 1H), 2.43–2.38 (m, 1H), 2.23 (s, 3H), 2.19 (s, 3H), 2.10–2.05 (m, 1H), 2.04 (s, 3H), 1.73 (s, 3H). ^{13}C NMR δ: 175.7, 161.8, 160.9, 145.4, 144.8, 128.2, 122.3, 121.5, 121.3, 118.5, 117.1, 114.1, 98.7, 74.1, 55.3, 31.4, 27.3, 20.7, 12.2, 11.9, 11.2. HRMS: calcd for: $C_{23}H_{26}NO_4$ $(M+H)^+$ 380.1856. Found: 380.1842.

5.1.2.17. 2-(5-(3,4-Dihydro-6-hydroxy-2,2,7,8-tetramethyl-2*H*-1-benzopyran-5-yl)isoxazol-3-yl)-3,4-dihydro-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (34). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 79%, yellow oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 5.86 min, detection at 254 nm, purity 99.6%. ^{1}H NMR (600 MHz) δ: 6.25 (s, 1H, *H-isoxazole*), 5.55 (s, 1H, O*H*), 4.25 (s, 1H, O*H*), 2.70–2.65 (m, 3H, –C*H*$_2$), 2.55–2.52 (m, 3H, –C*H*$_2$), 2.20 (s, 3H, Ar–C*H*$_3$), 2.17 (s, 6H, Ar–C*H*$_3$), 2.14 (s, 3H, Ar–C*H*$_3$), 2.06 (s, 3H, Ar–C*H*$_3$), 1.74 (s, 3H, –C*H*$_3$), 1.69–1.66 (m, 2H, –C*H*$_2$), 1.30 (s, 6H, –C*H*$_3$). ^{13}C NMR δ: 168.8, 166.5, 145.7, 145.4, 145.1, 144.9, 129.5, 123.2, 122.33, 121.2, 118.6, 117.5, 116.1, 110.0, 103.1, 73.5, 73.0, 32.7, 31.9, 27.8, 26.8, 21.8, 20.9, 12.3, 12.2, 11.9, 11.2. HRMS: calcd for: $C_{29}H_{36}NO_5$ $(M+H)^+$ 478.2588. Found: 478.2576.

5.1.2.18. 2-{[(3-(phenyl)isoxazol-5-yl)methoxy]methyl}-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (38). Purification by column chromatography (pet. ether–AcOEt 9:1). Yield: 68% yellowish oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 3.45 min, detection at 254 nm, purity 98.7%. ^{1}H NMR (600 MHz) δ: 7.80–7.78 (m, 2H), 7.47–7.44 (m, 3H), 6.51 (s, 1H), 4.79–4.72 (m, 2H), 4.24 (s, 1H), 3.60 (dd, 2H, *J* = 26.8, 10.0 Hz), 2.63 (t, 2H, *J* = 6.7 Hz), 2.16 (s, 3H), 2.12 (s, 3H), 2.11 (s, 3H), 2.03–1.98 (m, 1H), 1.81–1.76 (m, 1H), 1.31 (s, 3H). ^{13}C NMR δ: 170.0, 162.3, 145.0, 144.9, 130.0, 128.9, 128.9, 126.8, 122.5, 121.2, 118.5, 117.2, 100.8, 76.2, 74.7, 64.6, 29.7, 28.6, 22.0, 20.3, 12.2, 11.9, 11.3. HRMS: calcd for: $C_{24}H_{28}NO_4$ $(M+H)^+$ 394.2013. Found: 394.1995.

5.1.2.19. 5-{[(3,4-Dihydro-6-methoxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-yl)methoxy]methyl}-3-(4-hydroxyphenyl) isoxazole (39). According to the general procedure described above a mixture of analogues **39** and **40** was obtained which was purified by column chromatography (pet. ether–AcOEt 8:2). Yield: 29% yellow oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 3.78 min, detection at 254 nm, purity 97.4%. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.67 (d, 2H, *J* = 8.3 Hz), 6.90 (d, 2H, *J* = 8.3 Hz), 6.46 (s, 1H), 5.53 (s, 1H), 4.76–4.70 (m, 2H), 3.63 (s, 3H), 3.65–3.56 (dd, 2H), 2.60 (t, 2H, *J* = 6.7 Hz), 2.18 (s, 3H), 2.13 (s, 3H), 2.09 (s, 3H), 2.03–1.98 (m, 1H), 1.80–1.75 (m, 1H), 1.31 (s, 3H). ^{13}C NMR δ: 169.8, 161.9, 157.3, 149.7, 147.3, 128.4, 128.0, 125.9, 122.8, 121.5, 117.5,

115.8, 100.7, 76.2, 75.0, 64.6, 60.4, 28.3, 22.1, 20.1, 12.6, 11.9, 11.7. HRMS: calcd: $C_{25}H_{30}NO_5$ $(M+H)^+$ 424.2118. Found: 424.2103.

5.1.2.20. 2-{[(3-(4-methoxyphenyl)isoxazol-5-yl)methoxy]-methyl}-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol (40). Yield: 8% yellow oil. HPLC: 8:2 MeOH–H_2O, flow rate: 1.5 mL/min, t_R: 3.53 min, detection at 260 nm, purity 99.8%. ^{1}H NMR (600 MHz) δ: 7.72 (d, 2H, J = 8.7 Hz, Ar*H*), 6.97 (d, 2H, J = 8.7 Hz, Ar*H*), 6.45 (s, 1H, *H-isoxazole*), 4.76–4.69 (m, 2H, –O–CH_2–), 4.20 (s, 1H, O*H*), 3.86 (s, 3H, –OCH_3), 3.59 (dd, 2H, J = 27.3, 10.0 Hz, –CH_2–O), 2.62 (t, 2H, J = 6.8 Hz, –CH_2), 2.16 (s, 3H, Ar–CH_3), 2.12 (s, 3H, Ar–CH_3), 2.10 (s, 3H, Ar–CH_3), 2.02–1.98 (m, 1H, –C*H*H), 1.80–1.76 (m, 1H, –C*H*H), 1.30 (s, 3H, –CH_3). ^{13}C NMR δ: 169.7, 161.9, 161.0, 145.1, 144.9, 128.2, 122.5, 121.5, 121.2, 118.5, 117.3, 114.3, 100.6, 74.7, 64.6, 55.3, 29.7, 28.6, 22.0, 20.3, 12.2, 11.9, 11.3. HRMS: calcd for: $C_{25}H_{30}NO_5$ $(M+H)^+$ 424.2118. Found: 424.2104.

5.2. Biology

5.2.1. Evaluation of the activity of chroman analogues to prevent oxytosis of HT22 hippocampal neurons

The new compounds were tested as previously described.[6,26] Briefly, HT22 cells were plated in a 96-well flat bottom plate at a density of 4000 cells per well in 100 μl of DMEM-Hepes-GlutaMAX medium containing 10% of foetal bovine serum. Twenty-four hours after plating, the cells were challenged with 5 mM glutamate in the presence of increasing concentrations (0.03–10 μM) of the chroman analogues or vehicle (⩽0.2% DMSO) in fresh medium for 24 h prior to assessing the relative numbers of living cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. MTT conversion to coloured formazan was assessed from the difference in optical density (dOD) at 550 and 670 nm. Direct interference of the test compounds with MTT conversion to formazan was excluded using mock cultures deprived of HT22 cells. Interference of the chromans with mitochondrial conversion of MTT to formazan was excluded using the trypan blue exclusion assay to directly determine the number living cells (see below). No challenged cells served to test cytotoxicity at different chroman concentrations, whereas challenged cells served to assess neuroprotective activity by comparison. Cells exposed only to vehicle (DMSO) or glutamate served as controls. Cell death (CD) in the absence of chroman analogues was calculated by $CD_{Vehicle} = [(dOD_{Vehicle} - dOD_{Glutamate}) * 100/dOD_{Vehicle}$, whereas cell death in their presence was calculated by $CD_{Compound} = [(dOD_{Compound} - dOD_{Compound+Glutamate}) * 100/dOD_{Compound}$. Neuroprotection (%) was calculated by $[(CD_{Vehicle} - CD_{Compound}) * 100/CD_{Vehicle}$. Values are mean ± SEM of at least three independent experiments, each carried out in triplicate.

5.2.2. Evaluation of cytotoxicity of chroman analogues

Cytotoxicity was assessed as already described.[26] Briefly, HT22 cells were plated in a 24-well flat bottom plates at a density of 10,000 cells per well in DMEM-Hepes-GlutaMAX medium containing 10% of foetal bovine serum. Twenty-four hours after plating, the cells were incubated with increasing concentrations (0.03–10 μM) of chroman analogues or vehicle (⩽0.1% DMSO) in fresh medium for 24 h. After the 24-h incubation, cell culture supernatants was removed, adherent cells were detached by trypsinization, collected in the respective supernatant fractions and combined adherent and floating cells (if any) were stained with 0.4% Trypan blue in phosphate buffered saline and trypan blue-stained and non-stained cells counted using a Neubauer haemocytometer. Trypan blue is known to be excluded by living cells, but to penetrate dead cells. Therefore, the Stained Cell Count (SCC) in the absence and presence of chroman analogues, given as percentages of the Total Cell Count (TCC) was used to measure% cytotoxicity as calculated by: $(SCC_{Compound} - SCC_{Vehicle})/TCC$. Innate cytotoxicity of HT22 cell culture (i.e., $SCC_{Vihecle}/TCC$) was <5%. Values are mean ± SEM of at least three independent experiments, each carried out in triplicate. In a few cases, cytotoxicity was assessed using the LDH release assay with largely similar results.

5.2.3. Statistics

The statistical significance of the differences observed was determined using t-test. Differences were considered significant for values of p <0.05.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.074.

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