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Design, synthesis and in vitro evaluation of novel chroman-4-one, chroman, and 2*H*-chromene derivatives as human rhinovirus capsid-binding inhibitors

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ABSTRACT

As part of an effort to generate broad-spectrum inhibitors of rhinovirus replication, novel series of (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones **1a–e**, (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones **2a** and **2b**, (*Z*)-3-[(*E*)-3-phenylallylidene]chromans **3a–e**, and (*E*)-3-(3-phenylprop-1-en-1-yl)-2*H*-chromenes **4a–d** were designed and synthesized. All the compounds were tested in vitro for their efficacy against infection by human rhinovirus (HRV) 1B and 14, two representative serotypes for rhinovirus group B and A, respectively. Most of the analogues were found to be potent and selective inhibitors of both HRVs, although HRV 1B was generally more susceptible than HRV 14. Mechanism of action studies of (*E*)-6-chloro-3-(3-phenylprop-1-en-1-yl)-2*H*-chromene **4b**, the most potent compound on HRV 1B infection, suggested that **4b** behaves as a capsid-binder probably acting at the uncoating level.

1. Introduction

Human rhinoviruses (HRVs), members of the Picornaviridae family, represent the most frequent etiological agents of the common cold and are associated with upper respiratory tract complications such as otitis media and sinusitis.[1] Although HRVs often induce a mild and self-limited respiratory illness in humans, the prevalence and the recurrent nature of HRV infections explain their high medical and socio-economic impact in term of healthcare and lost productivity. In addition, HRV infections can also affect the lower respiratory airways and can exacerbate chronic respiratory disorders such as asthma and chronic obstructive pulmonary disease.[2,3] Since the multiplicity of serotypes makes the development of an effective vaccine impracticable, constant efforts have been devoted to the discovery and development of efficacious antiviral agents. However, at present, no antiviral therapy has been approved for the treatment of rhinovirus infections.

Pursuing our researches on small molecules with anti-picornavirus activity, we recently devoted our attention to a series of (*Z*)-3-benzylidenechromans, 3-benzyl-2*H*-chromenes and 3-benzylchromans[4] related to the most active synthetic 3(2*H*)-isoflavenes[5–8] and homoisoflavones[9–11] previously studied by us. The antiviral potency of these compounds was evaluated in vitro against HRV 1B and 14, selected as representative serotypes for group B and A of HRVs, respectively. Several of these inhibitors presented submicromolar potency against HRV 1B coupled with high therapeutic index. On the contrary, HRV 14 infection was only weakly inhibited.[4] Similarly to related flavanoids,[8,12,13] these analogues behaved as capsid-binders and interfered with very early events of HRV multiplication.[4] The explanation for the different sensibility of HRV 1B and 14 to these chromans could reside in the size of the compound-binding site which differs depending upon the specific serotype. Previous research on capsid-binding compounds demonstrated that viral group B binding site accommodates molecules with shorter chains, while long-chained compounds are routinely more active against group A.[14]

To develop compounds with broad-spectrum activity, we modified the linker chain length between the heterocycle and the phenyl moiety. A linear, unsaturated chain containing two or four carbon atoms was introduced as a linker. Optimum activity against both HRV serotypes was achieved with the unsaturated 2-carbon chain analogues, (*E*)-3-styryl-2*H*-chromenes. Surprisingly, despite the larger size of the HRV 14 capsid binding site, elongation of the linker chain from two to four carbon atoms resulted in a loss of activity against this serotype.[15] Mechanism of action studies indicated that also these compounds behaved as capsid-binders interfering with the early stage(s) of rhinovirus infection.[15]

These observations prompted us to further explore the effect of some simple structural modification of this scaffold. In the present paper, we describe the synthesis, antiviral activity and mechanism of action of new analogues containing an unsaturated 3-carbon chain between the two rings. The antiviral potency of the new

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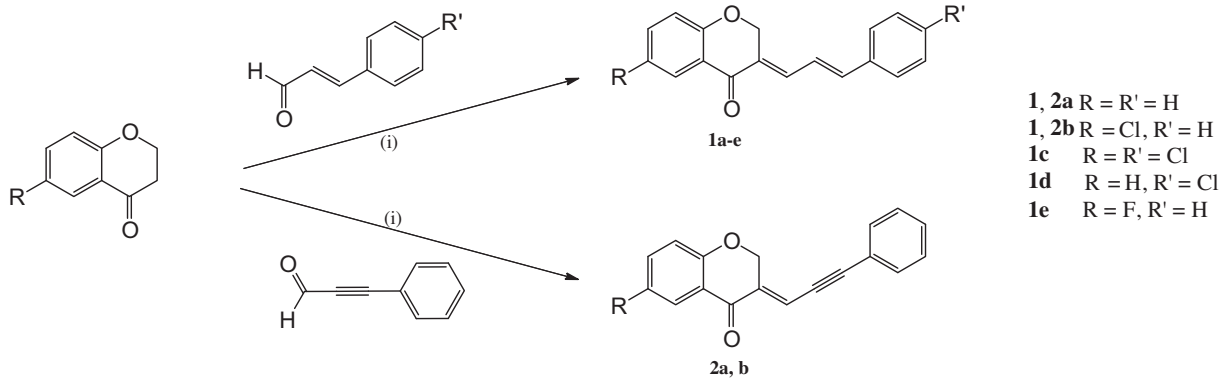


Scheme 1. Reagents and conditions: (i) 85% H_3PO_4, 80 °C, 4 h.

(*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones **1a–e**, (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones **2a** and **2b**, (*Z*)-3-[(*E*)-3-phenylallylidene]chromans **3a–e**, and (*E*)-3-(3-phenylprop-1-en-1-yl)-2*H*-chromenes **4a–d** was evaluated against infection of HeLa cells by HRV 1B and 14, representative serotypes for group B and A of HRVs, respectively.

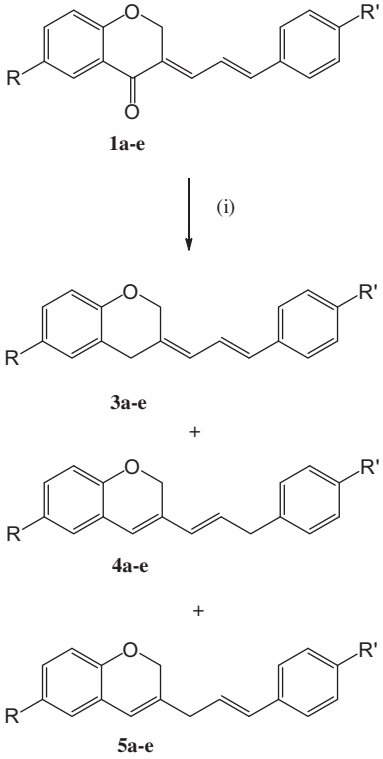
2. Results and discussion

2.1. Chemistry

(*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones **1a–e** and (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones **2a** and **2b** were prepared by acid-catalyzed condensation of chroman-4-ones with *trans*-cinnamaldehydes and 3-phenylpropiolaldehyde, respectively. The reaction was carried out by heating the mixture in 85% phosphoric acid for 4 h (Scheme 1). The ^{1}H NMR spectra of the new chromanones **1a–e** suggest the *trans*-configuration of the exocyclic double bond. In fact, the signals of Hα and Hβ appear around 7.5 ppm and 7.00 ppm, respectively. In the ^{1}H NMR spectra of the *cis*-isomers, these signals would be shifted at higher and lower field, respectively, due to the effect of carbonyl group. The coupling constant values of Hβ and Hγ ($J_{\beta-\gamma}$ from 15.3 to 15.5 Hz) indicate also the *trans*-configuration of this double bond. The stereochemistry of these compounds was confirmed by 2D NOESY experiments. The strong NOE cross peaks between H2 and Hβ and between Hα and Hγ demonstrate the *trans*-configuration for both double bonds.

In a similar manner, the chemical shift of the proton in the chain (around 7.00 ppm) indicates the *trans*-configuration of the exocyclic double bond of (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones **2a** and **2b**.

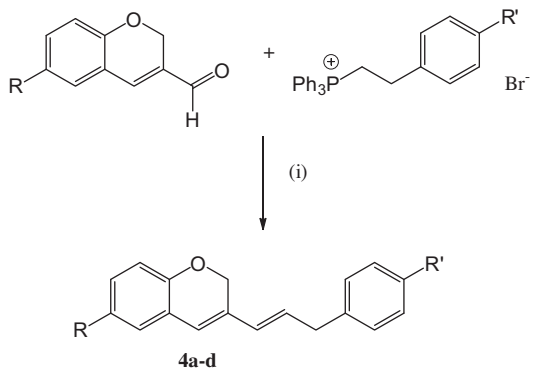
Subsequent reaction of (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones **1a–e** with lithium aluminum hydride in the presence of aluminum chloride, reduced the carbonyl group to a methylene group with some isomerization of the exocyclic double bond (**5a–e**) or both the double bonds (**4a–e**) (Scheme 2). The presence of small amounts of (*E*)-3-(3-phenylprop-1-en-1-yl)-2*H*-chromenes **4a–e** and 3-cinnamyl-2*H*-chromenes **5a–e** together with (*Z*)-3-[(*E*)-3-(3-chlorophenyl)allylidene]chromans **3a–e** was evident in the ^{1}H NMR spectra of the crude reaction products. The main product was isolated from the mixture by column chromatography and further purified by crystallization. The stereochemistry of the exocyclic double bond of (*Z*)-3-[(*E*)-3-(3-chlorophenyl)allylidene]chromans **3a–e** was established by 2D NOESY experiments. The strong NOE cross peaks between H4 and Hα unequivocally indicate the *cis*-configuration of this double bond. Therefore, the reduction of (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones **1a–e** to (*Z*)-3-[(*E*)-3-(3-chlorophenyl)allylidene]chromans **3a–e** occurs with the retention of the exocyclic double bond configuration, but the *E*/*Z* descriptors change because of the change in priority at C3. The coupling constant values of Hβ and Hγ ($J_{\beta-\gamma}$ = 15.3 or 15.4 Hz) in the ^{1}H NMR spectra suggest the *trans*-configuration of the second double bond. The strong NOE cross peaks between Hα and Hγ confirm this configuration.



1, **3**, **4**, **5a** R = R' = H
1, **3**, **4**, **5b** R = Cl, R' = H
1, **3**, **4**, **5c** R = R' = Cl
1, **3**, **4**, **5d** R = H, R' = Cl
1, **3**, **4**, **5e** R = F, R' = H

Scheme 2. Reagents and conditions: (i) $LiAlH_4$, $AlCl_3$, dry Et_2O, rt 1 h and reflux 2.5 h.

As shown in Scheme 3, (*E*)-3-(3-phenylprop-1-en-1-yl)-2*H*-chromenes **4a–d** were conveniently obtained by the Wittig reaction of the appropriate 2*H*-chromene-3-carbaldehyde and phenethyltriphenylphosphonium bromide, using sodium ethoxide as base. The coupling constant values of the vinylic protons ($J_{\alpha-\beta}$ = 15.9 Hz) indicate the *trans*-configuration of the double bond in the chain.



4a R = R' = H
4b R = Cl, R' = H
4c R = R' = Cl
4d R = H, R' = Cl

Scheme 3. Reagents and conditions: (i) EtONa, absolute EtOH, reflux, 2.5 h.

2.2. Antiviral tests

The results of the biological evaluation of the new compounds (**1a–e, 2a**, **2b**, **3a–e** and **4a–d**) are presented in Table 1. Each compound was first tested for its effects on morphology, viability and growth of HeLa cells, a human cell line suitable for the replication of HRVs. Morphological alterations were scored microscopically, and the action of the compounds on logarithmic cell growth was determined by the XTT colorimetric method.[16] The cytotoxicity of compounds is referred as maximum non-cytotoxic concentration (MNTC) and 50% cytotoxic concentration (TC_{50}). The MNTC is the highest dose that did not produce any toxic effect or reduction of cell growth after 3 day incubation at 37 °C. The TC_{50} is the concentration of compound reducing the cell viability by 50% as compared with the control. In general, all the new compounds exhibited low cytotoxicity showing TC_{50} ranging from 25 to 100 μM.

The dose-dependent inhibitory activity of compounds on HRV 1B and HRV 14 replication was evaluated in vitro by a plaque reduction assay, starting from the MNTC. A previous systematic evaluation of a panel of capsid-binding compounds against all HRVs established the existence of two virus groups, called groups A and B, with contrasting susceptibilities for these anti-rhinoviruses. Group B contains twice as many serotypes as group A, and accounts for five times as many colds as group A serotypes.[17,18] In our research, we utilized HRV 1B and 14 as representative serotypes for group B and A, respectively. The results of antiviral activity are expressed as compound concentration required to produce a 50% reduction of plaque number with respect to mock-treated virus-infected cultures (IC_{50}) (Table 1). When the IC_{50} value is not achieved up to the MNTC, the percentage inhibition at this dose is reported in parentheses. The therapeutic index (TI), expressed as TC_{50} versus IC_{50} ratio, was calculated and reported in Table 1. 4′,6-Dichloroflavan (**BW683C**), an inhibitor of group B serotypes, was included as a control.[19]

Although **BW683C** is at present one of the most active compounds against HRV 1B and group B serotypes, it was generally inactive against group A of HRVs.[17–19] None of the new compounds was more potent than the reference compound **BW683C** against HRV 1B. Differently, they were found to be effective also against serotype 14 infection indicating a wider anti-HRV spectrum.

Although HRV 1B was generally found more susceptible than HRV 14 to the action of the new compounds, several derivatives showed activity in the micromolar range against both HRV serotypes. Only compounds **1c**, **3c** and **4a** exhibited a higher potency against serotype 14.

In the (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-one series **1a–e**, all the analogues showed potent anti-HRV 1B activity (IC_{50}s ranging from 0.85 to 4.98 μM) and selectivity (TIs from 7.46 to 50.50). On the contrary, only 4′,6-dichloro and 6-fluoro derivatives (**1c** and **1e**) exhibited IC_{50}s lower than MNTCs against HRV 14. Notably, compound **1c** was the most potent and selective compound among all

Table 1
Cytotoxicity and anti-picornavirus activity of (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones **1a–e**, (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones **2a** and **2b**, (*Z*)-3-[(*E*)-3-phenylallylidene]chromans **3a–e**, and (*E*)-3-(3-phenylprop-1-en-1-yl)-2*H*-chromenes **4a–d**

Comp	R	R′	MNTC (μM)[a]	TC50 (μM)[b]	IC50 (μM)[c] HRV 1B	TI[d]	IC50 (μM)[c] HRV 14	TI[d]
1a	H	H	12.50	25.00	0.85	29.41	12.50 (7.2%)	–
1b	Cl	H	25.00	50.00	0.99	50.50	25.00 (48.6%)	–
1c	Cl	Cl	12.50	25.00	3.35	7.46	1.42	17.61
1d	H	Cl	25.00	50.00	2.54	19.68	25.00 (17.1%)	–
1e	F	H	25.00	50.00	4.98	10.04	17.02	2.94
2a	H	H	25.00	50.00	9.30	5.38	20.00	2.50
2b	Cl	H	50.00	75.00	7.89	9.51	30.41	2.47
3a	H	H	50.00	100.00	0.99	101.01	47.59	2.10
3b	Cl	H	25.00	50.00	2.79	17.92	25.00 (30.3%)	–
3c	Cl	Cl	12.50	25.00	11.49	2.18	8.27	3.02
3d	H	Cl	25.00	50.00	1.39	35.97	6.02	8.31
3e	F	H	50.00	75.00	5.57	13.46	36.51	2.05
4a	H	H	50.00	75.00	13.59	5.52	11.09	6.76
4b	Cl	H	12.50	25.00	0.42	59.52	12.50 (23.0%)	–
4c	Cl	Cl	25.00	100.00	9.73	10.28	25.00 (27.0%)	–
4d	H	Cl	50.00	100.00	3.61	27.70	45.10	2.22
BW683C	–	–	25.00	>25.00[e]	0.026	>961	NA[f]	–

[a] The maximum non-cytotoxic concentration (MNTC) was the highest dose tested that did not produce any cytotoxic effect and reduction in viability of HeLa cells, or on cell growth after 3 days of incubation at 37 °C. At this concentration, residual ethanol in culture medium was verified not to be cytotoxic.
[b] The TC_{50} value was the concentration of compound which reduced the HeLa cell viability by 50%, as compared with the control. At this concentration, residual ethanol in culture medium was verified not to be cytotoxic.
[c] The IC_{50} value was the dose of compound reducing the plaque number by 50% and was calculated by plotting the drug concentration versus the percentage of plaque reduction. When a 50% reduction was not achieved, the percent of inhibition obtained at the MNTC was reported in parentheses.
[d] The therapeutic index (TI) value was equal to TC_{50}/IC_{50}.
[e] The saturation concentration in cell culture medium was found to be lower than TC_{50}.
[f] Not active up to the highest concentration tested (MNTC).

the new derivatives against serotype 14 (IC_{50} = 1.42 μM, TI = 17.61) while it was about twofold less potent and selective against serotype 1B (IC_{50} = 3.35 μM, TI = 7.46).

When the chroman-4-one ring in **1a-e** series was replaced by a chroman ring to give analogues **3a–e**, the inhibitory activity against serotype 1B was generally retained (IC_{50}s ranging from 0.99 to 11.49 μM), and only the 6-chlorochroman **3b** exhibited an IC_{50} higher than MNTC against serotype 14.

In both (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-one and (*Z*)-3-[(*E*)-3-(3-chlorophenyl)allylidene]chroman series, the replacement of the chlorine at the 6 position in compounds **1b** and **3b** with fluorine to give the compounds **1e** and **3e** resulted in a marked reduction in activity against HRV 1B. On the contrary, the potency against serotype 14 was enhanced by this substitution, although the 6-fluoro analogues **1e** and **3e** displayed a higher activity against serotype 1B. A similar behaviour was observed against both serotypes when the double bond in the chain of (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones **1a** and **1b** was replaced by a triple bond to give the corresponding (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones **2a** and **2b**.

The isomerisation of both double bonds in (*Z*)-3-[(*E*)-3-(3-chlorophenyl)allylidene]chromans **3a–d** to provide inhibitors **4a–d**, generally led to a significant loss in potency or a modest improvement in activity. The only exception to this generalization was the 6-chloro analogue **4b** which was >sixfold more potent than corresponding **3b** against HRV 1B and showed the highest inhibitory activity (IC_{50} = 0.42 μM) coupled with remarkable selectivity (TI = 59.52).

2.3. Mechanism of action studies

(*E*)-6-Chloro-3-(3-phenylprop-1-en-1-yl)-2*H*-chromene **4b**, the most potent compound against HRV 1B (IC_{50} = 0.42 μM), was selected to clarify the mechanism of antiviral action by evaluating the effects produced on both virus particles and multiplication.

The virus-neutralizing effect of **4b** on HRV 1B infectivity was investigated by incubating a virus suspension at high titre with the compound at a concentration of 42 μM (100 times the IC_{50}). After serial 10-fold dilutions to achieve non inhibitory concentrations of free compound, the infectivity titers of mock- and **4b**-treated virus suspensions were found to be similar (5.33×10^6 PFU/mL and 5.11×10^6 PFU/mL, respectively). These results indicate that **4b** does not damage virus particles.

In stabilization studies, **4b** (42 μM) significantly protected HRV 1B infectivity against inactivation by both mild acid and heat treatments. As shown in Figure 1, in the absence of **4b**, the infectivity of control virus decreased significantly when exposed to either pH 5 (Fig. 1A) or 56 °C (Fig. 1B) (3.8 and 2.9 log PFU/mL, respectively). In the presence of **4b**, the drop in virus infectivity was significantly reduced (2.8 and 1.8 log PFU/mL, respectively) and the protective effect towards low pH and thermal inactivation was 1.0 and 1.1 log, respectively. Exposure of HRVs to mild acid or heat induces conformational changes of the virion capsid structure similar to those produced during the uncoating of the viral particles inside the host cells.[20,21] The presence of capsid-binders within the hydrophobic pocket of VP1 protein results in resistance to acid and thermal inactivation due to a reduction of capsid flexibility.[22,23] Taken together, our data suggest that **4b** could act as a capsid-binder. However, binding of **4b** was reversible by dilution as indicated by results on virus infectivity. A similar behaviour has already been described for (*Z*)-3-(4-chlorobenzylidene)chroman and (*E*)-3-styryl-2*H*-chromene, two related compounds recently studies by us.[4,15]

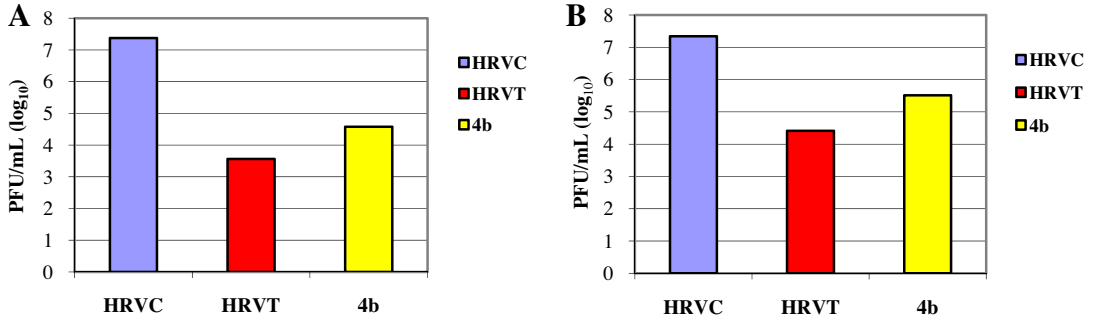
The antiviral action of **4b** (42 μM) towards different stages of HRV 1B multiplication in HeLa cells was investigated under one-step growth conditions. **4b** was present: (i) during the entire time of virus replication, (ii) during virus binding to the cell membrane only, (iii) added or removed at different time intervals after virus attachment to the cells in the cold.

The highest level of inhibition (88.5%) was reached when **4b** was added to cells together with the virus inoculum and maintained until the end of virus multiplication. A similar effect was observed when **4b** was added immediately after virus adsorption period (1 h at 4 °C, time 0). Also the addition of **4b** 15, 30, 45 or 60 min after virus binding still produced a high reduction (above 80%) in virus yield. Instead, when **4b** was added at later times (2, 4 or 6 h after virus binding), inhibition significantly dropped (31%, 12% and 1%, respectively, Fig. 2).

Pretreatment of HeLa cells with **4b** before HRV infection or the presence of **4b** during the time of virus adsorption only (1 h, 4 °C) caused only a minimal reduction in virus yield (6% and 16%, respectively) (data not shown). Both results indicate that **4b** does not hinder cellular receptors for virus nor interfere with HRV 1B attachment to the host cell membrane.

In experiments where **4b** was added at the end of virus adsorption (0 time) and removed after 30 min or 1 h treatment of infected cells (33 °C), it produced a reduction in virus yield of approximately 35%. A time-dependent increase of inhibition was observed when the compound was removed after longer incubation times and the highest inhibition (93%) was achieved when **4b** was removed after 6 h of incubation with the infected cells (Fig. 3).

Taken together, our results demonstrate that also (*E*)-6-chloro-3-(3-phenylprop-1-en-1-yl)-2*H*-chromene (**4b**) exerts an early activity during HRV multiplication, although it does not interfere



HRVC: untreated virus,
HRVT: virus exposed to pH 5.0,
4b: virus exposed to pH 5.0 in the presence of **4b**.

HRVC: untreated virus,
HRVT: virus heated at 56 °C,
4b: virus heated at 56 °C in the presence of **4b**.

Figure 1. Protective effect of (*E*)-6-chloro-3-(3-phenylprop-1-en-1-yl)-2*H*-chromene **4b** on acid (A) and thermal inactivation (B) of HRV 1B infectivity.

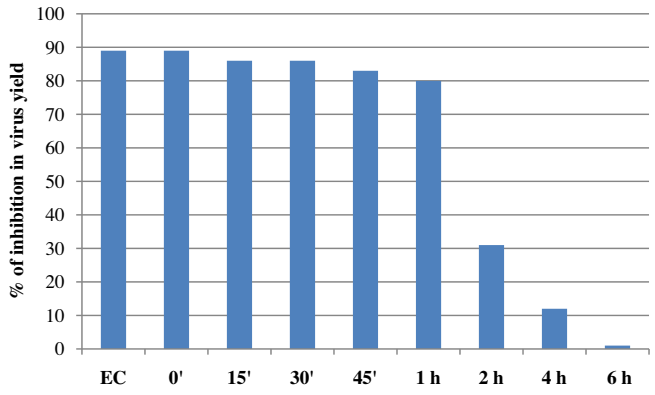


Figure 2. Effect of varying the time of addition of **4b** (42 μM) on the inhibition of HRV 1B multiplication under one-step growth conditions. Virus yield was determined by plaque assay. Virus control titre was 2.4×10^4 PFU/mL. EC: **4b** was present during the entire infection cycle (1 h at 4 °C and 10 h at 33 °C). 0′, 15′, 30′, 45′, 1, 2, 4 and 6 h: compound was added at different times (0′, 15′, 30′, 45′, 1, 2, 4, and 6 h) after the virus adsorption period (1 h at 4 °C, time 0) and maintained until the end of virus multiplication (up to 10 h).

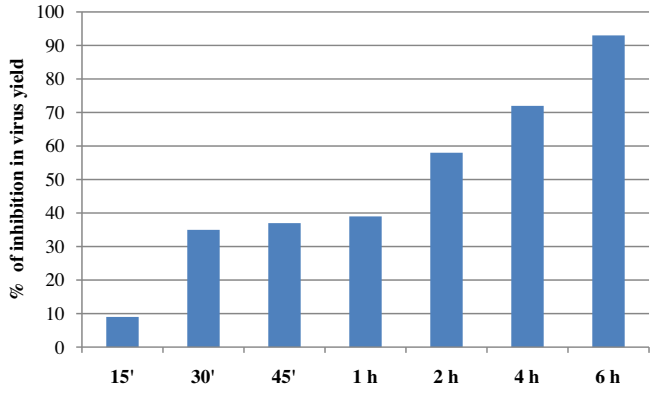


Figure 3. Effect of varying the time of removal of **4b** (42 μM) on the inhibition of HRV 1B replication under one-step growth conditions. Virus yield was determined by plaque assay. Virus control titre was 2.4×10^4 PFU/mL. 15′, 30′, 45′, 1, 2, 4 and 6 h: compound **4b** was added after virus adsorption (1 h at 4 °C, time 0) and removed after different lengths of incubation (15′, 30′, 45′, 1, 2, 4 and 6 h) at 33 °C.

with cell receptor recognition by the virus. Maximal reduction in virus yield is achieved when **4b** is added to infected cells within the first hour of infection or removed from infected cells after 6 h of treatment. These data suggest an interference by **4b** during the uncoating process.

The results obtained with the chromene **4b** significantly differ from those previously described for related compounds such as (*Z*)-3-(4-chlorobenzylidene)chroman and (*E*)-3-styryl-2*H*-chromene.[4,15] Maximal inhibition of virus yield was observed when both these compounds were present during the time of virus adsorption only. A similar reduction was noticed when compounds were removed 30 min after virus binding.[4,15] On the contrary, both analogues did not modify virus yield when added only 1 h after virus infection. Therefore, modification in shape and length of the backbone as in the new (*E*)-6-chloro-3-(3-phenylprop-1-en-1-yl)-2*H*-chromene **4b** does not allow interaction of this molecule with virus capsid structures involved in receptor recognition.

3. Conclusion

In the present study we focused our attention onto the design, synthesis and anti-HRV activity of new series of (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones **1a–e**, (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones **2a** and **2b**, (*Z*)-3-[(*E*)-3-phenylallylidene]chromans **3a–e**, and (*E*)-3-(3-phenylprop-1-en-1-yl)-2*H*-chromenes **4a–d**. As expected several compounds exhibited a wide spectrum of anti-HRV activity coupled with a high therapeutic index. The data obtained from stabilization and time of addition/removal studies are in agreement with the capsid-binder hypothesis. Time of addition/removal experiments suggest an interference with the uncoating process of viral genome without an action on HRV adsorption to the host cell membrane.

4. Experimental

4.1. Chemistry

Chemicals were purchased from Sigma–Aldrich or Alfa Aesar and used without further purification. Melting points were determined on a Stenford Research Systems OptiMelt (MPA-100) apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were detected with a Bruker AM-400 spectrometer, using TMS as internal standard. IR spectra were recorded on a FT-IR PerkinElmer Spectrum 1000. All compounds were routinely checked by thin-layer chromatography (TLC) and ^{1}H NMR. TLC was performed on silica gel or aluminium oxide fluorescent coated plates. Components were visualised by UV light. Elemental analyses (C, H, Cl) of all new compounds were within ±0.4% of theoretical values. 2*H*-Chromene-3-carbaldehyde,[15] 6-chloro-2*H*-chromene-3-carbaldehyde,[15] phenethyltriphenylphosphonium bromide[24] and 4-chlorophenethyltriphenylphosphonium bromide[24] were synthesized following the procedure previously described.

4.1.1. General procedure for the synthesis of the (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-ones (1a–e) and (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones (2a,b)

A mixture of the appropriate chroman-4-one (10 mmol) and *trans*-cinnamaldehyde (10 mmol) or 3-phenylpropiolaldehyde (10 mmol), in 85% phosphoric acid (63 mL) was heated at 80 °C for 4 h while stirring. After cooling the mixture was diluted with ice and water. The precipitate was filtered off, washed with water and crystallized.

4.1.1.1. (*E*)-3-[(*E*)-3-Phenylallylidene]chroman-4-one (1a). Yield: 80%, mp = 115–119 °C from ethyl alcohol. IR (KBr): 1664 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.01 (dd, 1H, H5, J_{5-6} = 7.8 Hz, J_{5-7} = 1.8 Hz), 7.54–7.45 (m, 4H, H2′, H6′, H7, Hα), 7.40–7.34 (3H, H3′–H5′), 7.09 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.5 Hz), 7.08–6.97 (m, 3H, H6, H8, Hβ), 5.26 (d, 2H, H2, $J_{2-\alpha}$ = 1.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 181.92, 161.44, 143.30, 136.06, 135.97, 135.56, 129.52, 128.92, 127.92, 127.67, 127.45, 122.45, 121.88, 121.64, 117.86, 66.99. Anal. calcd for $C_{18}H_{14}O_2$: C, 82.42; H, 5.38. Found: C, 82.58; H, 5.46.

4.1.1.2. (*E*)-6-Chloro-3-[(*E*)-3-phenylallylidene]chroman-4-one (1b). Yield: 85%, mp = 159–160 °C from ethyl alcohol. IR (KBr): 1669 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.96 (d, 1H, H5, J_{5-7} = 2.7 Hz), 7.54–7.49 (3H, H2′, H6′, Hα), 7.43–7.35 (m, 4H, H7, H3′–H5′), 7.11 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.4 Hz), 6.99 (dd, 1H, Hβ $J_{\alpha-\beta}$ = 11.6 Hz, $J_{\beta-\gamma}$ = 15.4 Hz), 6.95 (d, 1H, H8, J_{7-8} = 8.8 Hz), 5.26 (d, 2H, H2, $J_{2-\alpha}$ = 1.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 180.81, 159.85, 143.98, 136.79, 135.93, 135.35, 129.71, 128.96, 128.82, 127.54, 127.38, 127.31, 123.27, 121.46, 119.58, 67.14. Anal. calcd for $C_{18}H_{13}ClO_2$: C, 72.85; H, 4.42; Cl, 11.95. Found: C, 72.97; H, 4.45; Cl, 11.80.

4.1.1.3. (*E*)-6-Chloro-3-[(*E*)-3-(4-chlorophenyl)allylidene]chroman-4-one (1c). Yield: 81%, mp = 168–176 °C from ethyl acetate. IR (KBr): 1654 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.95 (d, 1H, H5, J_{5-7} = 2.7 Hz), 7.50–7.44 (3H, H2′, H6′, Hα), 7.41

(dd, 1H, H7, J_{7-8} = 8.8 Hz, J_{5-7} = 2.7 Hz), 7.36 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.5 Hz), 7.05 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.3 Hz), 7.03–6.92 (m, 2H, H8, Hβ), 5.25 (d, 2H, H2, $J_{2-\alpha}$ = 1.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 180.80, 159.85, 142.34, 136.35, 135.60, 135.47, 134.42, 129.23, 128.72, 128.64, 127.46, 127.23, 123.20, 121.97, 119.61, 67.10. Anal. calcd for $C_{18}H_{12}Cl_2O_2$: C, 65.28; H, 3.65; Cl, 21.41. Found: C, 65.59; H, 3.73; Cl, 21.64.

4.1.1.4. (*E*)-3-[(*E*)-3-(4-Chlorophenyl)allylidene]chroman-4-one (1d). Yield: 83%, mp = 160–163 °C from ethyl acetate. IR (KBr): 1665 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.01 (dd, 1H, H5, J_{5-6} = 7.8 Hz, J_{5-7} = 1.8 Hz), 7.51–7.43 (4H, H7, H2′, H6′, Hα), 7.34 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.6 Hz), 7.07 (ddd, 1H, H6, J_{5-6} = 7.8 Hz, J_{6-7} = 7.2 Hz, J_{6-8} = 1.1 Hz), 7.02–6.93 (m, 3H, H8, Hβ, Hγ), 5.26 (d, 2H, H2, $J_{2-\alpha}$ = 1.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 181.88, 161.45, 141.66, 135.66, 135.51, 135.31, 134.57, 129.68, 129.19, 128.56, 127.94, 122.41, 122.17, 121.96, 117.90, 66.97. Anal. calcd for $C_{18}H_{13}ClO_2$: C, 72.85; H, 4.42; Cl, 11.95. Found: C, 73.09; H, 4.37; Cl, 11.78.

4.1.1.5. (*E*)-6-Fluoro-3-[(*E*)-3-phenylallylidene]chroman-4-one (1e). Yield: 90%, mp = 182–185 °C from ethyl acetate. IR (KBr): 1669 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.65 (dd, 1H, H5, J_{5-F} = 8.3 Hz, J_{5-7} = 2.9 Hz), 7.54–7.49 (m, 3H, H2′, H6′, Hα), 7.42–7.33 (3H, H3′–H5′), 7.19 (ddd, 1H, H7, J_{7-8} = 7.8 Hz, J_{7-F} = 9.0 Hz, J_{5-7} = 2.9 Hz), 7.11 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.5 Hz), 7.04–6.95 (m, 2H, H8, Hβ), 5.24 (d, 2H, H2, $J_{2-\alpha}$ = 1.3 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 181.20, 157.64, 157.62 (d, J = 244 Hz), 143.86, 136.55, 135.96, 129.68, 128.96, 128.47, 127.53, 123.07 (d, J = 7 Hz), 122.99 (d, J = 23 Hz), 121.54, 119.49 (d, J = 7 Hz), 112.87 (d, J = 23 Hz), 67.12. Anal. calcd for $C_{18}H_{13}FO_2$: C, 77.13; H, 4.67; F, 6.78. Found: C, 77.44; H, 4.59.

4.1.1.6. (*E*)-3-(3-Phenylprop-2-yn-1-ylidene)chroman-4-one (2a). Yield: 50%, mp = 140 °C from *n*-hexane. IR (KBr): 2183, 1662 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.00 (dd, 1H, H5, J_{5-6} = 7.8 Hz, J_{5-7} = 2.6 Hz), 7.55–7.48 (m, 3H, H2′, H6′, H7), 7.42–7.35 (m, 3H, H3′–H5′), 7.07 (ddd, 1H, H6, J_{5-6} = 7.8 Hz, J_{6-7} = 8.0 Hz, J_{6-8} = 1.1 Hz), 7.03–7.05 (m, 2H, H8, Hα), 5.34 (d, 2H, H2, $J_{2-\alpha}$ = 1.9 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 180.43, 161.78, 139.54, 136.13, 132.00, 129.66, 128.60, 127.89, 122.12, 122.07, 121.68, 118.14, 116.92, 104.71, 85.28, 68.60. Anal. calcd for $C_{18}H_{12}O_2$: C, 83.06; H, 4.65. Found: C, 83.37; H, 4.50.

4.1.1.7. (*E*)-6-Chloro-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-one (2b). Yield: 60%, mp = 127–131 °C from *n*-hexane. IR (KBr): 2185, 1661 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.95 (d, 1H, H5, J_{5-7} = 2.6 Hz), 7.53 (dd, 2H, H2′, H6′, $J_{2'-3'}$ = 7.9 Hz, $J_{2'-5'}$ = 1.6 Hz), 7.44 (dd, 1H, H7, J_{7-8} = 9.0 Hz, J_{5-7} = 2.6 Hz), 7.41–7.36 (m, 3H, H3′–H5′), 7.03 (t, 1H, Hα, $J_{2-\alpha}$ = 1.9 Hz), 6.97 (d, 1H, H8, J_{7-8} = 9.0 Hz), 5.33 (d, 2H, H2, $J_{2-\alpha}$ = 1.9 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 179.39, 160.18, 138.60, 135.93, 132.04, 129.81, 128.63, 127.59, 127.12, 122.46, 121.96, 119.86, 117.78, 105.45, 85.16, 68.73. Anal. calcd for $C_{18}H_{11}ClO_2$: C, 73.35; H, 3.76; Cl, 12.03. Found: C, 73.08; H, 3.91; Cl, 11.95.

4.1.2. General procedure for the synthesis of the (*Z*)-3-[(*E*)-3-phenylallylidene]chromans (3a–e)

A solution of the appropriate (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-one (1a-e) (10 mmol) in dry ethyl ether (120 mL) was added dropwise to a suspension of lithium aluminium hydride (17.5 mmol) and aluminium chloride (35.0 mmol) in dry ethyl ether (20 mL). After complete addition, the mixture was stirred at room temperature for 1 h and refluxed for 2.5 h. After cooling, excess of reducing reagent was destroyed by adding ethyl acetate at 0 °C, and the mixture was poured into 2 N hydrochloric acid. The organic layer was washed with saturated aqueous sodium bicarbonate and brine, than it was dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was chromatographed on silica gel column eluting with ethyl acetate/light petroleum (1:5 for **3a** and **3e**, 1:8 for **3b**, 1:10 for **3c**, 1:20 for **3d**) and purified by crystallization.

4.1.2.1. (*Z*)-3-[(*E*)-3-Phenylallylidene]chroman (3a). Yield: 54%, mp = 102–104 °C from *n*-hexane. 1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.43 (dd, 2H, H2′, H6′, $J_{2'-3'}$ = 8.2 Hz, $J_{2'-4'}$ = 1.3 Hz), 7.34–7.23 (m, 3H, H3′–H5′), 7.11–7.05 (m, 2H, H5, H7), 7.03 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 11.1 Hz, $J_{\beta-\gamma}$ = 15.4 Hz), 6.91–6.84 (m, 2H, H6, H8), 6.58 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.4 Hz), 6.32 (dd, 1H, Hα, $J_{\alpha-\beta}$ = 11.1 Hz, $J_{2-\alpha}$ = 0.9 Hz), 4.86 (d, 2H, H2, $J_{2-\alpha}$ = 0.9 Hz), 3.59 (s, 2H, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.86, 137.25, 133.20, 131.87, 128.93, 128.67, 128.73, 127.31, 126.44, 126.31, 123.15, 122.79, 121.02, 116.78, 65.33, 34.23. Anal. calcd for $C_{18}H_{16}O$: C, 87.06; H, 6.49. Found: C, 87.36; H, 6.63.

4.1.2.2. (*Z*)-6-Chloro-3-[(*E*)-3-phenylallylidene]chroman (3b). Yield: 49%, mp = 121–124 °C from *n*-hexane. 1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.42 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 7.7 Hz), 7.35–7.22 (m, 3H, H3′–H5′), 7.07–7.04 (m, 2H, H5, H7), 7.00 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 11.2 Hz, $J_{\beta-\gamma}$ = 15.3 Hz), 6.78 (d, 1H, H8, J_{7-8} = 8.4), 6.60 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.3 Hz), 6.33 (d, 1H, Hα, $J_{\alpha-\beta}$ = 11.2 Hz), 4.84 (s, 2H, H2), 3.56 (s, 2H, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.50, 137.10, 133.68, 130.56, 128.69, 128.48, 127.85, 127.28, 126.85, 126.48, 125.67, 124.31, 122.88, 118.07, 65.39, 34.03. Anal. calcd for $C_{18}H_{15}ClO$: C, 76.46; H, 5.35; Cl, 12.54. Found: C, 76.68; H, 5.38; Cl, 12.30.

4.1.2.3. (*Z*)-6-Chloro-3-[(*E*)-3-(4-chlorophenyl)allylidene]chroman (3c). Yield: 36%, mp = 142–146 °C from ethyl acetate/light petroleum. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.34 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.5 Hz), 7.29 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.5 Hz), 7.07–7.04 (m, 2H, H5, H7), 6.97 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 11.1 Hz, $J_{\beta-\gamma}$ = 15.4 Hz), 6.76 (d, 1H, H8, J_{7-8} = 8.0), 6.54 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.4 Hz), 6.31 (d, 1H, Hα, $J_{\alpha-\beta}$ = 11.1 Hz), 4.83 (s, 2H, H2), 3.56 (s, 2H, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.42, 135.57, 133.44, 132.26, 131.24, 128.87, 128.48, 127.60, 127.32, 126.58, 125.71, 124.17, 123.42, 118.07, 65.31, 34.05. Anal. calcd for $C_{18}H_{14}Cl_2O$: C, 68.15; H, 4.45; Cl, 22.35. Found: C, 67.91; H, 4.62; Cl, 22.46.

4.1.2.4. (*Z*)-3-[(*E*)-3-(4-Chlorophenyl)allylidene]chroman (3d). Yield: 42%, mp = 135–136 °C from ethyl acetate/light petroleum. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.34 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.5 Hz), 7.29 (d, 2H, H3', H5', $J_{2'-3'}$ = 8.5 Hz), 7.14–7.05 (m, 2H, H5, H7), 6.98 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 11.1 Hz, $J_{\beta-\gamma}$ = 15.4 Hz), 6.92–6.84 (m, 2H, H6, H8, J_{7-8} = 8.0), 6.53 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.4 Hz), 6.31 (d, 1H, Hα, $J_{\alpha-\beta}$ = 11.1 Hz), 4.86 (s, 2H, H2), 3.61 (s, 2H, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.74, 135.70, 133.11, 131.78, 131.55, 130.95, 129.47, 128.84, 127.57, 127.35, 126.08, 123.66, 121.09, 116.77, 65.21, 34.23. Anal. calcd for $C_{18}H_{15}ClO$: C, 76.46; H, 5.35; Cl, 12.54. Found: C, 76.71; H, 5.23; Cl, 12.74.

4.1.2.5. (*Z*)-6-Fluoro-3-[(*E*)-3-phenylallylidene]chroman (3e). Yield: 40%, mp = 121–123 °C from ethyl acetate/light petroleum. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.42 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 7.9 Hz), 7.35–7.22 (m, 3H, H3′–H5′), 7.00 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 11.2 Hz, $J_{\beta-\gamma}$ = 15.4 Hz), 6.81–6.76 (m, 3H, H5, H7, H8), 6.59 (d, 1H, Hγ, $J_{\beta-\gamma}$ = 15.4 Hz), 6.32 (d, 1H, Hα, $J_{\alpha-\beta}$ = 11.2 Hz), 4.83 (s, 2H, H2), 3.57 (s, 2H, H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 157.30 (d, J = 236 Hz), 150.97, 137.17, 133.53, 131.13, 128.69, 127.82, 126.56, 126.47, 124.12 (d, J = 8 Hz), 122.98, 117.66 (d,

J = 8 Hz), 114.79 (d, J = 23 Hz), 113.95 (d, J = 23 Hz), 65.48, 34.22. Anal. calcd for $C_{18}H_{15}FO$: C, 81.18; H, 5.68; F, 7.13. Found: C, 81.23; H, 5.72; Cl, 7.00.

4.1.3. General procedure for the synthesis of the (*E*)-3-(3-phenylprop-1-en-1-yl)-2*H*-chromenes (4a–d)

A 0.5 M solution of sodium ethoxide (25 mL) was added dropwise to a stirred suspension of the appropriate phenethyltriphenylphosphonium bromide (10 mmol) in ethyl alcohol (50 mL). After stirring for 45 min at room temperature, a solution of the appropriate 2*H*-chromene-3-carbaldehyde (10 mmol) in ethyl alcohol (85 mL) was added dropwise and the mixture was refluxed for 2.5 h. After cooling, water was added, ethyl alcohol was removed under reduced pressure and the residue was extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was purified by column chromatography on silica gel eluting with ethyl acetate/light petroleum (1:4). The product was further purified by crystallization from *n*-hexane.

4.1.3.1. (*E*)-3-(3-Phenylprop-1-en-1-yl)-2H-chromene (4a). Yield: 45%, mp = 89–90 °C from *n*-hexane. 1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.31 (t, 2H, H3′, H5′, $J_{2'-3'} = J_{3'-5'} = 7.5$ Hz), 7.25–7.19 (m, 3H, H2′, H4′, H6′), 7.07 (ddd, 1H, H7, $J_{7-8} = 8.0$ Hz, $J_{6-7} = 7.4$ Hz, $J_{5-7} = 1.6$ Hz), 6.97 (dd, 1H, H5, $J_{5-6} = 7.4$ Hz, $J_{5-7} = 1.6$ Hz), 6.85 (dt, 1H, H6, $J_{5-6} = J_{6-7} = 7.4$ Hz, $J_{6-8} = 1.1$ Hz), 6.78 (dd, 1H, H8, $J_{7-8} = 8.0$ Hz, $J_{6-8} = 1.1$ Hz), 6.32 (s, 1H, H4), 6.19 (d, 1H, Hα, $J_{\alpha-\beta} = 15.9$ Hz), 5.74 (dt, 1H, Hβ, $J_{\alpha-\beta} = 15.9$ Hz, $J_{\beta-\gamma} = 6.9$ Hz), 4.93 (s, 2H, H2), 3.50 (d, 2H, Hγ, $J_{\beta-\gamma} = 6.9$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 159.86, 142.39, 136.26, 135.54, 135.02, 134.42, 129.23, 128.73, 128.64, 127.56, 126.59, 123.20, 121.73, 119.52, 67.09, 42.91. Anal. calcd for $C_{18}H_{16}O$: C, 87.06; H, 6.49. Found: C, 86.95; H, 6.63.

4.1.3.2. (*E*)-6-Chloro-3-(3-phenylprop-1-en-1-yl)-2H-chromene (4b). Yield: 43%, mp = 80–81 °C from *n*-hexane. 1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.34 (t, 2H, H3′, H5′, $J_{2'-3'} = J_{3'-5'} = 7.9$ Hz), 7.26–7.19 (m, 3H, H2′, H4′, H6′), 7.02 (dd, 1H, H7, $J_{7-8} = 8.5$ Hz, $J_{5-7} = 2.5$ Hz), 6.95 (d, 1H, H5, $J_{5-7} = 2.5$ Hz), 6.72 (d, 1H, H8, $J_{7-8} = 8.5$ Hz), 6.25 (s, 1H, H4), 6.19 (d, 1H, Hα, $J_{\alpha-\beta} = 15.9$ Hz), 5.78 (dt, 1H, Hβ, $J_{\alpha-\beta} = 15.9$ Hz, $J_{\beta-\gamma} = 6.8$ Hz), 4.94 (s, 2H, H2), 3.52 (d, 2H, Hγ, $J_{\beta-\gamma} = 6.8$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.00, 139.52, 137.16, 131.73, 130.25, 128.92, 128.66, 128.61, 128.22, 126.41, 126.06, 124.10, 120.95, 116.59, 65.79, 39.53. Anal. calcd for $C_{18}H_{15}ClO$: C, 76.46; H, 5.35; Cl, 12.54. Found: C, 76.71; H, 5.22; Cl, 12.31.

4.1.3.3. (*E*)-6-Chloro-3-[3-(4-chlorophenyl)prop-1-en-1-yl]-2H-chromene (4c). Yield: 41%, mp = 135–143 °C from *n*-hexane. 1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.28 (d, 2H, H3′, H5′, $J_{2'-3'} = 8.1$ Hz), 7.12 (d, 2H, H2′, H6′, $J_{2'-3'} = 8.1$ Hz), 7.01 (dd, 1H, H7, $J_{7-8} = 8.5$ Hz, $J_{5-7} = 2.5$ Hz), 6.95 (d, 1H, H5, $J_{5-7} = 2.5$ Hz), 6.71 (d, 1H, H8, $J_{7-8} = 8.5$ Hz), 6.25 (s, 1H, H4), 6.16 (d, 1H, Hα, $J_{\alpha-\beta} = 15.9$ Hz), 5.72 (dt, 1H, Hβ, $J_{\alpha-\beta} = 15.9$ Hz, $J_{\beta-\gamma} = 6.7$ Hz), 4.91 (s, 2H, H2), 3.46 (d, 2H, Hγ, $J_{\beta-\gamma} = 6.7$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.06, 137.96, 132.28, 131.52, 130.01, 129.49, 129.33, 128.74, 128.37, 126.14, 124.02, 121.31, 116.64, 65.77, 38.79. Anal. calcd for $C_{18}H_{14}Cl_2O$: C, 68.15; H, 4.45; Cl, 22.35. Found: C, 68.35; H, 4.25; Cl, 22.51.

4.1.3.4. (*E*)-3-[3-(4-Chlorophenyl)prop-1-en-1-yl]-2H-chromene (4d). Yield: 48%, mp = 90–91 °C from *n*-hexane. 1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.28 (d, 2H, H3′, H5′, $J_{2'-3'} = 8.3$ Hz), 7.13 (d, 2H, H2′, H6′, $J_{2'-3'} = 8.3$ Hz), 7.08 (dd, 1H, H7, $J_{7-8} = 8.2$ Hz, $J_{6-7} = 7.4$ Hz, $J_{5-7} = 1.6$ Hz), 6.98 (dd, 1H, H5, $J_{5-6} = 7.4$ Hz, $J_{5-7} = 1.6$ Hz), 6.86 (dt, 1H, H6, $J_{5-6} = J_{6-7} = 7.4$ Hz, $J_{6-8} = 1.1$ Hz), 6.78 (dd, 1H, H8, $J_{7-8} = 8.2$ Hz, $J_{6-8} = 1.1$ Hz), 6.32 (s, 1H, H4), 6.17 (d, 1H, Hα, $J_{\alpha-\beta} = 15.9$ Hz), 5.68 (dt, 1H, Hβ, $J_{\alpha-\beta} = 15.9$ Hz, $J_{\beta-\gamma} = 6.9$ Hz), 4.92 (s, 2H, H2), 3.46 (d, 2H, Hγ, $J_{\beta-\gamma} = 6.8$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.77, 138.25, 131.43, 130.96, 129.06, 128.66, 128.21, 128.17, 127.49, 126.76, 125.56, 123.29, 121.56, 115.49, 67.00, 42.52.Anal. calcd for $C_{18}H_{15}ClO$: C, 76.46; H, 5.35; Cl, 12.54. Found: C, 76.63; H, 5.21; Cl, 12.30.

4.2. Virology

4.2.1. Cells

HeLa (Ohio) cells were grown at 37 °C using Eagle's Minimum Essential Medium (MEM) supplemented with 100 μg/mL of streptomycin and 100 U/mL of penicillin G and 8% heat-inactivated foetal calf serum (FCS) (growth medium). The concentration was reduced to 2% for cell maintenance (maintenance medium).

4.2.2. Compounds

Stock solutions were made up in ethanol (1, 0.5 or 0.1 mg/mL) and further diluted in cell culture medium shortly before use.

4.2.3. Virus

Reference strains of HRV type 1B and 14 were purchased from American Type Culture Collection (ATCC). Virus stocks were prepared infecting hela (Ohio) cell monolayers at a multiplicity of infection of 0.1 PFU/cell. Infected cells were incubated at 33 °C. When the viral-induced cytopathic effect involved most of the cells, the cultures were freeze-thawed three times and the clarified supernatants titrated by plaque assay, essentially as described by Fiala and Kenny.[25] The virus was stored at −80 °C until used

4.2.4. XTT assay for cellular cytotoxicity

A tetrazolium-based (XTT) colorimetric assay was used to measure the cytotoxicity of compounds, as previously described.[16] Briefly, HeLa cells were seeded in 96-well tissue culture plates (2×10^3 cells/well) in 100 μL of growth medium with or without compounds in twofold dilutions, starting from the maximum soluble concentration in cell culture medium. Triplicate wells were used for each drug concentration to be tested. In parallel, media containing the same concentrations of ethanol were used as control in order to evaluate the residual toxicity of ethanol. The plates were incubated at 37 °C in 5% CO_2-air until the untreated monolayers were confluent (3 days). Then, 50 μL of XTT labelling mixture was added to each well (final XTT concentration 0.15 mg/mL) and the cells incubated for 4 h at 37 °C. The spectrophotometric absorbance of the samples was measured using an ELISA reader at 492 nm with a reference wavelength at 690 nm. Cytotoxicity was also scored microscopically as morphological alterations on the third day of incubation in the presence of compounds. The highest concentration of compound that did not produce any modification of morphology and viability on 100% of cells was the maximum non-cytotoxic concentration. The 50% cytotoxic concentration (TC_{50}) was indicated as the concentration of compound reducing the cell viability by 50%, as compared with mock-treated cells.

4.2.5. Determination of the 50% inhibitory concentration (IC_{50})

The IC_{50} values were determined as described previously.[7] Briefly, monolayers of HeLa cells in 6-well plates were infected with a virus suspension producing approximately 100 plaques per well. After 1 h of incubation at 33 °C, the virus inoculum was removed and the cells were overlaid with medium for plaques, in the presence or absence of fourfold dilutions of drugs. After three days of incubation at 33 °C, the cells were stained with a neutral red solution (0.2 mg/mL) in pH 7.4 phosphate buffered saline (PBS) and the plaques were counted. The IC_{50} was expressed as the concentration of drug reducing the plaque number by 50% as

compared with mock-treated control. It was calculated from a dose/response curve obtained by plotting the percentage of plaque reduction, with respect to the control plaque count, versus the logarithm of compound dose. Triplicate wells were utilized for each drug concentration.

4.2.6. Virus inactivation and stabilization

For virus inactivation studies, HRV 1B suspensions with or without **4b** (42 μM) were incubated at 33 °C for 1 h. After serial 10-fold dilutions, virus titres were measured by plaque assay on HeLa cell monolayers.

For virus stabilization studies, the virus was incubated with or without **4b** (42 μM) for 1 h at 33 °C before mild acid or thermal treatment. For mild acid treatment, the pH of the mixtures was adjusted to 5 by adding 0.2 M acetate buffer (pH 5). After incubation at 33 °C for 30 min, the mixtures were neutralized with 0.85 M Tris base. For thermal treatment, the mixtures were incubated for 20 min at 56 °C (pH 7.2) and then refrigerated on ice. All samples were diluted 10-fold serially and titrated by plaque assay on HeLa cell monolayers.

4.2.7. Virus yield reduction assays

Confluent monolayers of HeLa cells in 24-well plates were infected at a multiplicity of five in the presence or absence of **4b** (42 μM). The infection was synchronized by allowing HRV 1B to bind in the cold (4 °C). After 1 h, the inoculum was removed by washing thrice with cold PBS. The end of virus binding is indicated as 0 time. Then, MEM with or without the compound (42 μM) was added and the temperature raised to 33 °C to permit internalization. Single-cycle conditions were achieved by incubating the cells at 33 °C for 10 h post-infection (p.i.). The cultures were freeze-thawed three times, cell debris removed by low-speed centrifugation in the cold and the supernatants titrated by plaque assay on HeLa cell monolayers. To determine which stage of virus replication was affected by **4b**, the drug was added or removed from HRV-infected cells at various times p.i. (0, 15′, 30′, 45′, 1, 2, 4 and 6 h) and the cultures incubated at 33 °C up to 10 h p.i. The virus yield was determined as above.

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