SAPIENZA
UNIVERSITÀ DI ROMA

Dipartimento di Chimica e Tecnologie del Farmaco

PhD Thesis in Pharmaceutical Sciences

XXV cycle

NOVEL 4*H*-CHROMEN-4-ONE, *2H*-CHROMENE AND CHROMAN DERIVATIVES: DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION

Tutor:
Chiar.ma Prof.ssa Nicoletta Desideri

PhD student:
Luca Proietti Monaco

2009-2012

INDEX

Chapter I

4*H*-CHROMEN-4-ONE, 2*H*-CHROMENE AND CHROMAN DERIVATIVES AS ANTI-PICORNAVIRUS INHIBITORS

Chapter I

4*H*-CHROMEN-4-ONE, 2*H*-CHROMENE AND CHROMAN DERIVATIVES AS ANTI-PICORNAVIRUS INHIBITORS

1. INTRODUCTION

The picornavirus family represent one of the largest families of human and animal viral pathogens. Based on similarities in virion physico-chemicals properties, RNA sequence and genome organization these viruses are currently classified in twelve established and five proposed genera.
The established genera are:

- *Enterovirus* (Human enterovirus A, B, C, D (HEV-A, B, C, D), unclassified Simian enterovirus A (SEV-A), Bovine enterovirus A, B, Porcine enterovirus B, Human rhinovirus A, B, C (HRV-A, B, C));
- *Hepatovirus* (Hepatitis A virus (HAV));
- *Parechovirus* (Human parechovirus (HPeV) and Ljungan virus);
- *Kobuvirus* (Aichi virus, Bovine kobuvirus, Porcine kobuvirus);
- *Cardiovirus* (Encephalomiocarditis virus (EMCV), Theilovirus (ThV));
- *Aphtovirus* (Foot-and-mouth disease virus (FMDV), Equine rhinitis A virus, Bovine rhinitis A and B virus);
- *Erbovirus* (Equine rhinitis B virus);
- *Teschovirus* (Porcine Teschovirus);
- *Sapelovirus* (Porcine sapelovirus, Simian sapelovirus and Avian sapelovirus);
- *Avihepatovirus* (Duck hepatitis A virus);
- *Senecavirus* (Seneca Valley virus);
- *Tremovirus* (Avian encephalomyelitis virus);

The proposed genera are:

- *Cosavirus* (Human Cosavirus (HCoSV A) and a number of candidate species);
- *Aquamavirus* (Aquamavirus A);
- *Dicipivirus* (Cadicivirus A);
- *Megrivirus* (Melegrivirus A);
- *Salivirus* (Salivirus A).

Six genera of this family include pathogens infecting humans: *Enterovirus, Parechovirus, Hepatovirus, Cardiovirus, Kobuvirus, Aphtovirus*.

2. VIROLOGY

Picornaviruses are small, non–enveloped, positive stranded RNA viruses whose diameter varies between 28 and 34 nm.[1] The RNA is surrounded by a capsid consisting of a densely-packed icosahedral arrangement of 60 *protomers*, each constituted by 4 proteins, called VP1 (viral protein 1), VP2, VP3 and VP4. The three larger proteins (VP1, VP2, VP3, ~30 kDa each) are located on the external surface of the virus and one smaller protein (VP4, ~7 kDa) lies on the inner surface, interfacing with VP1-3 and RNA.[2]

The complete virion derives from the assembly of the different protein units (figure 1):

- VP1, VP2, VP3 and VP4 form a *protomer*, the smallest subunit of capsid;
- Five protomers form a *capsomer*;
- Twelve capsomers form the icosahedral capsid.

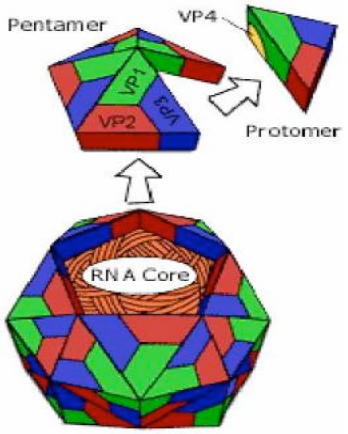


Figure. 1. Structure of capsid

The major capsid proteins VP1 to VP3 are each folded into eight-stranded antiparallel β-sheets with a jelly-roll topology. These β-barrels of five copies of VP1 are located around the fivefold axis, while VP2 and VP3 are around the threefold axis. The smaller protein VP4 have a less ordered structure and, being located on the inner surface of the capsid, anchors the RNA genoma (figure 2).

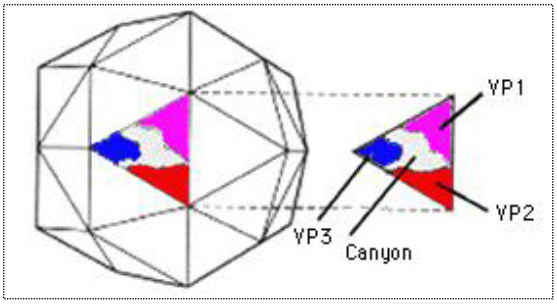


Figure 2. Structure of protomer

The virion's surface presents a plateau star-shaped at the top of each axis of pentaicosahedric symmetry, surrounded by a deep depression called "canyon" (figure 3). The canyon is formed by the folding of aminoacid chains with highly conserved sequences. Under the floor of the canyon, inside VP1, there is a hydrophobic pocket that represents the binding site for anti-picornavirus compounds called capsid-binders.[3] Moreover, attachment of the virus to the host cell occurs through binding of the cellular receptors into this canyon.

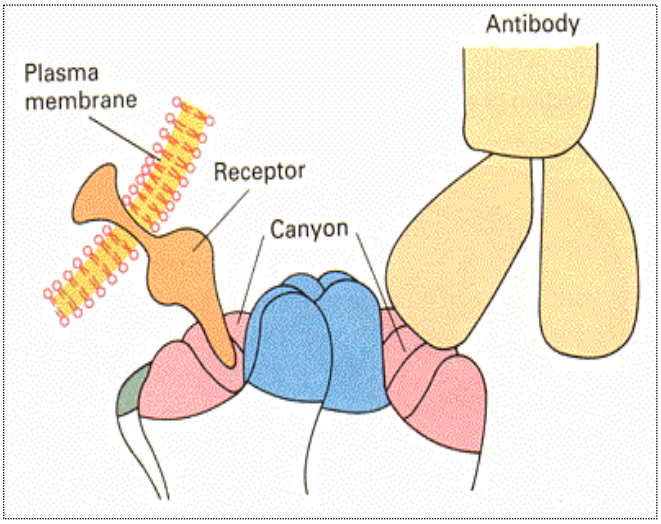


Figure 3. Canyon's structure. To the left: it is shown the binding to the receptor; to the right: it is shown the binding to the antibodies.

2.1. Viral genome

All picornaviruses share a similar genomic organization that consists of a single-stranded, positive sense RNA molecule of approximately

6700-8850 nucleotides[4-9] (figure 4). The genome contains a single open reading frame (ORF) preceded by a long 5'-untranslated region (5' UTR) whose sequences tend to be strongly conserved among the different viral species. In a subset of picornaviruses, a region L follows 5' UTR. A relatively short region 3'-UTR follows the ORF, it varies in length from 14 to 126 bases and probably contains signals for the binding of the replicase, essential to initiate the synthesis of new RNA chains. At its end there is a polyadenilated tract (poly-A), heterogeneous in length (on average 35-100 residues), important for the synthesis of the negative-stranded RNA.[10]

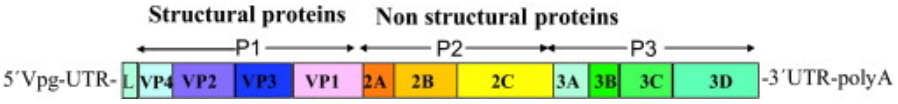


Figure 4. Principal organization of picornavirus genomes. The actual genome organization may deviate in some picornaviruses

A small viral protein, VPg, is covalently linked to the 5' end of the viral genome. The VPg is involved in the replication of the viral genome but it is not essential for infectivity, in fact if it is removed by treatment with viral protease the viral infectivity is not reduced.[11]

The ORF is translated into a long polyprotein (approximately 2200 amino-acid residues) that was cleaved nascently by viral proteases into structural and non-structural proteins, divided into three primary precursors (P1, P2, and P3). The viral capsid proteins VP1-VP4 are formed by the cleavage of the P1 region. Seven non-structural proteins involved in the replication of the genome or with enzymatic activity (2A- 2C and 3A, 3B- 3D) are formed from P2 and P3 cleavage, respectively.

2.2. Replication cycle

Picornavirus replication occurs entirely in the cytoplasm in a single-step growth. The viral multiplication cycle begins with the attachment of the virus to the host cell surface through interaction with cellular receptors.

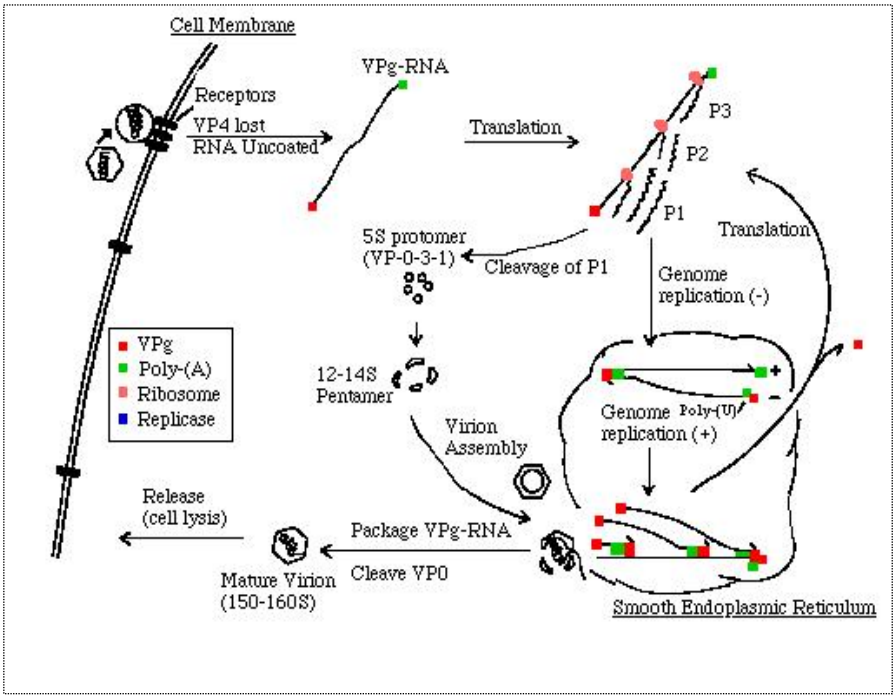


Figure 5. Replicative cycle of the Picornavirus.

During the last few year, several cellular receptors for different groups of picornaviruses have been identified (table 1). The major group of HRVs and several coxsackieviruses use intercellular adhesion molecule 1 (ICAM-1) as cellular receptor, while poliovirus (PV) bounds to poliovirus receptor (PVR or CD155). These receptors belong to the immunoglobulin superfamily (IgSF) whose extracellular regions consist of between two to five Ig domains. The N-terminal domain binds to the viral surface, and the carboxy-terminal domain comprises a transmembrane portion and a short cytoplasmic region. The sites of attachment on the viral surface are represented by canyons inside VP1, as demonstrated by studies of site-specific mutagenesis.[12] The change of even a single amino acid in the portion forming the canyon shows an altered pattern of affinity for the cellular receptor. The receptor ICAM-1 is expressed on the surface of many tissues, including the nasal epithelium that is the site of penetration of the rhinoviruses. The expression of ICAM-1 is controlled by cellular factors, such as cytokines.[13] The infection caused by rhinoviruses increases the expression of ICAM-1. The antibodies anti-ICAM-1 can

block the infection of HeLa cells caused by rhinoviruses. On the basis of these observations, a soluble form of ICAM-1, that can inhibit viral growth, in vitro, in high and specific way, was produced and purified.[14,15]

Table 1. Different picornavirus cellular receptor.

Virus:	Serotypes:	Receptor:	Description:
Human Rhinovirus	**91**	ICAM-1 (Intracellular Adhesion Molecule 1)	Immunoglobulin-like molecule; 5 domains
Human Rhinovirus	**10**	LDLR (Low Density Lipoprotein Receptor)	
Poliovirus	**3**	CD155	Immunoglobulin-like molecule; 3 domains
Coxsackie A	**3**	ICAM-1	
Echo	**2**	VLA-2	Integrin-like molecule
Echo	**6**	DAF (Decay Accelerating Factor)	-
EMCV	**1**	VCAM-1 (Vascular Cell Adhesion Molecule)	-

Binding of cellular receptors into the canyon induces the release of the pocket factor, if present, and induces conformational changes in the capsid that initiate the uncoating process. After attachment, the viral genomic RNA enters the cytoplasm of the host cell and serves as a template for viral protein translation and RNA replication. RNA replication occurs in small membranous vesicles. A single negative strand intermediate serves as a template for the production of several positive strand RNAs. New viral particles are formed by association of new positive strand RNA and viral structural protein, and are subsequently released by lysis of the host cell.

The minor group of rhinoviruses (12 serotypes) interacts with several members of the superfamily of low-density lipoproteins receptor (LDLR). These receptors are highly conserved between species and almost ubiquitously expressed. The LDLRs do not belong to the IgSF and interact with the outer part of the canyons without interfering with the stability of the capsid and uncoating. Therefore, the release of the RNA genome for minor group of HRVs is triggered

by the internal acidity of the endosome that causes conformational changes of the virion surface.

The time required for a complete cycle of replication, generally ranges from 5 to 12 hours and it depends on virus strain and on some factors like pH, temperature and host cell type.

The Picornavirus infectivity is not modified by organic solvents while it is sensitive to the pH variation and the stability to the different pH characterizes the different picornaviruses. Enteroviruses A, B, C and D, Cardiovirus, Parechovirus and Epatovirus can survive to pH = 1-3, therefore they are capable of overcoming the stomach acidity and to reach the intestine where they can multiply. Rhinoviruses or Aphthoviruses are fleeting at pH below 6 because they replicate in the nose and in the oropharynx (pH = 6.8-7.3) and do not require stability in an acid environment. Moreover, rhinoviruses are more thermostable than the other enteroviruses. It can survive up to 50 °C even if, in order to obtain an optimal propagation in cell cultures, an incubation temperature of 33 °C is required (table 2).

Table 2. Properties of Rhino- and other Entero- viruses.

	pH sensitivity	Optimum growth temperature	Detergent sensitivity	Serotypes	Transmission	Site of primary infection
Rhino viruses	labile to acid pH	33 degrees C (approx)		>100	aerosol	upper respiratory tract
Other Entero viruses	resistant to acid pH	37 degrees C (approx)	Resistant	72	oro-fecal	gut

References

1. Knipe D.M., Howley P.M., *Fields Virology*, 4th Ed. Vol.1, **2001**.
2. Rossmann M.G.; Arnold E.; Erickson J.W., Frankenberger E.A., Griffith J.P., Hecht H.J., Johnson J.E., Kamer G., Luo M., Mosser A.G., Rueckert R.R., Sherry, B., Vriend G., *Nature*, **1985**, *317*, 145.
3. Smith T.J., Kremer M.J., Luo M., Vriend G., Arnold E., Kamer G., Rossmann M.G., McKinlay M.A., Diana G.D., Otto M.J. *Science*, **1986**, *233*, 1286.
4. Kitamura N., Semler B.L., Rothberg P.G., Larsen G.R., Adler C.J., Dorner A.J., Emini E.A., Hanecak R., Lee J.J., van der W.S., Anderson C.W., Wimmer E., *Nature*, **1981**, *291*, 547.
5. Callahan P.L., Mizutani S., Colonno R.J., *Proc. Natl. Acad. Sci USA*, **1985**, *82*, 732.

6. Chua B.H., McMinn P.C., Lam S.K., Chua K.B., *J. Gen. Virol*. **2001**, *82*, 2629.
7. Kaku Y., Sarai A., Murakami Y., *J. Gen. Virol*., **2001**, *82*, 417.
8. Shih S.R., Ho M.S., Lin K.H., Wu S.L., Chen Y.T., Wu C.N., Lin T.Y., Chang L.Y., Tsao K.C., Ning H.C., Chang P.Y., Jung S.M., Hsueh C., Chang K.S., *Virus Res.*, **2000**, *68*, 127.
9. Lindberg A.M., Johansson S., Andersson A., *Virus Res*., **1999**, *59*, 75.
10. Yogo Y., Wimmer E., *Proc. natn. Acad. Sci. U.S.A.,* **1972**, *69*, 1877.
11. Racaniello V.R., Baltimore D., *Proc. Natl. Acad. Sci. USA*, **1981**, *78*, 4887.
12. Liao S., Racaniello V., *J. Virol.*, **1997**, *71*, 9770.
13. Xiao C., Tuthill T.J., Bator Kelly C.M., Challinor L.J., Chipman P.R., Killington R.A., Rowlands D.J., Craig A., Rossmann M.G., *J. Virol.*, **2004**, *78*, 10034.
14. Kolatkar P.R., Bella J., Olson N.H., Bator C.M., Baker T.S., Rossmann M.G., *EMBO J.*, **1999**, *18*, 6249.
15. Neubauer C., Frasel L., Kuechler, E., BLAAS, D., *Virol*., **1987**, *158*, 255.

3. PATHOGENESIS AND CLINICAL SIGNIFICANCE

The family Picornaviridae is one of the largest family of viral pathogens, implicated in an extensive range of clinical manifestations that affect humans as well animals. The syndromes caused by Picornaviruses range from mild upper respiratory tract infections such as common colds, fever and rush to more serious and life-threatening infections such as meningitis, poliomyelitis, myocarditis, pericarditis and neonatal sepsis, just to name a few.[1,2] Enterovirus infections in their most virulent forms, are physically debilitating and can be fatal for immuno-suppressed or elderly patients or in early childhood.

Among the picornavirus genera, Aphthovirus, Cardiovirus, Parechovirus, Hepatovirus and Enterovirus are the most clinically and economically significant.

3.1 Aphthovirus

The genus Aphthovirus consist of four species, foot-and-mouth disease virus (FMDV), bovine rhinitis A virus (BRAV), bovine rhinitis B virus (BRBV) and equine rhinitis A virus (ERAV).

The major member of this genus is FMDV which causes severe infection in cloven-hoofed animals, including domestic and wild bovids. The disease is characterized by acute fever, followed by the development of blisters chiefly in the mouth and on the feet.[3] Unfortunately, the inactivated vaccines presently available are not entirely effective due to the high variability of the virus. Vaccination blocks disease symptoms but does not always block transmission of the virus to other animals. FMDV is transmissible to humans, but it crosses the species barrier with difficulty[4] and the experience of the human infection is limited. Symptoms in humans are mostly mild and self limiting, mainly uncomfortable tingling blisters on the hands but also fever, sore throat, and blisters on the feet and in the mouth, including tongue.[4]

3.2 Cardiovirus.

The genus Cardiovirus consist of two species, Encephalomyocarditis virus (EMCV) and Theiloviruses (ThV). EMCV is represented by a single serotype with the same name. This virus is an important pathogen of rodents associate with myocarditis and encephalitis. EMCV infection in man has been rarely reported.

ThVs are classified into four types: Theiler's murine encephalomyelitis virus (TMEV), Thera virus (TRV) Vilyuisk human encephalomyelitis virus (VHEV), and Saffold virus (SAF-V) 1-8. The last two types infect humans causing gastroenteritis, respiratory diseases, myocarditis, acute and chronic encephalitis. There was no reliable evidence of cardiovirus infection in humans until 2007 when a virus distantly related to TMEV was identified in a fecal sample dating back to 1982 from an infant with fever of unknown origin.[5] The virus was named Saffold virus. In the following two years eight types of Saffold-like cardioviruses were found mainly in patients with gastroenteritis.[6-9] Serologic studies, in respiratory secretions from children with respiratory problems and in stools of both healthy and diarrheic children, indicate that asymptomatic cardiovirus infection is highly prevalent in infants below two years old.[6]

3.3 Parechovirus

The Parechovirus genus is comprised of two species, Human parechoviruses (HPeV) and Ljungan virus. People of all ages are infected by HPeVs, although these infections occur usually in young children, with a seroprevalence of 88% in children of two years.[10] Recent reports show that epidemiological and clinical features of HPeVs are similar to human EVs. Commonly, HPeVs cause mild gastrointestinal or respiratory illness, but it is occasionally implicated in severe disease conditions, such as flaccid paralysis, myocarditis and encephalitis.[11] Probably, the associated spectrum of diseases is underestimated, since many serotypes of HPeVs have been only recently identified.

3.4 Hepatovirus

The genus Hepatovirus consists of a single species, Hepatitis A virus (HAV). HAV causes hepatitis A in humans and primates.[12] The infection is usually transmitted by ingestion of contaminated food or water.[13-15] The incubation period is 10-50 days and fever and jaundice are the main symptoms of the infection. In developing countries, the diffusion of HAV infection is high among children. However, only 10% of infected children are symptomatic, whereas the severity of the disease increases with age.[16] HAV, normally, causes an acute self-limiting disease that does not progress to a chronic phase, and

manifestations of the disease are generally restricted to the liver. Although a few cases of permanent liver damage have been reported, 99% of the cases recover completely and the fatalities are less than 0.1%. An effective vaccine is now available for prevention of HAV infection.

3.5. Enteroviruses

Enteroviruses (EVs) are the best known human and animal pathogens into the picornavirus family.

Clinical manifestations may vary diversely with one serotype, while multiple serotypes can present identical symptoms and may mimic bacterial infections. Most enterovirus infections cause benign, self-limiting disease. However, they can also produce severe and sometimes fatal illnesses such as meningitis, encephalitis, myocarditis, neonatal sepsis and poliomyelitis.[17,18]

This genus includes polioviruses (PV 1, 2, 3), coxsackieviruses (CV-A 1-22, 24, CV-B 1-6), echoviruses (E 1-21, 24-33), numbered enteroviruses (EV 67-71, 73) rhinoviruses (HRV-A, B, C), and hand foot-and-mouth disease virus (HFMDV).

The high infectivity of HRVs is mainly due to direct contact between infected and susceptible host, particularly in people who do not have specific immnunoglobuline A (IgA) in nasal secretions. These viruses infect the host through the nose, mouth or eyes and they reache the pharynx through mucous ciliary action. The HRVs are unable to replicate in the gastrointestinal tract because they are unstable to acidic pH. Moreover, they preferentially grow at a temperature of 33 °C into the nasal cavity.

The other EVs are able to replicate in both oropharynx and intestine. The infection usually causes mild manifestations (fever and malaise) into the initial site of multiplication and rapid healing. However, the virus can spread through the lymph circulation and blood circulation to target organs giving clinical situations debilitating and potentially fatal (figure 6).

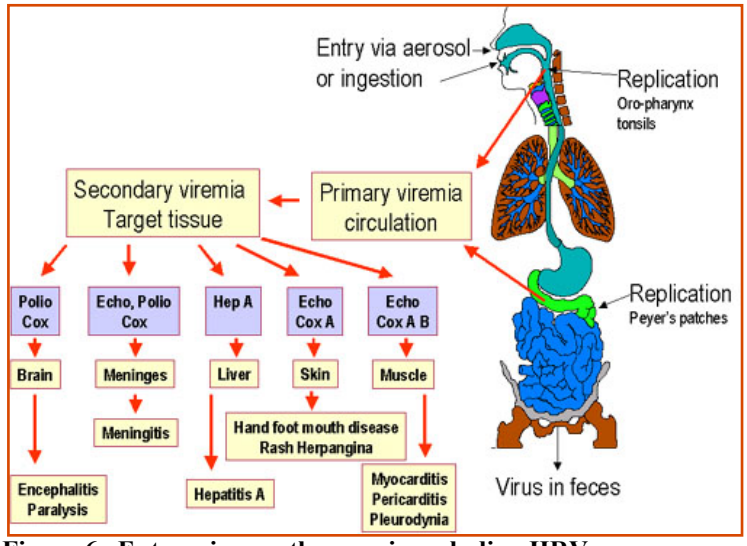


Figure 6. Enterovirus pathogenesis excluding HRVs.
The viruses infect the host by the respiratory tract or food, they multiply in the oropharynx and intestine (primary viremia), and can spread through lymph circulation and blood circulation generating infection in target organs such as the meninges, skin, myocardium, etc. (Secondary viremia).

3.5.1. Poliovirus (PV 1, 2, 3).

Polioviruses are highly contagious viruses that are the etiologic agents of poliomyelitis, also known as infantile paralysis. There are three serotypes of polioviruses, PV 1, PV 2 and PV 3. Most disease results from type 1 poliovirus while types 2 and 3 are less neurovirulent. Polioviruses are spread through fecal-oral or oral transmission, and viral replication occurs in the gastrointestinal tract. Initial clinical symptoms may include fever, fatigue, headache, vomiting, constipation (or less commonly diarrhea). In a minority of patients the virus invades the central nervous system and destroys the motor neurons leading to temporary or permanent paralysis. In rare cases, paralytic poliomyelitis leads to death for respiratory arrest. Following the introduction of two effective vaccines, poliomyelitis has been eradicated from most of the world.[19]

3.5.2. Coxsackievirus A and B (CVA-B).

Group A and B of Coxsackieviruses are the principal cause of many clinical event including vescicular pharyngitis called

"erpangina", a severe feverish pharyngitis accompanied by vomiting and ulcerative lesions of the soft palate and uvula, and foot and mouth disease, a rash vesicular type.[19,20] Aseptic meningitis is a more severe syndrome, characterized by headache and irritation of the meninges (stiff-neck) accompanied by skin rash and petechiae. The majority of children with Coxsackievirus infections completely resolves the symptoms in about 10-12 days. The development of autoimmune diabetes mellitus has been recently associated with alterations of the pancreatic β cells caused by CV infections.[21]

3.5.3. Echovirus (E)

Echoviruses (Enteric Cytopathic Human Orphan viruses) are the principal causes of rash, pharyngitis and acute feverish diseases in infants and children. Moreover, they are the most common cause of aseptic meningitis. Infection of an infant may cause severe systemic diseases, and is associated with high infant mortality rates.[22] In this population, death usually results from overwhelming liver failure or myocarditis rather than infection of the central nervous system. The fecal-oral route is the predominant mode of transmission, although transmission sometimes occurs via respiration of oral secretions such as saliva. An indirect transmission occurs through water, food, and objects.

3.5.4. Enterovirus 68-71, 73 (EV)

EV 68-71 and EV73 have been associated with vesicular rash, meningo-encephalitis and respiratory infections such as pneumonia and bronchiolitis.[23,24] The EV70 apparently does not replicate in the intestinal tract but it is responsible for an eye infection commonly called "acute hemorrhagic conjunctivitis" (AHC), characterized by swelling, irritation, conjunctival congestion and subconjunctival hemorrhage. The acute hemorrhagic conjunctivitis presents an incubation period of 24 hours and resolution in 1-2 weeks.[25] In some cases, the infection can lead to a neurological disease indistinguishable from acute poliomyelitis, suggesting that the tissue tropism of EV70 also includes the central nervous system.[26] After the eradication of poliovirus from developed countries, EV71 has emerged as major cause of neurologic threat in the world. Although EV71 usually causes hand, foot and mouth disease, a mild

exanthematous infection occurring mainly in young children, it is also associated with acute neurological diseases including aseptic meningitis, acute flaccid paralysis and even fatal neurogenic pulmonary edema.[1] In the last decades a significant increase in epidemic activity of EV71 has been observed and severe outbreaks have been reported in various parts of the world, particularly in the Asia-Pacific region.[27-30]

3.5.5. Rhinovirus (HRV)

Because of high similarity in genome sequence and genome organization, human rhinoviruses were only recently added to the genus Enterovirus. The rhinoviruses are commonly isolated from respiratory secretions of people with acute colds. In fact, their name refers the privileged place of infection, the nasal mucosa.

The isolation of rhinoviruses was performed by Pelon and Price inoculating nasopharyngeal secretions of patients with colds in tissue cultures of kidney of rhesus monkey.[31,32] The first strain to be isolated was named JH 2060, this strain was similar to echovirus type 28. It was subsequently classified as rhinovirus type 1. The isolation of other serotypes and the classification of rhinoviruses were facilitated by the reduced temperature of cells incubation at 33 °C and the use of culture media at pH about 7.00.[33]

The genome was characterized in 1984[34], and the introduction of molecular biology techniques, such as RT-PCR, facilitated the identification of other serotypes.[35,36]

HRVs are the leading agents of upper respiratory tract infections in both adults and children. Although HRV-induced respiratory illnesses are usually mild and self-limiting, their diffusion and recurrent nature can explain the high medical and socio-economic impact in term of healthcare and lost productivity. HRVs are also responsible for respiratory tract complications such as sinusitis and otitis media, and severe lower respiratory tract illnesses in infants, holder adults and immune-compromised hosts. In patients with chronic respiratory disorders such as asthma and chronic obstructive pulmonary disease (COPD), HRVs are the major factors in the induction of exacerbations.[37,38] In adults, COPD exacerbations are predicted by the World Health Organization to be the world's third leading cause of death by the year 2030.

References

1. Pallansch M.A., Roos R.P., In *Virology*; Fields B.N., Knipe D.M., (Eds). 4th ed.; Lippnicott Williams and Wilkins: Philadelphia, PA, **2001**; p. 723.
2. Ehrenfeld, E., Domingo, E., Roos, R.P. (Eds.), **2010**. The Picornaviruses. ASM Press, Washington, p. 493.
3. Health Protection Report, *HPR-CDR Weekly*, **2011**, *11*, 1-2.
4. Bauer K., *Arch. Virol.* [suppl.], **1997**, *13*, 95.
5. Jones M.S., Lukashov V.V., Ganac R.D., Schnurr D.P., *J. Clinic. Microbiol.*, **2007**, *45*, 2144.
6. Zoll J., Erkens Hulshof S.E., Lanke K., Lunel F.V., Melchers W.J.G., Van der Ven E.S., Roivainen M.R., Galama J.M.D., Kuppeveld F.J.M., *PLoS Pathog*, **2009**, *5*, e1000416.
7. Abed Y., Boivin G.. *Emerg. Infecti. Dis.*, **2008**, *14*, 834.
8. Blinkova O., Kapoor A., Victoria J., Jones M., Wolfe N., Naeem A., Shaukat S., Sharuf S., Alam M.M., Angez M., Zaidi S., Delwart E.L., *J. Virol*., **2009**, *83*, 4631.
9. Drexler J.F., Luna L.K., Stocker A. Lameida P.S., Ribeiro T.C., Petersen N., *Emerging Infectious Diseases,* **2008**, *14*, 1398.
10. Abed Y., Wolf D., Dagan R., Boivin G., *J. Clinic. Microbiol.,* **2007**, *45*, 2037.
11. Wolthers, K.C., Benschop, K.S., Schinkel, J., Molenkamp, R., Bergevoet, R.M., Spijkerman, I.J., Kraakman, H.C., Pajkrt, D., *Clin. Infect. Dis*., **2008**, *47*, 358.
12. Costa-Mattioli M., Ferre V., Casane D., Perez-Bercoff R., Imbert-Marcille B.M., Andre E.C., Bressollette-Bodin C., Billaudel S., Cristina J., *Virol.*, **2003**, *311*, 51.
13. Lee C.S., Lee J.H., Kwon K.S., *J. Infect Dis.*, **2008**, *61*, 239.
14. Pontrelli G., Boccia D., Di Renzi M., Massari M., Giugliano F., Celentano L.P., Taffon S., Genovese D., Di Pasquale S., Scalise F., Rapicetta M., Croci L., Salmaso S., *Epidemiol. Infect.*, **2007**, *136*, 1027.
15. Sattar S.A., Jason T., Bidawid S., Farber J., *J. Infect. Dis.*, **2000**, *11*, 159.
16. Brundage S.C., Fitzpatrick A.N., *Am., Fam., Physician*, **2006**, *73*, 2162.
17. Chapman N.M., Kim K.S., *Curr. Top. Microbiol.,Immunol.*, **2008**, *323*, 275.
18. Huber S., *Curr. Top. Microbiol. Immunol.*, **2008**, *323*, 199.
19. Shin-Ru S., Shu-Jen C., Gholam Hossein H., Hsing-Jang L., Chen-Tso T., Kak-Sha S., *Med. Res. Rev.,* **2004**, *24*, 449.
20. Chang L.Y.,Lin T.Y., Hsu K.H., Huang Y.C., Lin K.L., Hsueh C. Shih S.R. Ning H.C., Hwang M.S., Wang H.S., Lee C.Y., *Lancet*, **1999**, *354*, 1682.
21. Horwitz M.S., Bradley L.M, Harbertson J., Krahl T., Lee J., Sarvennick N., *Nature Med.*, **1998**, *4*, 781.
22. Ajmal D., Suhail A., Widad A.N., *J. Virol.*, **2010**, *7*, 236.
23. Chang J.Y., King C.C., Hsu K.H., Ning H.C., Tsao K.C., Li C.C., Huang Y.C. Shih S.R., Chiou S.T., Chen P.Y., Chang H.J., Lin T.Y.. *Pediatrics*, **2002**, *109* (6), E88.
24. Oberste M.S., Maher K., Schnurr D., Flemister M.R., Lovchik J.C., Peters H., Sessions W., Kirk C., Chatterjee N., Fuller S., Hanauer J.M., and Mark A.. *J. Gen. Virol.*, **2004**, *85*, 2577.

25. Mirkovic R.R., Kono R., Yin-Murphy M., Soher R., Schmidt N.J., Melnik..J.L., *Bull World Health Organ*, **1973**, *49* (4), 341.
26. Schmidt N.J., Lennette E.H., Ho H.H.. *J. Infect. Dis*., **1974**, *129*, 304.
27. Ho M., Chen E. R., Hsu K. H., Twu S. J., Chen K. T., Tsai S. F., Wang J. R., Shih S., *R. N. Eng. J. Med*. **1999**, *341*, 929.
28. AbuBakar S., Chee H. Y., Al-Kobaisi M. F., Xiaoshan J., Chua K. B., Lam S. K., *Virus Res*. **1999**, *61*, 1.
29. McMinn P.C., Lindsay K., Perera D., Chan H. M., Chan K. P., Cardosa M. J., *J. Virol*. **2001**, *75*, 7732.
30. McMinn P. C., Stratov I., Nagarajan L., Davis S., *Clin. Infect. Dis*. **2001**, *32*, 236.
31. Pelon W., Mogabgab W.J., Phillips I.A., Pierce W.E.. *Proc Soc Exp Biol Med*., **1957**, *94* (2), 262.
32. Price W.H., *Proc. Natl. Acad. Sci U.S.A*. **1956**, *42* (12), 892.
33. Tyrrell D. A., Parsons R., *Lancet*, **1960**, *1*, 239.
34. Stanway G., Hughes P. J., Mountford R. C., Minor P. D., Almond J.W., *Nucleic Acids Research*, **1984** *12*(20), 7859.
35. Johnston S. L., *Am. J. Resp. And Critical Care Med*., **1998**, *152* (4 Pt 2), S46.
36. Makela M.J., Puhakka T., Ruuskanen O., Leinonen M., Saikku P., Kimpimaki M., Blomqvist S., Hyypia T., Arstila P., *J. Clinic. Microbiol*., **1998**, *36* (2), 539.
37. Hershenson M.B., Johnston S.L., *Am. J. Respir. Crit. Care Med.*, **2006**, *174*, 1284.
38. Mallia P., Contoli M., Caramori G., Pandit A., Johnston S.L., Papi A., *Curr. Pharm. Des.*, **2007**, *13*, 73.

4. SMALL MOLECULES INHIBITORS OF PICORNAVIRUS REPLICATION

The spread and the medical and economic importance of infections caused by picornaviruses have stimulated the research for effective agents in the prevention or treatment of these infections. Vaccination is effective for the prevention of polioviruses, hepatitis A, and foot and mouth virus infections, but this approach is impractical for other enteroviruses owing to the multiplicity of serotypes (more than 100 HRV serotypes) and the high mutation rate during virus replication.

For these reasons, antiviral chemotherapy remains the only option. Moreover, a drug for the treatment of diseases caused by picornaviruses should possess not only a high power and low toxicity, but also a broad spectrum of action.

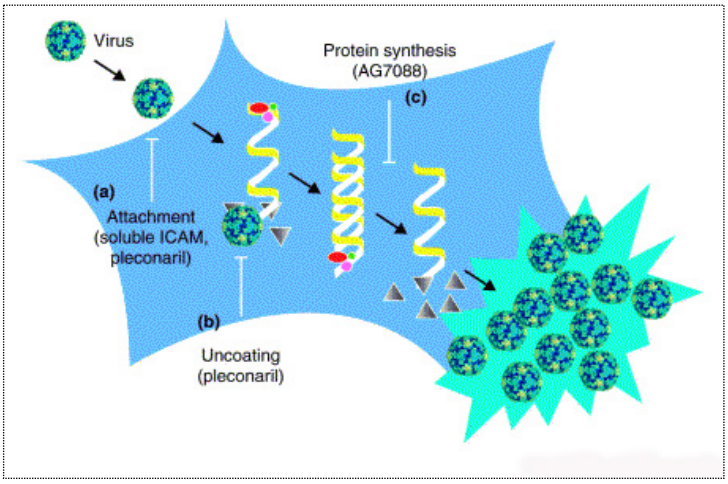


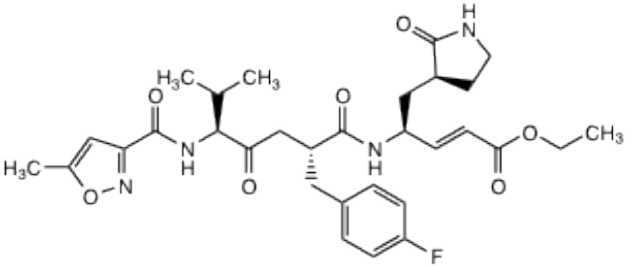
Figure 7. Objectives of anti-picornavirus therapy.
Agents that prevent (**a**) viral anchorage to the cellular receptor of the host cell, (**b**) inhibitors of viral uncoating (**c**) inhibitors of viral protein synthesis.

Although extensive efforts have been devoted to the search for effective agents, to date no drug has been approved by the FDA for clinical use, and the patient care remains symptomatic. Each step during the life cycle of Picornavirus has been considered a potential target for antiviral compounds, including virus adsorption, uncoating, RNA translation, polyprotein cleavage, RNA replication, and particle assembly (figure 7). However, only the inhibition of capsid functions

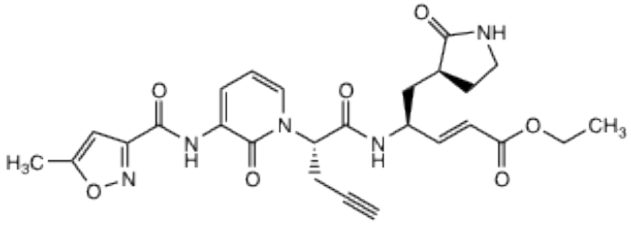
and the interruption of viral protein synthesis resulted in antiviral agents with demonstrated clinical efficacy. Since it is well recognized that the structures of viral capsid and 3C protease are relatively conserved among different serotypes, molecules acting on each of these targets could be promising anti-picornavirus agents with a broad-spectrum of activity.[1]

4.1 3C protease inhibitors

Picornaviruses translate their genomic information into a polyprotein that is co-translationally processed by virus-encoded proteases $2A^{pro}$, $3C^{pro}/3CD^{pro}$. The generation of mature structural protein and enzymes is essential for the viral replication. The viral proteases, in particular 3C protease, are attractive targets for the development of anti-picornavirus agents due to the highly conserved residues into the catalytic site and the poor sequence similarities with cellular proteases.



AG 7088 (Rupintrivir)



AG 7404 (Compound 1)

Figure 8. Structure of 3C protease inhibitors.

During the last years, a variety of peptidic and nonpeptidic protease inhibitors (figure 8) have been discovered and developed. Ruprintrivir, developed by Pfizer, is a potent and irreversible peptidomimetic inhibitors of 3C protease.[2-5] This compounds showed in vitro low cytotoxicity and a wide range of anti-EV activity. For these reasons, Ruprintivir was progressed to clinical trials.

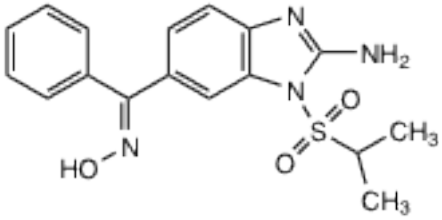
Administered intranasally for its poor oral bioavailability, Rupintrivir was able to reduce severity of illness and viral load in HRV challenge trials. However, this compound was terminated for clinical development (phase II) due to its unsatisfying results in naturally infected patients.

In order to identify orally bioavailable analogues of Rupintrivir, a series of non peptidic inhibitors of 3C protease were designed. Compound I exhibited a potent and broad spectrum anti-EV activity in vitro. In phase I clinical studies, it was well tolerated by healthy volunteers.[6] However, no further clinical development of this compound was planned.

4.2 3A protein inhibitors

Nonstructural protein 3A is a small, hydrophobic protein highly conserved among enteroviruses.[7] This indispensable protein is implicated in many functions including RNA replication,[8] cellular tropism and pathogenicity.[9]

Enviroxime (figure 9) is a benzimidazole derivative with a broad spectrum of anti-EV activity, developed by Lilly Pharmaceuticals.[10,11] It probably inhibits the plus-strand RNA synthesis by targeting 3A protein. Despite its potency in vitro, Enviroxime resulted in modest or no benefit in clinical studies. Poor bioavailability and gastrointestinal side-effects resulted in discontinuance of the program.



Enviroxime

Figure 9. Structure of Enviroxime

4.3 Compounds that bind to the capsid (capsid binders)

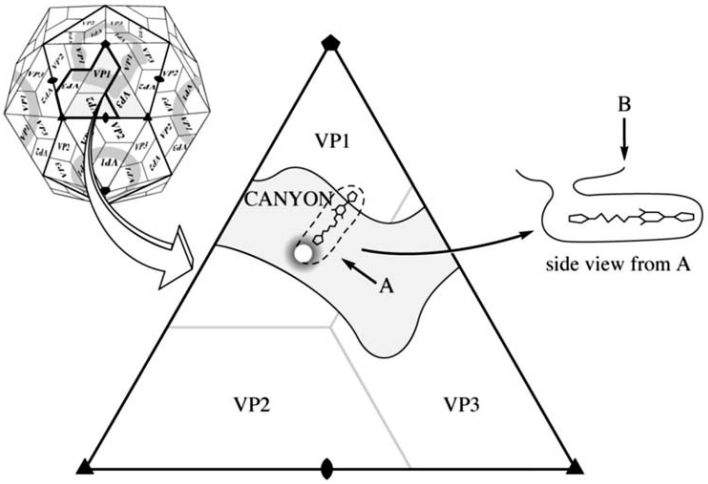


Figure 10. Diagrammatic view of picornaviruses with enlargement of one icosahedral asymmetric unit showing the outline of the canyon and the entrance to the antiviral-binding pocket.

As previously mentioned, capsid binding molecules are able to inhibit virus infectivity blocking uncoating and/or the attachment of the virus to the host cellular receptors. The binding site for these compounds is an hydrophobic pocket placed inside VP1 under the canyon floor (figure 10). Uncoating of a viral particle requires a certain degree of capsid flexibility. The insertion of a capsid binder in this pocket leads to an increase of viral particle stability, preventing conformational changes that are essential for the uncoating. The bottom of the canyon is also the most important binding site for cellular receptors, such as intercellular adhesion molecule-1 (ICAM-1) recognized by the major group of rhinoviruses, the poliovirus receptor (PVR or CD155) by polioviruses, the coxsackie-adenovirus receptor (CAR) by Coxsackie B viruses and vascular cell adhesion molecule-1 (VCAM-1) by encephalomyocarditis virus (EMCV). The binding of these inhibitors in the VP1 pocket can induce conformational changes in the bottom of the canyon preventing virus adsorption to the host cell (figure 11).

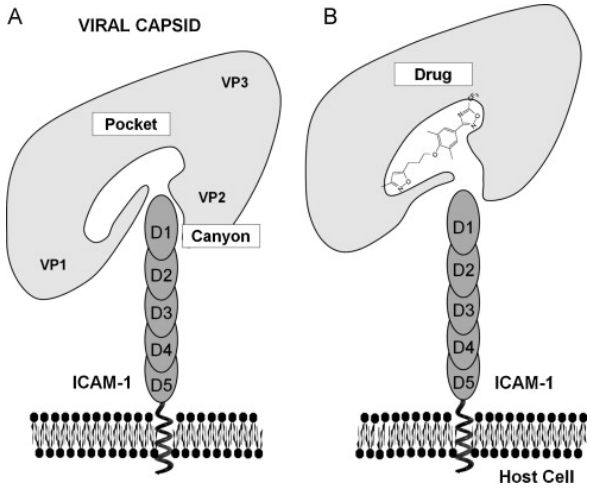


Figure 11. Mechanism of action of capsid binders. Schematic representation of the interaction between ICAM-1 and major group rhinoviruses. Left (A): ICAM-1 binds into the canyon surrounding each fivefold axis, inducing conformational changes that eventually lead to uncoating of the virus and release of the viral RNA. Right (B): Binding of a "capsid binder" into the hydrophobic pocket underneath the canyon floor. This binding event induces conformational changes, thereby (i) increasing the rigidity of the virion (preventing uncoating and subsequent release of viral RNA) and at the same time (ii) decreasing the ability of the virion to interact with its receptor.

4.3.1 WIN compounds.

The WIN series of capsid-binding compounds, developed by Sterling Winthrop, played a leading role in the development of antiviral agents effective against human EV infections (figure 12). Disoxaril, also known as Win 51711, was the first compound of this series to reach clinical trials. *In vitro*, Disoxaril showed high inhibitory potency against many enteroviruses including rhinoviruses.[12-17] When administered orally to mice, this molecule effectively prevented the paralysis and death of mice infected with poliovirus-2 and echovirus-9. However clinical studies were interrupted because of the appearance of crystallurea in phase I clinical studies.

An analogue, Win 54954, was assessed (Phase II) against two rhinovirus (HRV 23 and HRV 39) and Coxsackievirus A21.[17,18] This compound was able to reduce *in vivo* only the viral titer and the severity of the infection caused by Coxsackievirus A21 but it did not show efficacy against rhinoviruses. Win 54954 had also a very short half-life, presumably due to instability of the oxazoline ring in acidic

condition, and it was not further studied because of the onset of side effects (redness, skin rashes and reversible hepatitis).

Afterward, the oxazoline ring was substituted in order to obtain metabolically stable compounds. These studies led to analogs containing a 2-methyltetrazole ring.[19] The Win 61605 was the most promising compound of the series. Unfortunately, it caused hepatotoxicity after oral administration to dogs. Its toxicity is presumably due to the products of the tetrazole ring metabolism and led to the suspension of the compound evaluation *in vivo*.

Bioisosteric replacement of the 2-methyltetrazole ring with a 5-methyloxadiazoline ring, led to Win 61893 that was rapidly metabolized and induced reversible hepatitis.[20]

Replacement of the methyl group at the oxadiazole ring with a trifluoromethyl group resulted in Win 63843 (pleconaril).[20] This new analogue showed greater metabolic stability and a broader anti-EV spectrum than its precursors.[20,21,22] In a phase III clinical trial, pleconaril was able to reduce the duration and the severity of common cold symptoms.[23] However, the FDA did not approve pleconaril for marketing due to its safety profile. [24]

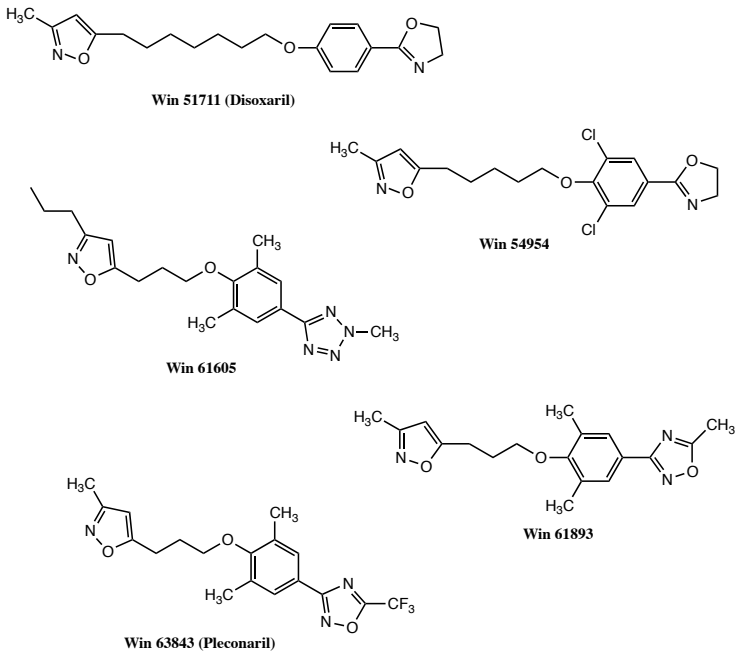


Figure 12. Structures of WIN compounds.

4.3.2 Pirodavir and related compounds

Pirodavir (R 77975) and its predecessor R 61837 (figure 13) are two pyridazine analogues developed by the Janssen Foudation.[25-27] Pirodavir was 500-times more potent *in vitro* than R 61837 and it inhibits approximately 80% of 100 HRV serotypes at concentration of 0.1 μg/mL or less. Its administration as a nasal spray, six times a day for 5 days, induces a significant reduction of the spread of the virus, but without a real clinical benefit. The lack of clinical efficacy of Pirodavir could be partially due to its poor water solubility that makes difficult its administration in an aqueous formulation compatible with secretions present in the airways. Moreover, the ester function rapidly undergoes hydrolysis to the corresponding inactive acid.

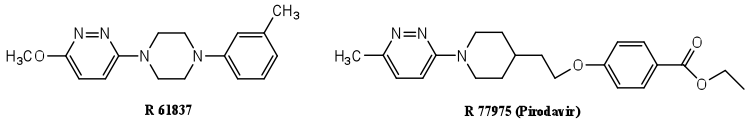


Figure 13. Structure of Pirodavir and R 61837.

Although, R 61837 is less active than Pirodavir against several rhinovirus serotypes, it was prophylactically effective in preventing colds in human volunteers infected with HRV-9.[27]

To circumvent the problem of the metabolic instability of ester function, a series of oxime ether analogs of pirodavir was synthesized at Biota Pharmaceuticals (figure 14). BTA 39 and BTA 188 showed potent activity against 56 HRV serotypes with IC_{50} ranging from 0.5 to 6.7 nM.[28]

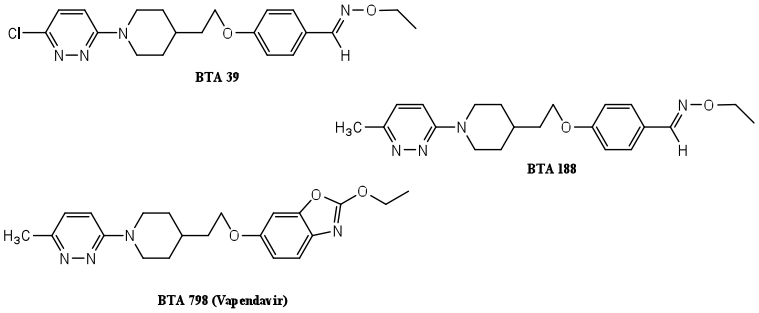


Figure 14. Structure of BTA series.

Afterwards, the benzaldehyde oxime ether moiety was replaced by a 2-ethoxybenzoxazole. BTA798 emerged as the most interesting compound within this series.[29] This analogue was a potent, orally bioavailable, broad spectrum inhibitor of HRVs and other EVs. BTA798 is currently undergoing Phase II clinical trials for the treatment of HRV infections in asthmatic patients.

4.4. Flavanoids, flavonoids and analogues.

Flavonoids and flavanoids are important groups of oxygenated heterocycles widespread in the plant kingdom and daily consumed in the human diet. They contribute to the colour of many flowers and vegetables, and are involved in defensive system of plants. In addition, several medicinal plants and herbs used in folk medicine contain flavanoids and flavonoids.[30] Natural compounds, together with a number of synthetic analogues, are responsible for a great variety of biological and pharmacological activities, including antipicornavirus properties.[30-32]

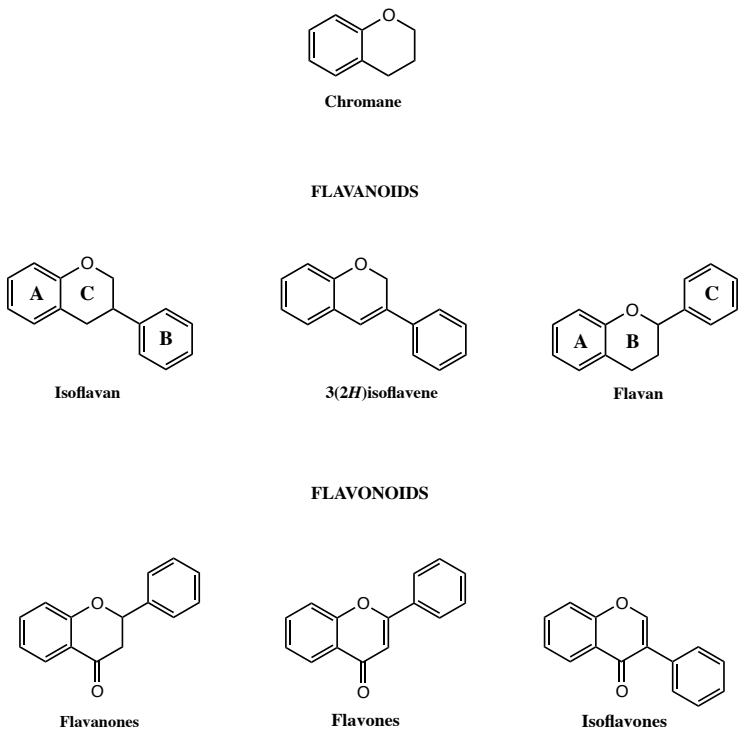


Figure 15. Structure of flavanoids and flavonoids

The structure of these compounds is derived from a heterocyclic hydrocarbon, chromane, by substitution in position 2 or 3 with a phenyl group forming flavan and isoflavan, respectively. Then, the basic structure consists of 15 carbon atoms arranged in three rings (C6-C3-C6), which are labelled A, B, and C ring. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds whitin a class differ in the pattern of substitution of the A and B rings (figure 15). In addition, chalcones, homoisoflavones, and 2-styrylchromones are classified as flavanoids. Chalcones are bicyclic compounds with C ring opened while, in homoisoflavonoids and styrylchromones, the B ring is not directly linked to the heterocycle (figure 16).

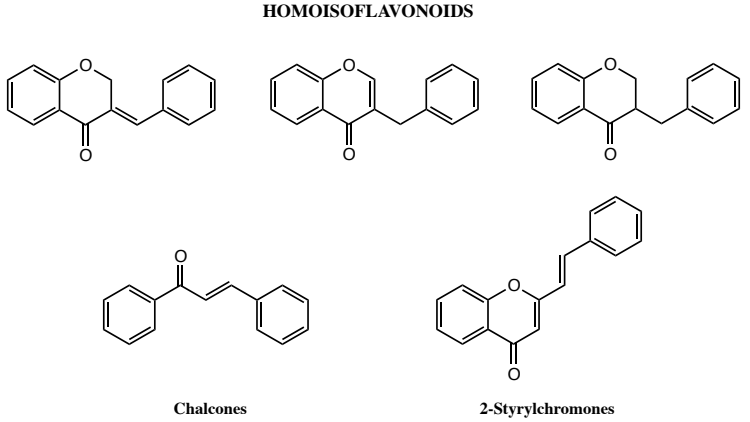
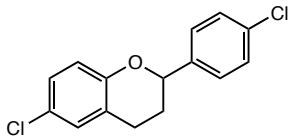


Figure 16. Structure of chalcones, homoisoflavones, and 2-styrylchromones

4',6-Dichloroflavano (**BW683C**), was the first synthetic flavanoids showing a potent antirhinovirus activity.[32] This compound inhibited the prevalent serotypes of HRV (1A, 1B, 2, 15, 29 and 31) with IC_{50} values ranging from 0.007 to 0.17 μM. Studies on the mechanism of action of **BW683C** suggested that it blocks viral replication inhibiting a stage immediately following the entrance of virus into the host cell.[32]



4', 6 dichloroflavano (BW683C)

Unfortunately, the clinical trial produced disappointing results. In fact 4',6 dichloroflavan was unable to prevent the HRV infection after oral or intranasal administration.[33]

Among the different classes of flavonoids and flavanoids, we studied the antipicornavirus activity of flavans, isoflavans, isoflavenes, flavones, flavanones, homoisoflavones and 2-styrylchromones. Several synthetic flavanoids and flavonoids exhibited a broad antipicornavirus spectrum, although flavonoids were found less potent then flavanoids.[34-40]

Among flavanoids, 3(2*H*)-isoflavenes generally were more potent against EVs, while isoflavans against HRVs and hepatitis A virus. Among flavans, 4',6-dicyanoflavan was selectively active against HRVs, while 6-chloro-4'-cyanoflavan and 4'-chloro-6-cyanoflavan showed a broad spectrum of antipicornavirus activity in the micro or submicromolar range.[34-38]

Studies on the mechanism of action of selected flavanoids on HRV and PV infections suggested that they specifically interfered with some early events of viral replication. In the case of HRV, 4',6-dicyanoflavan delayed the uncoating kinetic of neutral-red encapsidated virus and prevented mild acid or thermal inactivation of virus infectivity, suggesting an action at the uncoating level due to a stabilising effect on virus capsid conformation.[41] In the case of poliovirus, 3(2*H*)-isoflavene was found to exert its action during the uncoating step.[42] Analysis of mutations conferring resistance provides strong evidence that 3(2*H*)-isoflavene inserts into the hydrophobic pocket inside capsid VP1, increasing the stability of the viral particle and rendering the virus more resistant to uncoating.[43]

Also flavonoids presented a wide spectrum of anti-picornavirus activity. While flavanones were generally less effective than flavones, 3-methoxy or 3-hydroxyflavones and their esters with natural occurring hydroxybenzoic and hydroxycinnamic acids were more active than 3-unsubstituted flavones.[39,40]

Afterwards, we synthesized analogues of two small classes of natural flavonoids: homoisoflavonoids (3-benzylidenchroman-4-ones, 3-benzylchroman-4-ones, and 3-benzyl-4-chromanones) and 2-styrylchromones.[44-46]

Homoisoflavonoids were weakly effective against poliovirus 2, while they exhibited a variable degree of activity against HRV 1B and 14, selected as representative serotypes for groups B and A of HRVs, respectively. The two groups of HRVs were identified by a different susceptibility of all the serotypes to a panel of antirhinovirus

compounds. Group B contains twice as many serotypes as group A, and accounts for five times as many colds as group A.[47,48] HRV 1B was much more susceptible than HRV14 to the action of homoisoflavonoids, and the presence of one or more chlorine atoms increased the antiviral effect. Only the activity of 3-benzyl-4',5,7-trichlorochroman-4-one and of chloro-substituted 3-benzyl-4-chromanones extended to both HRV serotypes tested and to poliovirus.[44]

2-Styrylchromones were synthesized as vinylogues of flavones, in order to assess whether the replacement of 2-phenyl substituent on the chromone ring by the 2-styryl would affect the antiviral properties. In addition, the influence of the substituent in position 3 was also evaluated for this class of flavonoids, since the introduction of a 3-hydroxy or 3-methoxy group was previously found to positively affect the antipicornavirus potency of flavones.[31,39] Antiviral results indicate that 2-styrylchromones can be considered a new class of antirhinovirus flavonoids with activity against both rhinovirus groups A and B.[45,46] As expected, the introduction of a 3-hydroxy or a 3-methoxy group improves antirhinovirus activity.[46]

Flavans, isoflavans, and 3-benzyl-4-chromanones are characterised by the presence of a chiral centre in position 2 or 3 of the chromane ring. Since they were initially synthesized and tested as racemates, the mixtures of selected compounds were successfully resolved by chiral HPLC in order to define the anti-rhinovirus activity of each stereoisomer. The results of the antiviral tests showed that the difference of activity between the two antipodes is variable and depend on the kind of virus tested.[49-52]

References

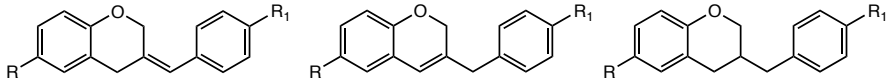
1. De Palma A.M., Vliegen I., De Clercq E., Neyts J., *Med. Res. Rev*. **2008**, *28*, 823.
2. Matthews, D.A., Dragovich, P.S., Webber S.E., Fuhrman S.A., Patick A.K., Zalman L.S. Hendrickson T.F., Love R.A., Prins T.J., Marakovits J.T., Zhou R., Tikhe J., Ford C.E., Meador J.W., Ferre R.A., Brown E.L., Binford S.L., Brothers M.A., DeLisle, D.M., Worland S.T., *Proc. Natl. Acad. Sci U.S.A.*, **1999**, *96*, 11000.
3. Binford S.L., Maldonade F., Brothers M.A., Weady P.T., Zalman L.S., Meador III J.W., Matthews D.A., Patick A.K., *Antimicrob. Agents Chemother*. **2005**, *49*, 619.
4. Binford S.L., Weady P.T., Maldonado F., Brothers M.A., Matthews D.A., Patick A.K., *Antimicrob. Agents Chemother*., **2007**, *51*, 4366.

5. Patick A.K., Binford S.L., Brothers M.A., Jackson R.L., Ford C.E., Diem M.D., Maldonado F., Dragovich P.S., Zhou R., Prins T.J., Fuhrman S.A., Meador J.W., Zalman L.S., Matthews D.A., Worland S.T., *Antimicrob. Agents Chemothe*r., **1999**, *43*, 2444.
6. Patick A.K., Brothers M.A., Maldonado F., Binford S., Maldonado O., Fuhrman S., Petersen A., Smith G.J. III, Zalman L.S., Burns-Naas L.A., Tran J.Q.. *Antimicrob Agents Chemother*, **2005**, *49*, 2267.
7. Wessels E., Duijsings D., Lanke K.H., van Dooren S.H., Jackson C.L., Melchers W.J., et al. *J. Virol.* **2006**, *80*, 11852.
8. Fujita K., Krishnakumar S.S., Franco D., Paul A.V., London E., Wimmer E.. *Biochemistry,* **2007**, *46*, 5185.
9. Pacheco J.M., Henry T.M., O'Donnell V.K., Gregory J.B., Mason P.W. *J. Virol.* **2003**, *77*, 13017.
10. Heinz B.A., Vance L.M., *J. Virol*., **1995**, *69*, 4189.
11. Heinz B.A., Vance L.M., *J. Virol*., **1996**, *70*, 4854.
12. Diana G.D., McKinlay M.A., Otto M.J., Akullian V., Oglesby C.J. *J. Med. Chem.*, **1985**, *28*, 1906.
13. Fox M.P., Otto M.J., Shave W.J., McKinlay M.A.. *Antim. Agents Chemother.*, **1986**, *30*, 110.
14. Diana G.D., Otto M.J., Treasurywala A.M., McKinlay M.A., Oglesby R.C., Maliski E.G., Rossmann M.G., Smith T.J., *J. Med. Chem*., **1988**, *31*, 540.
15. Diana G.D., Cutcliffe D., Oglesby R.C., Otto M.J., Mallamo J.P., Akullian V., McKinlay M.A.. *J. Med. Chem*., **1989**, *32*, 450.
16. Diana G.D., Cutcliffe D., Volkots D.L., Mallamo J.P., Bailey T.R., Vescio N., Oglesby R.C., Nitz T.J., Wetzel J., Giranda V., Pevear D.C., Dutko F.J.. *J. Med. Chem*., **1993**, *36*, 3240.
17. Woods M.G., Diana G.D., Rogge M.C., Otto M.J., Dutko F.J., McKinlay M.A.. *Antim. Agents Chemother*., **1989**, *33*, 2069.
18. Bailey T.R., Diana G.D., Kowalczyk P.J., Akullian V., Eissenstat M.A., Cutcliffe D., Mallamo J.P., Carabateas P.M., Pevear D.C.. *J. Med. Chem*., **1992**, *35*, 4628.
19. Diana G.D., Volkots D.L., Nitz T.J., Bailey T.R., Long M.A., Vescio N., Aldous S., Pevear D.C., Dutko F.J., *J. Med. Chem*., **1994**; *37*, 2421.
20. Diana G.D., Rudewicz P., Pevear D.C., Nitz T.J., Aldous S.C., Aldous D.J., Robinson D.T., Draper T., Dutko F.J., *J. Med. Chem.* **1995**, *38*, 1355.
21. Pevear D.C., Tull T.M., Seipel M.E., Groarke J.M. *Antimicrob. Agents Chemother.*, **1999**, *43*, 2109.
22. Florea N.R., Maglio D., Nicolau D.P., *Pharmacother*. **2003**, *23*, 339.
23. Hayden F.G., Herrington D.T., Coats T.L., Kim K., Cooper E.C., Villano S.A., Liu S., Hudson S., Pevear D.C., Collett M., McKinlay M., *Clin Infect Dis*, **2003**, *36*, 1523.
24. Senior K., *Lancet. Infect. Dis*., **2002**, *2*, 264.
25. Chapman M.S., Minor I., Rossmann M.G.. Diana G.D., Andries K., *J. Mol. Biol.,* **1991**, *217*, 455.
26. Hayden F.G., Andries K., Janssen P.A.J.. *Antimic. Agents and Chemother*., **1992**, *36*, 727
27. Al Nakib W., Higgins P.G., Barrow G.I., Tyrrell D.A., Andries K., Vanden Bussche G., Taylor N., Janssen P.A.. *Antimicrob. Agents Chemother.*, **1989**, *33*, 522.

28. Barnard D.L., Hubbard V.D., Smee D.F., Sidwell R.W., Watson K.G., Tucker S.P., Reece P.A., *Antimicrob Agents Chemother*., **2004**, *48*, 1766.
29. Brown R.N., Cameron R., Chalmers D.K., Hamilton S., Luttick A., Krippner G.Y., McConnell D.B., Nearn R., Stanislawski P.C., Tucker S.P., Watson K.G., *Bioorg. Med. Chem. Lett*., **2005**, *15*, 2051.
30. Lin J.K., Tsai S.H., Lin-Shiau S.Y., *Drugs of the Future* **2001**, *26*, 145.
31. De Meyer N., Haemers A., Mishra L., Pandey H.K., Pieters L.A.C., Vander Berghe, D.A., Vlietinck A.J., *J. Med. Chem*., **1991**, *34*, 736.
32. Bauer D.J., Selway J.W.T., Batchelor J.F., Tisdale M., Caldwell J.C., Young D.A.B.. *Nature*, **1981**, *292*, 369.
33. Phillpotts R.J., Wallace J., Tyrrel D.A.J., Freestone D.S., Shepherd W.M. et al.. *Arch. Virol.*, **1983**, *75*, 115.
34. Burali C., Desideri N., Stein M.L., Conti C. & Orsi N. *Eur. J. Med. Chem.* **1987**, *22*, 119.
35. Conti C., Desideri N., Orsi N., Sestili I., Stein M.L.. *Eur.. J. Med. Chem.* **1990**; *25*, 725.
36. Desideri N., Sestili I., Stein M. L., Conti C., Tomao P., Orsi N. *Antiviral Chem. Chemother*. **1990**, *1*, 307.
37. Desideri N., Conti C., Sestili I., Tomao P., Stein M. L., Orsi N. *Antiviral Chem. Chemother*. **1992**, *3*, 195.
38. Superti F., Seganti L., Orsi N., Divizia M., Gabrielli R., Panà A., Stein M.L.. *Antiviral Res.* **1989**; *11*, 247.
39. Desideri N., Conti C., Sestili I., Tomao P., Stein M.L. & Orsi N., *Antiviral Chem. Chemother.* **1995**, *6*, 298.
40. Conti C., Mastromarino P., Sgro R., Desideri N.. *Antiviral Chem. Chemother.* **1998**, *9*, 511.
41. Conti C., Tomao P., Genovese D., Desideri N., Stein M.L., Orsi N., *Antimic. Agents Chemother.* **1992**, *36*, 95.
42. Genovese D., Conti C., Tomao P., Desideri N., Stein M. L., Catone S., Fiore L.. *Antiviral Res*. **1995**, *27*, 123.
43. Tait S., Salvati A. L., Desideri N., Fiore L., *Antiviral Res*. **2006**, *72*, 252.
44. Desideri N., Olivieri S., Stein M. L., Sgro R., Orsi N., Conti C.. *Antiviral Chem. Chemother*. **1997**, *8*, 545.
45. Desideri N., Conti C., Mastromarino P., Mastropaolo F., *Antiviral Chem. Chemother.* **2000**, *11*, 373.
46. Desideri N., Mastromarino P., Conti C.. *Antiviral Chem. Chemother.* **2003**, *14*, 195.
47. Andries K., Dewindt B., Snoeks J., Wouters L., Moereels Lewi P.J. & Janssen P.A.J.. *J. Virology*, **1990**, *64*, 1117.
48. Andries K., Dewindt B., Snoeks J., Willebrords R., Stokbroekx R., *Antiviral Res.* **1991**, *16*, 213.
49. Quaglia M.G., Desideri N., Bossù E., Melchiorre P., Conti C.. *J. Pharm. Biomed. Anal.,* **1991**, *9*, 811.
50. Quaglia M.G., Desideri N., Bossù E., Sestili I., Conti C.. *Chirality*, **1992**, *4*, 65.
51. Quaglia M.G., Desideri N., Bossù E., Sestili I., Tomao P., Conti C., Orsi N. *Chirality***, 1993**, *5*, 356.
52. Quaglia M.G., Desideri N., Boss E., Sgro R., Conti C.. *Chirality*, **1999**, *11*, 495.

5. SUBJECT OF PRESENT RESEARCH

A novel series of (*Z*)-3-benzylidenechromans, 3-benzyl-2*H*-chromenes and 3-benzylchromans,[1] related to the most active synthetic 3(2*H*)-isoflavenes[2-5] and homoisoflavones[6-8] previously studied, were recently prepared in our laboratory.[1]

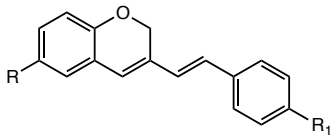


(*Z*)-3-benzylidenechromans **3-benzyl-2*H*-chromenes** **3-benzylchromans**

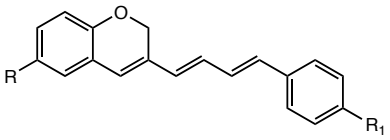
Several of these inhibitors presented submicromolar potency against HRV 1B coupled with high therapeutic index. On the contrary, HRV 14 infection was weakly inhibited.[1] Similarly to related flavanoids,[5,9,10] these analogues behaved as capsid-binders and interfered with very early events of HRV multiplication.[1] 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.[11]

To develop compounds with broad-spectrum activity, the linker chain length between the heterocycle and the phenyl moiety was modified. 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.[12] Mechanism of action studies indicated that also these compounds behaved as capsid-binders interfering with the early stage(s) of rhinovirus infection.[12]

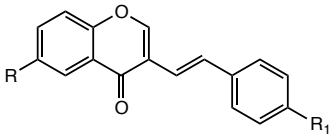
Starting from these results, we focused our attention on (*E*)-3-styryl-2*H*-chromenes as the most promising analogues to perform a systematic structure optimization study.



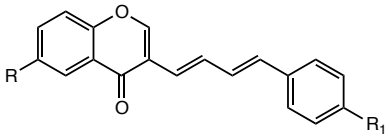
(*E*)-3-styryl-2*H*-chromenes



3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-2*H*-chromenes



(*E*)-3-styryl-4*H*-chromen-4-ones



3-[(1*E*,3*E*)-4-phenylbutan-1,3-dienyl]-4*H*-chromen-4-ones

In particular, we examined the impact on cytotoxity and activity of:

- nature, number and position of substituents on both aromatic rings;
- level of oxidation of the heterocyclic ring;
- modification of the linker between the two cycles;
- bioisosteric substitution of the benzene ring with a pyridine ring.

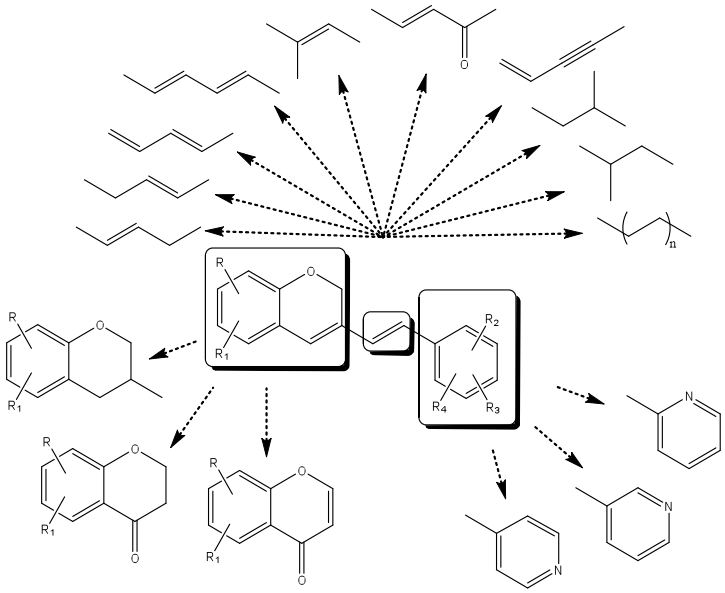


Figure 1. Systematic structure optimization on (*E*)-3-styryl-2*H*-chromenes.

The main targets of this research were:

- to synthesize new small molecules with high activity and selectivity against picornaviruses;
- to select compounds exhibiting a broad spectrum of anti-picornavirus activity;
- to study the mechanism of anti-picornavirus action of selected compounds.

References

1. Conti, C.; Desideri, N. *Bioorg. Med. Chem.* **2009**, *17*, 3720.
2. Burali, C.; Desideri, N.; Stein, M.L.; Conti, C.; Orsi, N. *Eur. J. Med. Chem.* **1987**, *22***,** 119.
3. Conti, C.; Desideri, N.; Orsi, N; Sestili, I.; Stein, M.L. *Eur. J. Med. Chem.* **1990**, *25***,** 725.
4. Desideri, N.; Conti, C.; Sestili, I.; Tomao, P.; Stein, M.L; Orsi, N. *Antiviral Chem. Chemother.* **1992**, *3***,** 195.
5. Genovese, D.; Conti, C.; Tomao, P.; Desideri, N.; Stein, M. L.;Catone, S.; Fiore, L. *Antiviral Res*. **1995**, *27*, 123.
6. Desideri, N.; Olivieri, S.; Stein, M. L.; Sgro, R.; Orsi, N.; Conti, C. *Antiviral Chem. Chemother.* **1997**, *8*, 545.
7. Quaglia, M. G.; Desideri, N.; Bossù, E.; Sgro, R.; Conti, C. *Chirality* 1**999**, *11*, 495.
8. Tait, S.; Salvati, A. L.; Desideri, N.; Fiore L. *Antiviral Res*., **2006**, *72*, 252.
9. Conti, C.; Tomao, P.; Genovese, D.; Desideri, N.; Stein, M.L.; Orsi, N. *Antimicrob. Agents Chemother.* **1992**, *36*, 95.
10. Salvati, A. L.; De Dominicis, A.; Tait, S.; Canitano, A.; Lahm, A. ; Fiore, L. *Antimicrob. Agents Chemother.* **2004**, *48*, 2233.
11. Diana, G. D. *Curr. Med. Chem.-Anti-Infective Agents.* **2003***, 2*, 1.
12. Conti, C.; Desideri, N. *Bioorg. Med. Chem.* **2010**, *18*, 6480.

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

Cinzia Conti[a], Luca Proietti Monaco[b] and Nicoletta Desideri[b]
Bioorg. Med. Chem. 19 (2011) 7357-7364.

[a]*Istituto Pasteur Fondazione Cenci Bolognetti, Dipartimento di Scienze di Sanità Pubblica, Sezione di Microbiologia, Università "La Sapienza" di Roma, P.le A. Moro, 5, 00185 Roma, Italy*
[b]*Istituto Pasteur Fondazione Cenci Bolognetti, Dipartimento di Chimica e Tecnologie del Farmaco, Università "La Sapienza", P.le A. Moro, 00185 Rome, Italy*

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 (*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.

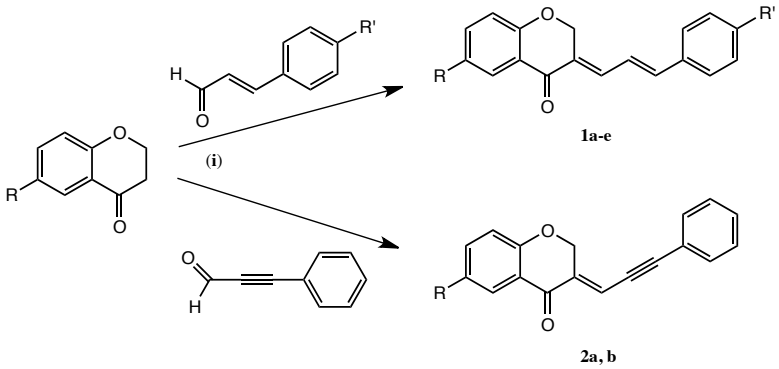
5.1.1. Results and discussion

5.1.1.1. 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 4h (scheme 1). The ^{1}HNMR 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}HNMR 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 ($J_{\beta\text{-}\gamma}$ 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.

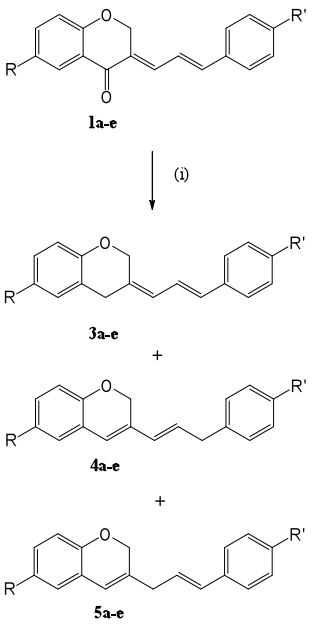
In a similar manner, the chemical shift of the proton in the chain (around 7.00 ppm) indicates the *trans*-configuration of the exocyclic double bond of (*E*)-3-(3-phenylprop-2-yn-1-ylidene)chroman-4-ones **2a** and **2b**.



1,2a	R = R' = H
1,2b	R = Cl, R' = H
1c	R = R' = Cl
1d	R = H, R' = Cl
1e	R = F, R' = H

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

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}HNMR 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.

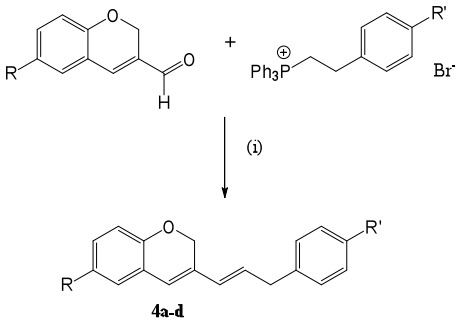


1, **3**, **4**, **5a** R = R' = H
1, **3**, **4**, **5b** R = Cl, R' = H
1, **3**, **4**, **5c** R = R' = Cl
1, **3**, **4**, **5d** R = H, R' = Cl
1, **3**, **4**, **5e** R = F, R' = H

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

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 ($J_{\beta\text{-}\gamma}$ = 15.3 or 15.4 Hz) in the ^{1}HNMR spectra suggest the *trans* configuration of the second double bond. The strong NOE cross peaks between Hα and Hγ confirm this configuration.

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 ($J_{\alpha\text{-}\beta}$ = 15.9 Hz) indicate the *trans* configuration of the double bond in the chain.



4a-d

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, 2.5h.

5.1.1.2. 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.[1] 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 μM 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.[2,3] 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.[4]

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 **4 a-d**.

Comp.	R	R'	MNTC (μM)[a]	TC_{50} (μM)[b]	IC_{50} (μM)[c] HRV 1B	TI[d]	IC_{50} (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.

[b]The TC_{50} value was the concentration of compound which reduced the Hela cell viability by 50%, as compared with the control.

[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).

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.[2-4] 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 μM 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 the new derivatives against serotype 14 (IC_{50} = 1.42 μM, TI = 17.61) while it was about two-fold 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 >six-fold 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).

5.1.1.3. 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. 1 A) or 56 °C (fig. 1 B) (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.[5,6] 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. [7,8] 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.[9,10]

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.

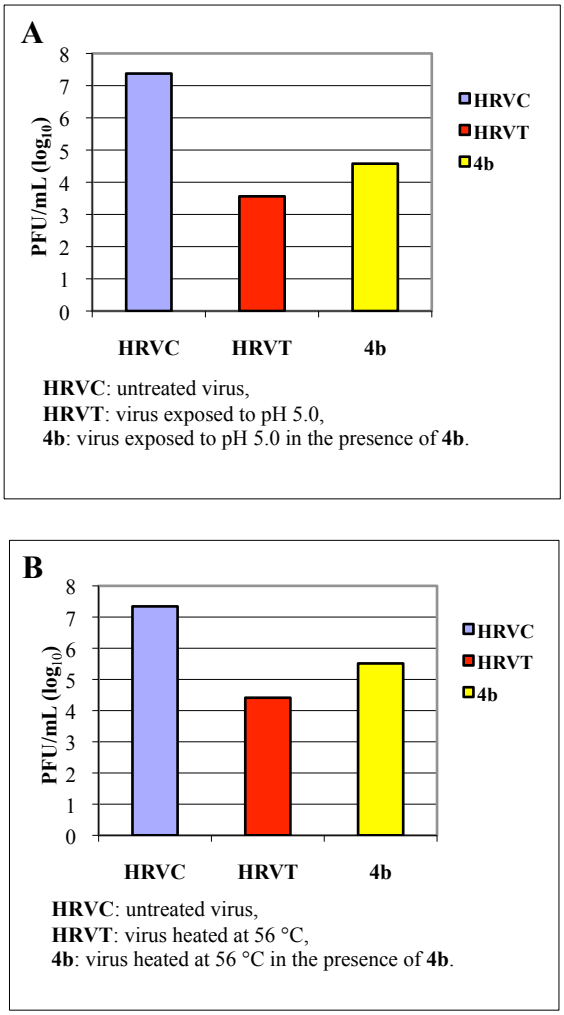


Figure 1. Protective effect of (*E*)-6-chloro-3-(3-phenylprop-1-en-1-yl)-2*H*-chromene **4b** on acid (**A**) and thermal inactivation (**B**) of HRV 1B infectivity.

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 minutes 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 hours after

virus binding), inhibition significantly dropped (31%, 12% and 1%, respectively, figure 2).

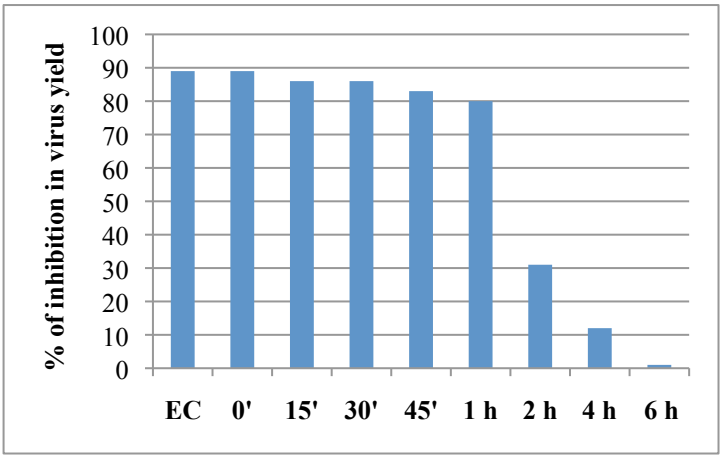


Figure 2. Effect of varying the time of addition of **4b** (42 µM) on the inhibition of HRV1B multiplication under one-step growth conditions. Virus yield was determined by plaque assay. Virus control titre was $2.4 \cdot 10^4$ PFU/mL. **EC**: **4b** was present during the entire infection cycle (1 h at 4°C and 10 h at 33°C). **0', 15', 30', 45', 1 h, 2 h, 4 h, 6 h**: compound was added at different times (0', 15', 30', 45', 1 h, 2 h, 4 h, 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).

Pretreatment of HeLa cells with **4b** before HRV infection or the presence of **4b** during the time of virus adsorption only (1h, 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 minute 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 (figure 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 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 hours of treatment. These data suggest an interference by **4b** during the uncoating process.

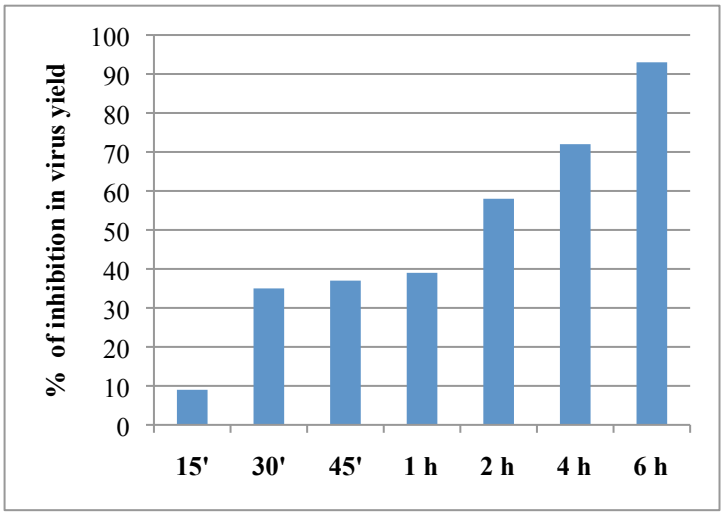


Figure 3. Effect of varying the time of removal of **4b** (42 µM) on the inhibition of HRV1B replication under one-step growth conditions. Virus yield was determined by plaque assay. Virus control titre was $2.4 \cdot 10^4$ PFU/mL. **15', 30', 45', 1 h, 2 h, 4 h, 6 h**: compound **4b** was added after virus adsorption (1 h at 4°C, time 0) and removed after different lengths of incubation (15', 30', 45', 1 h, 2 h, 4 h, 6 h) at 33 °C.

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.[9,10] 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.[9,10] 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.

5.1.2. 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.

5.1.3. Experimental

5.1.3.1. 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[10], 6-chloro-2*H*-chromene-3-carbaldehyde[10], phenethyltriphenylphosphonium bromide[11] and 4-chlorophenethyltriphenylphosphonium bromide[11] were synthesized following the procedure previously described.

5.1.3.1.1. 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.

5.1.3.1.1.1. (*E*)-3-[(*E*)-3-Phenylallylidene]chroman-4-one (1a). Yield: 80%, mp = 115-119 °C from ethyl alcohol. IR (KBr): 1664 cm^{-1}. ^1H 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.

5.1.3.1.1.2. (*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}. ^1H 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.

5.1.3.1.1.3. (*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}. ^1H 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.

5.1.3.1.1.4. (*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}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.01 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.8 Hz, $J_{5\text{-}7}$ = 1.8 Hz), 7.51-7.43 (4H, H7, H2', H6', Hα), 7.34 (d, 2H, H3', H5', $J_{2'\text{-}3'}$ = 8.6 Hz), 7.07 (ddd, 1H, H6, $J_{5\text{-}6}$ = 7.8 Hz, $J_{6\text{-}7}$ = 7.2 Hz, $J_{6\text{-}8}$ = 1.1 Hz), 7.02-6.93 (m, 3H, H8, Hβ, Hγ), 5.26 (d, 2H, H2, $J_{2\text{-}\alpha}$ = 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.

5.1.3.1.1.5. (*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}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.65 (dd, 1H, H5, $J_{5\text{-}F}$ = 8.3 Hz, $J_{5\text{-}7}$ = 2.9 Hz), 7.54-7.49 (m, 3H, H2', H6', Hα), 7.42-7.33 (3H, H3'-H5'), 7.19 (ddd, 1H, H7, $J_{7\text{-}8}$ = 7.8 Hz, $J_{7\text{-}F}$ = 9.0 Hz, $J_{5\text{-}7}$ = 2.9 Hz), 7.11 (d, 1H, Hγ, $J_{\beta\text{-}\gamma}$ = 15.5 Hz), 7.04-6.95 (m, 2H, H8, Hβ), 5.24 (d, 2H, H2, $J_{2\text{-}\alpha}$ = 1.3 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 181.20, 157.64, 157.62 (d, J = 244.0 Hz), 143.86, 136.55, 135.96, 129.68, 128.96, 128.47, 127.53, 123.07 (d, J = 7.0 Hz), 122.99 (d, J = 23.0 Hz), 121.54, 119.49 (d, J = 7.0 Hz), 112.87 (d, J = 23.0 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.

5.1.3.1.1.6. (*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}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.00 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.8 Hz, $J_{5\text{-}7}$ = 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, $J_{5\text{-}6}$ = 7.8 Hz, $J_{6\text{-}7}$ = 8.0 Hz, $J_{6\text{-}8}$ = 1.1 Hz), 7.03-7.05 (m, 2H, H8, Hα), 5.34 (d, 2H, H2, $J_{2\text{-}\alpha}$ = 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.

5.1.3.1.1.7. (*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}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.95 (d, 1H, H5, $J_{5\text{-}7}$ = 2.6 Hz), 7.53 (dd, 2H, H2', H6', $J_{2'\text{-}3'}$ = 7.9 Hz, $J_{2'\text{-}5'}$ = 1.6 Hz), 7.44 (dd, 1H, H7, $J_{7\text{-}8}$ = 9.0 Hz, $J_{5\text{-}7}$ = 2.6 Hz), 7.41-7.36 (m, 3H, H3'-H5'), 7.03 (t, 1H, Hα, $J_{2\text{-}\alpha}$ = 1.9 Hz), 6.97 (d, 1H, H8, $J_{7\text{-}8}$ = 9.0 Hz), 5.33 (d, 2H, H2, $J_{2\text{-}\alpha}$ = 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.

5.1.3.1.2. 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 2N 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.

5.1.2.1.2.1. (*Z*)-3-[(E)-3-Phenylallylidene]chroman (3a). Yield: 54%, mp = 102-104 °C from n-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.43 (dd, 2H, H2', H6', $J_{2'\text{-}3'}$ = 8.2 Hz, $J_{2'\text{-}4'}$ = 1.3 Hz), 7.34-7.23 (m, 3H, H3'-H5'), 7.11-7.05 (m, 2H, H5, H7), 7.03 (dd, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 11.1 Hz, $J_{\beta\text{-}\gamma}$ = 15.4 Hz), 6.91-6.84 (m, 2H, H6, H8), 6.58 (d, 1H, Hγ, $J_{\beta\text{-}\gamma}$ = 15.4 Hz), 6.32 (dd, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 11.1 Hz, $J_{2\text{-}\alpha}$ = 0.9 Hz), 4.86 (d, 2H, H2, $J_{2\text{-}\alpha}$ = 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.

5.1.3.1.2.2. (*Z*)-6-Chloro-3-[(*E*)-3-phenylallylidene]chroman (3b). Yield: 49%, mp = 121-124 °C from n-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.42 (d, 2H, H2', H6', $J_{2'\text{-}3'}$ = 7.7 Hz), 7.35-7.22 (m, 3H, H3'-H5'), 7.07-7.04 (m, 2H, H5, H7), 7.00 (dd, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 11.2 Hz, $J_{\beta\text{-}\gamma}$ = 15.3 Hz), 6.78 (d, 1H, H8, $J_{7\text{-}8}$ = 8.4), 6.60 (d, 1H, Hγ, $J_{\beta\text{-}\gamma}$ = 15.3 Hz), 6.33 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 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.

5.1.3.1.2.3. (*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.

5.1.3.1.2.4. (*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.

5.1.3.1.2.5. (*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 = 236.0 Hz), 150.97, 137.17, 133.53, 131.13, 128.69, 127.82, 126.56, 126.47, 124.12 (d, J = 8.0 Hz), 122.98, 117.66 (d, J = 8.0 Hz), 114.79 (d, J = 23.0 Hz), 113.95 (d, J = 23.0 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.

5.1.3.1.3. 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 2H-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.

5.1.3.1.3.1. (*E*)-3-(3-Phenylprop-1-en-1-yl)-2*H*-chromene (4a). Yield: 45%, mp = 89-90 °C from n-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.31 (t, 2H, H3', H5', $J_{2'\text{-}3'} = J_{3'\text{-}5'} = 7.5$ Hz), 7.25-7.19 (m, 3H, H2', H4', H6'), 7.07 (ddd, 1H, H7, $J_{7\text{-}8} = 8.0$ Hz, $J_{6\text{-}7} = 7.4$ Hz, $J_{5\text{-}7} = 1.6$ Hz), 6.97 (dd, 1H, H5, $J_{5\text{-}6} = 7.4$ Hz, $J_{5\text{-}7} = 1.6$ Hz), 6.85 (dt, 1H, H6, $J_{5\text{-}6} = J_{6\text{-}7} = 7.4$ Hz, $J_{6\text{-}8} = 1.1$ Hz), 6.78 (dd, 1H, H8, $J_{7\text{-}8} = 8.0$ Hz, $J_{6\text{-}8} = 1.1$ Hz), 6.32 (s, 1H, H4), 6.19 (d, 1H, Hα, $J_{\alpha\text{-}\beta} = 15.9$ Hz), 5.74 (dt, 1H, Hβ, $J_{\alpha\text{-}\beta} = 15.9$ Hz, $J_{\beta\text{-}\gamma} = 6.9$ Hz), 4.93 (s, 2H, H2), 3.50 (d, 2H, Hγ, $J_{\beta\text{-}\gamma} = 6.9$ 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.

5.1.3.1.3.2. (*E*)-6-Chloro-3-(3-phenylprop-1-en-1-yl)-2*H*-chromene (4b). Yield: 43%, mp = 80 -81 °C from n-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.34 (t, 2H, H3', H5', $J_{2'\text{-}3,} = J_{3'\text{-}5'} =$ 7.9 Hz), 7.26-7.19 (m, 3H, H2', H4', H6'), 7.02 (dd, 1H, H7, $J_{7\text{-}8} = 8.5$ Hz, $J_{5\text{-}7} = 2.5$ Hz), 6.95 (d, 1H, H5, $J_{5\text{-}7} = 2.5$ Hz), 6.72 (d, 1H, H8, $J_{7\text{-}8} = 8.5$ Hz), 6.25 (s, 1H, H4), 6.19 (d, 1H, Hα, $J_{\alpha\text{-}\beta} = 15.9$ Hz), 5.78 (dt, 1H, Hβ, $J_{\alpha\text{-}\beta} = 15.9$ Hz, $J_{\beta\text{-}\gamma} = 6.8$ Hz), 4.94 (s, 2H, H2), 3.52 (d, 2H, Hγ, $J_{\beta\text{-}\gamma} = 6.8$ 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.

5.1.3.1.3.3. (*E*)-6-Chloro-3-[3-(4-chlorophenyl)prop-1-en-1-yl]-2*H*-chromene (4c). Yield: 41%, mp = 135-143 °C from n-hexane. ^{1}H 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.

5.1.3.1.3.4. (*E*)-3-[3-(4-Chlorophenyl)prop-1-en-1-yl]-2*H*-chromene (4d). Yield: 48%, mp = 90-91 °C from n-hexane. ^{1}H 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.

5.1.3.2. Virology

5.1.3.2.1. 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).

5.1.3.2.2. 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.

5.1.3.2.3. 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.[12] The virus was stored at -80 °C until used.

5.1.3.2.4. XTT assay for cellular cytotoxicity

A tetrazolium-based (XTT) colorimetric assay was used to measure the cytotoxicity of compounds, as previously described.[1] Briefly, HeLa cells were seeded in 96-well tissue culture plates (2 x 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.

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

The IC_{50} values were determined as described previously.[13] 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.

5.1.3.2.6. 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.

5.1.3.2.7. 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 h, 2 h, 4 h, 6 h) and the cultures incubated at 33 °C up to 10 h p.i.. The virus yield was determined as above.

Acknowledgements

We gratefully acknowledge the financial support from "Istituto Pasteur-Fondazione Cenci Bolognetti", Università degli Studi di Roma "La Sapienza". We also thank Dr. Ivano Pindinello for technical assistance.

References

1. Scudiero, D. A.; Shoemaker, R. H.; Paull, K.D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827.
2. Andries, K.; Dewindt, B.; Snoeks, J.; Wouters, L.; Moereels, H.; Lewi, P. J.; Janssen, P. A. J. *J. Virol.* **1990**, *64*, 1117.
3. Andries, K.; Dewindt, B.; Snoeks, J.; Willebrords, R.; Stokbroekx, R; Lewi, P. J. *Antiviral Res.* **1991**, *16*, 213.
4. Bauer, D.J.; Selway, J.W.T.; Batchelor, J.F.; Tisdale, M.; Caldwell, J.C.; Young, D.A.B. *Nature*. **1981**, *292*, 369.
5. Lonberg-Holm, K.; Korant, B.D. *J. Virol.***1972**, *9*, 29.
6. 6. Lonberg-Holm, K.; Yin, F.H. *J. Virol.***1973**, *12*, 114.
7. Rombaut, B.; Vrijsen, R.; Boeye, A**.** *Antiviral Res.* **1985**, Suppl. 1, 67.
8. Rombaut, B.; Andries, K.; Boeye, A. *J. Gen. Virol.* **1991**, *72*, 2153.
9. Conti, C.; Desideri, N. *Bioorg. Med. Chem.* **2009**, *17*, 3720
10. Conti, C.; Desideri, N. *Bioorg. Med. Chem.* **2010**, *18*, 6480.
11. Morgan, T. K. Jr. U.S. Patent 45993441, 1986.
12. Fiala, M.; Kenny, G.E. *J.Bacteriol.*, **1966**, *92***,** 1710.
13. Desideri, N.; Conti, C.; Sestili, I.; Tomao, P.; Stein, M.L; Orsi, N. *Antiviral Chem. Chemother.* **1992**, *3***,** 195.

5.2. Synthesis, anti-rhinovirus efficacy and mechanism of action studies of novel 3-[2-(pyridinyl)vinyl]-substituted 2*H*-chromenes and 4*H*-chromen-4-ones.

Cinzia Conti[a], Luca Proietti Monaco[b] and Nicoletta Desideri[b]
Submitted

[a]*Istituto Pasteur Fondazione Cenci Bolognetti, Dipartimento di Scienze di Sanità Pubblica, Sezione di Microbiologia, Università "La Sapienza" di Roma, P.le A. Moro, 5, 00185 Roma, Italy*
[b]*Istituto Pasteur Fondazione Cenci Bolognetti, Dipartimento di Chimica e Tecnologie del Farmaco, Università "La Sapienza" di Roma, P.le A. Moro, 00185 Rome, Italy*

In this paper, we describe the synthesis of a new series of [2-(2*H*-chromen-3-yl)vinyl]pyridines (**5a, 5b, 6a, 6b, 7a, 7b, 8a, 9a**) and 3-[2-(pyridinyl)vinyl]-4*H*-chromen-4-ones (**11a, 11b, 12a, 12b, 13a, 13b**) designed to explore the impact on activity and/or selectivity of the bioisosteric replacement of the phenyl moiety by a pyridine ring. The antiviral potency of the new synthesized compounds has been evaluated against infection by HRV 1B and 14 in cell cultures. Among these derivatives, (*E*)-2-[2-(2*H*-chromen-3-yl)vinyl] pyridine **5a** has been selected for preliminary studies to elucidate their mechanism of action against both HRV serotypes.

5.2.1. Results and discussion

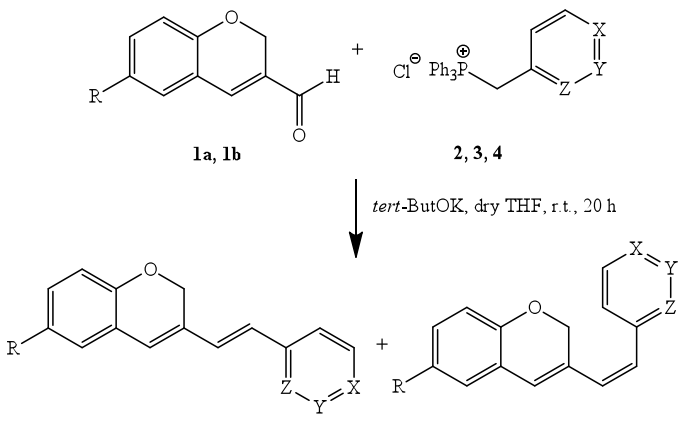
5.2.1.1. Chemistry

As described in scheme 1, the [2-(2*H*-chromen-3-yl)vinyl]pyridines (**5a, 5b, 6a, 6b, 7a, 7b, 8a, 9a**) were obtained by the Wittig reaction of the 2*H*-chromene-3-carbaldehydes (**1a**, **1b**) with the appropriate pyridilmethyltriphenylphosphonium chloride (**2**, **3**, **4**). The reaction was performed in dry THF using potassium *tert*-butoxide as base. *E*/*Z*-mixtures (**6a**/**8a** and **7a**/**9a**) were separated into their individual isomers by column chromatography on silica gel. For the analogues **5a, 5b, 6b** and **7b** only the *E* isomers could be isolated. The stereochemistry of compounds was established on the basis of the

coupling constant values of the protons in the chain (about 16 Hz and 12 Hz for *E* and *Z* isomers, respectively) (scheme 1).

Using the same synthetic strategy, the 3-[2-(pyridinyl)vinyl]-4*H*-chromen-4-ones (**11a, 11b, 12a, 12b, 13a, 13b**) were prepared starting from the commercially available 4-oxo-4*H*-chromen-4-ones (**10a, 10b**) and the appropriate pyridilmethyltriphenylphosphonium chloride (**2**, **3**, **4**).

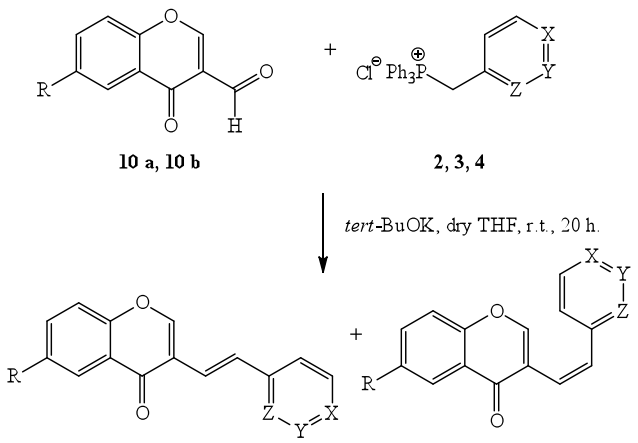
Scheme 1. Synthesis of (*E*)-2-[2-(2*H*-chromen-3-yl)vinyl]pyridines (**5a**, **5 b**), (*E*)-3-[2-(2*H*-chromen-3-yl)vinyl]pyridines (**6 a**, **6 b**), (*E*)-4-[2-(2*H*-chromen-3-yl)vinyl]pyridines (**7 a**, **7 b**), (*Z*)-3-[2-(2*H*-chromen-3-yl)vinyl]pyridines (**8a**) and (*Z*)-4-[2-(2*H*-chromen-3-yl)vinyl]pyridines (**9 a**).



1a:	R = H;	**5a:**	R = H, X = Y = CH, Z = N;
1b:	R = Cl;	**5b:**	R = Cl, X = Y = CH, Z = N;
2:	X = Y = CH, Z = N;	**6a, 8a:**	R = H, X = Z = CH, Y = N;
3:	X = Z = CH, Y = N;	**6b:**	R = Cl, X = Z = CH, Y = N;
4:	X = N, Y = Z = CH;	**7a, 9a:**	R = H, X = N, Y = Z = CH;
		7b:	R = Cl, X = N, Y = Z = CH.

The 2-(pyridin-3-yl)vinyl derivatives (**13 a, 13 b**) could be isolated by column chromatography only as *Z* isomers while, for the analogues **11 a, 11 b**, **12 a** and **12 b**, only the *E* isomers could be obtained from *E*/*Z*-mixtures. The stereochemical assignment was based on the coupling constant values of the protons in the chain (about 16 Hz and 12 Hz for *E* and *Z* isomers, respectively) (scheme 2).

Scheme 2. Synthesis of (*E*)-3-[2-(pyridin-2-yl)vinyl]-4*H*-chromen-4-ones (**11a, 11b**), (*E*)-3-[2-(pyridin-4-yl)vinyl]-4*H*-chromen-4-ones (**12a, 12b**), (*Z*)-3-[2-(pyridin-3-yl)vinyl]-4*H*-chromen-4-ones (**13a, 13b**).



10a: R = H;
10b: R = Cl;
2: X = Y = CH, Z = N;
3: X = Z = CH, Y = N;
4: X = N, Y = Z = CH;

11a: R = H, X = Y = CH, Z = N;
11b: R = Cl, X = Y = CH, Z = N;
12a: R = H, X = N, Y = Z = CH;
12b: R = Cl, X = N, Y = Z = CH;
13a: R = H, X = Z = CH, Y = N;
13b: R = Cl, X = Z = CH, Y = N.

5.2.1.2. Antiviral tests

In table 1 are reported the results of the biological evaluation of the new compounds (**5a, 5b, 6a, 6b, 7a, 7b, 8a, 9a**, **11a, 11b, 12a, 12b, 13a, 13b)**.

Initially, each compound was tested for its effects on morphology, viability and growth of HeLa cells, a human cell line suitable for HRV replication. The maximum non-cytotoxic concentration (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 50% cytotoxic concentration (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. For several analogues (**5a, 5b, 11a, 11b, 12a** and **13a**), both MNTC

and TC_{50} were found to be higher than the saturation concentration in cell culture medium.

Table 1. Cytotoxicity and anti-rhinovirus activity of [2-(2*H*-chromen-3-yl)vinyl]pyridines (**5a, 5b**, **6a, 6b**, **7a**, **7b, 8a**, **9a**) and 3-[2-(pyridinyl)vinyl]-4*H*-chromen-4-ones (**11a, 11b, 12a, 12b, 13a, 13b**).

Comp.	R	MNTC (µM)[a]	TC_{50} (µM)[b]	IC_{50} (µM)[c] HRV 1B	TI[d]	IC_{50} (µM)[c] HRV 14	TI[d]
5a	H	>50.00[e]	>50.00[e]	1.70	>29.41	2.93	>17.06
5b	Cl	>50.00[e]	>50.00[e]	1.69	>29.59	8.96	>5.58
6a	H	25.00	75.00	4.57	16.41	3.88	19.33
6b	Cl	25.00	37.50	4.34	8.64	4.27	8.78
7a	H	50.00	75.00	1.96	38.26	3.03	24.75
7b	Cl	50.00	75.00	4.74	15.82	6.71	11.17
8a	H	25.00	37.50	0.98	38.26	9.37	4.00
9a	H	25.00	50.00	0.71	70.42	2.90	17.24
11a	H	>200.00[e]	>200.00[e]	5.15	>38.84	7.08	>28.25
11b	Cl	>50.00[e]	>50.00[e]	1.60	>31.25	5.10	> 9.80
12a	H	>100.00[e]	>100.00[e]	>100 (22%)	-	92.12	>1.08
12b	Cl	100.00	150	19.05	7.87	15.62	9.60
13a	H	>200.00[e]	>200.00[e]	46.77	>4.28	15.41	>12.98
13b	Cl	25.00	50.00	4.26	11.74	10.12	4.94
BW683C		25.00	> 25.00[e]	0.025	>1000	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.
[b]The TC_{50} value was the concentration of compound which reduced the cell viability by 50%, as compared with the control.
[c] The IC_{50} value was the dose of compound reducing the plaque number by 50% and was calculated by plotting the log of 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 MNTC and TC_{50}.
[f] Not active up to the highest concentration tested (MNTC).

The dose-response effect of new compounds was evaluated on HRV 1B and HRV 14 plaque formation in HeLa cells. These serotypes have been selected as representatives for group B and A of HRVs, respectively, as defined on the bases of the contrasting susceptibility of all serotypes to a panel of capsid-binding compounds.[5,6] Group B contains twice as many serotypes as group A, and accounts for five times as many colds as group A serotypes.[5,6] In

table 1 are reported IC_{50} and therapeutic index (TI) values. 4',6-Dichloroflavan (**BW683C**), a well known inhibitor of group B serotypes, was included as a control.[7] All the new compounds interfered with the replication of both HRV serotypes at micromolar or submicromolar concentrations. Only for the chromen-4-one **12a**, the reduction of HRV 1B plaque number was found lower than 50% up to the NMTC, therefore the percentage inhibition at this dose is reported in parentheses. **12a** showed also low activity against serotype 14 (IC_{50} = 92.12 μM). Within the 3-[2-(pyridinyl)vinyl]-4*H*-chromen-4-one series (**11a, 11b, 12a, 12b, 13a** and **13b)**, the presence of a chlorine atom at the 6 position (**11b, 12b, 13b)** always resulted in a marked enhancement in activity against both serotypes compared to the unsubstituted analogues (**11a, 12a, 13a**). The same substitution in the [2-(2*H*-chromen-3-yl)vinyl]pyridine series (**5a, 5b, 6a, 6b, 7a, 7b, 8a** and **9a**) generally led to a significant loss in potency against serotype 14 and a modest improvement in activity against serotype 1B. The only exception to this generalization was the 6-chloro analogue **7b** that was more than 2-fold less potent than corresponding **7a** against both serotypes. Although HRV 1B was generally more susceptible than HRV 14 to the action of the [2-(2*H*-chromen-3-yl)vinyl]pyridines (**5a, 5b, 6a, 6b, 7a, 7b, 8a** and **9a**), all these compounds showed potent activity and selectivity against both serotypes. When the 2*H*-chromene ring in this series was replaced by a chroman-4-one ring to give analogues **11a, 11b, 12a, 12b, 13a** and **13b**, the inhibitory activity against both serotypes was generally reduced. Only the chroman-4-one **11b** exhibited a higher potency than the corresponding 2*H*-chromene **5b** against serotype 14.

A comparison among the activity of *E* and *Z* isomers (**6a**/**8a** and **7a**/**9a**) showed that the *Z* isomers (**8a** and **9a**) exhibited a higher activity than corresponding *E* isomers (**6a** and **7a**) against HRV 1B. On the contrary, the pair of isomers **7a**/**9a** presented almost the same potency, while the *Z* isomer **8a** was less active than *E* isomer **6a** toward serotype 14.

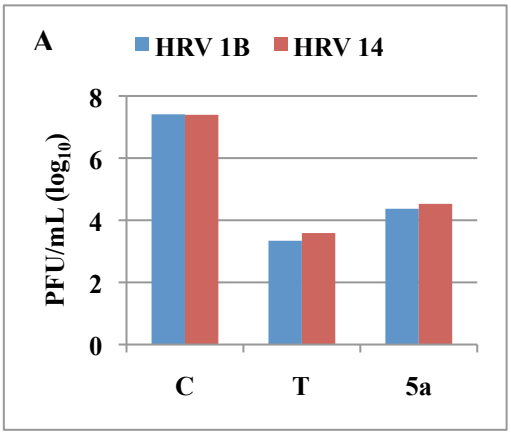
5.2.1.3. Mechanism of action studies

(*E*)-2-[2-(2*H*-chromen-3-yl)vinyl]pyridine **5a** was selected to investigate the mechanism of the antiviral action because of its potent in vitro activity against HRV serotype 1B and 14 (IC_{50} = 1.70 μM and 2.93 μM, respectively) and high therapeutic indexes (TI > 50.00 and > 17.06, respectively). The effects produced by **5a** either on virus particles or multiplication were evaluated at a concentration of 43 μM and 58 μM, approximately 25 and 20 times the IC_{50} against serotype 1B and 14, respectively.

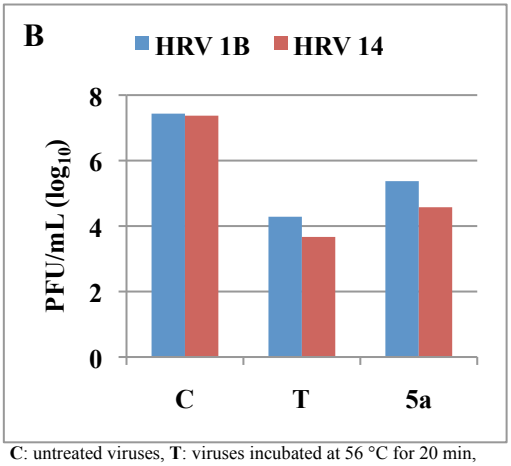
The neutralizing effect of **5a** on infectivity was investigated by incubating HRV 1B and 14 suspensions at high titre with the compound. After ten-fold dilutions to achieve no inhibitory concentrations of free compound, the infectivity titers of mock- and **5a**-treated virus suspensions were found to be similar (serotype 1B: 2.38×10^7 PFU/mL and 2.15×10^7 PFU/mL, respectively; serotype 14: 4.27×10^7 PFU/mL and 4.13×10^7 PFU/mL, respectively). Instead, in stabilization studies, **5a** significantly protected HRV infectivity against inactivation by both mild acid and heat treatments. As shown in figure 2 A, exposure to mild acid produced a drop in infectivity of 4.1 log and 3.8 log for serotype 1B and 14, respectively. In the presence of **5a**, the reduction in infectivity was lower and the protective effect was 1.0 log and 0.9 log, respectively. In a similar manner, the infectivity of both serotypes was significantly lowered by heat treatment (3.1 log and 3.7 log, respectively) and the presence of **5a** again exerted a protective effect of 1.1 log and 0.9 log, respectively (fig. 2 B). Therefore, data from these experiments strongly suggest that **5a** behaves as a capsid binder towards both serotypes although the action was reversible by dilution. Exposure to mild acid or heat is known to promote conformational changes of the rhinovirus capsid structure similar to those observed during the uncoating of the viral genome.[8,9] Binding of so-called capsid-binders in a pocket immediately underneath the floor of the canyon, results in resistance to acid and thermal inactivation due to a reduction of capsid flexibility.[10,11]

In order to determine whether **5a** interfered with a specific period of HRV life cycle, the action of compound addition or removal at different time intervals was investigated in infected HeLa cells under one-step growth conditions. Data represented in fig. 3 indicate that **5a** was significantly effective toward both serotypes when added at the beginning of infection or at the end of the adsorption period and maintained for the entire time of virus multiplication. At later times,

although the shape of inhibition curves was similar, half-maximal reduction of virus yield was observed at 45 min p.i. for serotype 1B and at more than 1 h for serotype 14. Susceptibility to compound disappeared when **5a** was added after 2 h for 1B serotype and 4 h for serotype 14. Similar behaviours were observed when **5a** was removed from HRV 1B or 14 infected cultures after different times of treatment (fig 4). Also under these experimental conditions, half maximal inhibition was recorded after 45 min of contact for serotype 1B and after more than 1 h for serotype 14. Maximal inhibition was achieved after a 4 h treatment for HRV 1B and a 6 h treatment in the case of serotype 14.



C: untreated viruses, **T**: viruses exposed to pH 5.0, **5a**: viruses exposed to pH 5.0 in the presence of **5a**



C: untreated viruses, **T**: viruses incubated at 56 °C for 20 min, **5a**: viruses incubated 56°C for 20 min in the presence of **5a.**

Figure 2. Protective effect of **5a** on acid (**A**) and thermal inactivation (**B**) of HRV 1B and HRV 14, respectively.

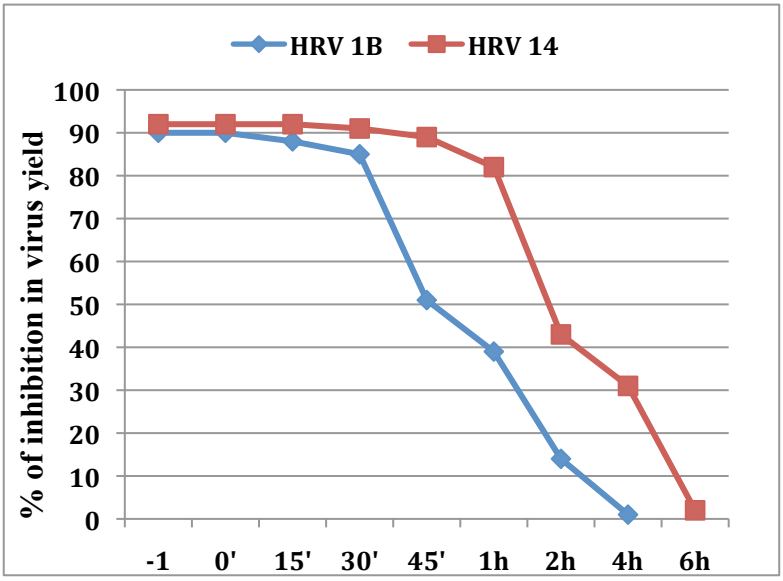


Figure 3. Effect of addition of **5a** at different times during HRV 1B and 14 multiplication under one-step growth conditions.
Virus yield was determined by plaque assay. Virus control titre was $3.04 \cdot 10^4$ PFU/mL and $2.83 \cdot 10^4$ PFU/mL for HRV 1B and HRV 14, respectivelly. **- 1**: **5a** was present during the entire infection cycle (1 h at 4°C and 10 h at 33°C). **0', 15', 30', 45', 1 h, 2 h, 4 h, 6 h**: **5a** was added at different times (0', 15', 30', 45', 1 h, 2 h, 4 h, 6 h, 8 h) after the virus adsorption (1 h at 4°C, time 0) and maintained until the end of virus multiplication (up to 10 h pi).

Both HRV 1B and 14 were shown to efficiently infect the cells when **5a** was applied to the cultures during the virus absorption period only (14% and 16% reduction of HRV 1B and HRV 14 yield, respectively) or for 1h before infection (24% and 1% reduction for HRV 1B and HRV 14, respectively) (data not shown). The slight reduction of virus yield observed during the binding step could be due to residual compound that could interfere with the following steps.

Collectively, all these data suggest that the action of **5a** is exerted on the early events of HRV 1B and 14 multiplication but not on cell membrane receptor recognition. More likely, interaction of viruses with **5a** could increase the stability of viral shell thus preventing disassembly of the capsid and subsequent release of the core RNA into the cell cytoplasm. In agreement, data from the time-course of inhibition suggest an action at the uncoating level. Therefore, in our experiments, interference with RNA uncoating by drug binding appears to be independent of receptor grouping. Receptors and low

endosomal pH are known to differently contribute to the uncoating of the various HRV serotypes.[12] Nevertheless, on infection, minor group HRVs undergo similar structural changes as do major group viruses although this is not triggered by the receptors.[13] Conversion in altered subviral A and B particles is believed to be an essential intermediate for cell entry, uncoating and successfull infection. Differences in time-course of inhibition curves could be related to a different dependence on receptor function for HRV1B and 14 and to a different pathway for internalization into host cells.[12,13]

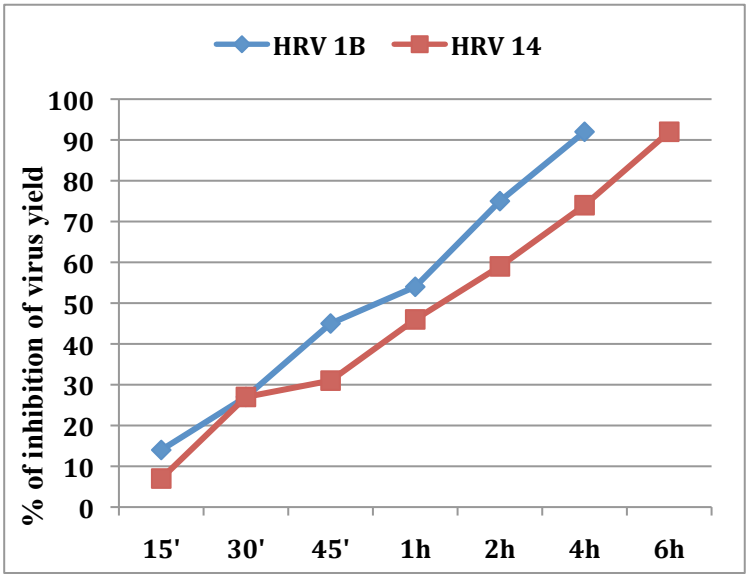


Figure 4. Effect of removal of **5a** at different times during HRV 1B and 14 multiplication under one-step growth conditions.
Virus yield was determined by plaque assay. Virus control titre was $3.04 \cdot 10^4$ PFU/mL and $4.68 \cdot 10^4$ PFU/mL for HRV 1B and HRV 14, respectively. **15', 30', 45', 1 h, 2 h, 4 h, 6 h: 5a** was added after virus adsorption (1 h at 4°C, time 0) and removed after different lengths of incubation (15', 30', 45', 1 h, 2 h, 4 h, 6 h) at 33 °C. Infected cultures were incubated at 33 °C up to 10h pi in maintenance medium.

A comparison of the mechanism of action of **5a** with previously studied (*E*)-3-styryl-2*H*-chromene[1] shows that both 2*H*-chromenes behaved as capsid-binders and interfered with early events of virus multiplication. However, (*E*)-3-styryl-2*H*-chromene was able to interfere also with virus binding to its cellular receptor. Therefore, the bioisosteric substitution of the phenyl with a pyridine ring abrogates the efficacy on binding.

5.2.2. Conclusion

A new series of potent and selective HRV 1B and 14 inhibitors has been designed and synthesized. Similarly to related 2*H*-chromenes[1], (*E*)-2-[2-(2*H*-chromen-3-yl)vinyl]pyridine **5a** behaves as a capsid-binder and interferes with early events of virus multiplication. However, differently from previously described (*E*)-3-styryl-2*H*-chromene, **5a** was unable to affect virus binding to its cellular receptors. Therefore, bioisosteric substitution of the phenyl with a pyridine ring causes a relevant change in inhibition mechanism.

5.2.3. Materials and methods

5.2.3.1. Chemistry

Chemicals were purchased from Sigma-Aldrich and used without further purification. Melting points were determined on a Stanford Research Systems OptiMelt (MPA-100) apparatus and are uncorrected. ^{1}H 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 (Fluka, DC-Alufolien Kieselgel or aluminum oxide F254). Components were visualised by UV light. Compounds purity was determined by elemental analysis and was confirmed to be >95% for all the tested compounds. Analytical results are within ±0.40% of the theoretical values. 2*H*-Chromene-3-carbaldehydes (**1a** and **1b**)[1] and pyridilmethyltriphenylphosphonium chlorides (**2, 3** and **4**)[2] were synthesized according to literature procedures.

5.2.3.1.1. General procedure for the synthesis of [2-(2*H*-chromen-3-yl)vinyl]pyridines (5a, 5b, 6a, 6b, 7 a, 7b, 8a, 9a) and 3-[2-(pyridinyl)vinyl]-4*H*-chromen-4-ones (11a, 11b, 12a, 12b, 13a, 13b)

Potassium *tert*-butoxide (20 mmol) was added to a stirred suspension of the appropriate pyridilmethyltriphenylphosphonium chloride (**2, 3** or **4**) (10 mmol) in dry THF (63 mL). After stirring for 10 min at room temperature, a solution of the appropriate 2*H*-

chromene-3-carbaldehyde (**1a** or **1b**) (10 mmol) or 4-oxo-4*H*-chromene-3-carbaldehyde (**10a** or **10b**) (10 mmol) in dry THF (43 mL) was added dropwise. The resulting mixture was stirred at room temperature for 20 h. After this period, water was added, THF was removed under reduced pressure and the residue was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous sodium sulphate, filtered and evaporated to dryness.

In the cases of 3-[2-(2H-chromen-3-yl)vinyl]pyridines (**6a, 8a**) and 4-[2-(2*H*-chromen-3-yl)vinyl]pyridines (**7a, 9a**), the obtained mixtures of (*E*) and (*Z*) isomers were separated by column chromatography on silica gel, eluting with ethyl acetate/light petroleum (1:1). The (*Z*) isomer was eluted at first. For the other compounds only one isomer was purified eluting with ethyl acetate/light petroleum (1:1) (**6b, 12b**), (1:2) (**5a**, **11a**), or (1:3) (**5b, 7b, 11b, 12a, 13a**,**13b**). Compounds **11a, 11b, 13a** and **13b** were crystallized as hydrochlorides.

5.2.3.1.1.1. (*E*)-2-[2-(2*H*-chromen-3-yl)vinyl]pyridine (5a). Yield: 47%, mp = 115-116°C from ethyl acetate. ^{1}H NMR (DMSO D6, 400MHz): δ (ppm) 8.58 (dd, 1H, J = 4.8, 2.0 Hz), 7.65 (dt, 1H, J = 7.7, 2.0 Hz), 7.37 (d, 1H, J =16.1 Hz), 7.32 (d, 1H, J = 7.8 Hz), 7.15 - 7.10 (m, 2H), 7.05 (dd, 1H, J = 7.5, 1.7 Hz), 6.89 (dt, 1H, J = 7.5, 1.0 Hz), 6.83 (d, 1H, J = 7.8 Hz), 6.63 (s, 1H), 6.51 (d, 1H, J =16.1 Hz), 5.11 (s, 2H). Anal. Calcd for $C_{16}H_{14}ClNO$: C, 70.72; H, 5.19; Cl, 13.05; N, 5.15. Found: C, 70.90; H, 5.32; Cl, 12.90; N, 5.04.

5.2.3.1.1.2. (*E*)-2-[2-(6-chloro-2*H*-chromen-3-yl)vinyl]pyridine (5b). Yield: 97%, mp = 156-161°C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400MHz): δ (ppm) 8.59 (dd, 1H, J = 4.8, 2.0 Hz), 7.65 (dt, 1H, J = 7.6, 2.0 Hz), 7.36 (d, 1H, J =16.0 Hz), 7.32 (d, 1H, J = 7.6 Hz), 7.15 (ddd, 1H, J = 7.6, 4.8, 1.2 Hz), 7.06 (dd, 1H, J = 8.4, 2.4 Hz), 7.02 (d, 1H, J = 2.4 Hz), 6.75 (d, 1H, J = 8.4 Hz), 6.56 (s, 1H), 6.53 (d, 1H, J =16.0 Hz), 5.10 (d, 2H, J = 1.2 Hz). Anal. Calcd for $C_{16}H_{12}ClNO$: C, 71.25; H, 4.48; Cl, 13.14; N, 5.19. Found: C, 71.33; H, 4.31; Cl, 12.96; N, 5.30.

5.2.3.1.1.3. (*E*)-3-[2-(2*H*-chromen-3-yl)vinyl]pyridine (6a). Yield: 32%, mp = 89-90°C. ^{1}H NMR ($CDCl_3$, 400MHz): δ (ppm) 8.61 (d, 1H, J = 2.4 Hz), 8.44 (dd, 1H, J = 4.8, 1.6 Hz), 7.68 (ddd, 1H, J = 8.0, 2.4, 2.0 Hz), 7.21 (dd, 1H, J = 8.0, 4.8 Hz), 7.10 (dt, 1H, J = 8.0, 8.0,

1.6 Hz), 7.00 (dd, 1H, *J* = 8.0, 1.6 Hz), 6.88 - 6.80 (m, 3H), 6.49 (s, 1H), 6.32 (d, 1H, *J* =16.8 Hz), 5.04 (s, 2H). Anal. Calcd for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95. Found: C, 81.84; H, 5.67; N, 6.12.

5.2.3.1.1.4. (*Z*)-3-[2-(2*H*-chromen-3-yl)vinyl]pyridine (8a). Yield: 24%, mp = 46-47 °C. ^{1}H NMR ($CDCl_3$, 400MHz): δ (ppm) 8.48-8.46 (m, 2H), 7.55 (dt, 1H, *J* = 7.6, 2.4 Hz), 7.19 (ddd, 1H, *J* = 8.0, 4.8, 0.8 Hz), 7.08 (dt, 1H, *J* = 7.6, 7.6, 1.6 Hz), 6.97 (dd, 1H, *J* = 7.6, 1.6 Hz), 6.86 (dt, 1H, *J* = 7.6, 7.6, 1.2 Hz), 6.75 (d, 1H, *J* = 8 Hz), 6.49 (s, 1H), 6.47 (d, 1H, *J* = 12.0 Hz), 6.30 (d, 1H, *J* = 12.0 Hz), 4.42 (d, 2H, *J* = 1.2 Hz). Anal. Calcd for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95. Found: C, 81.53; H, 5.72; N, 6.13.

5.2.3.1.1.5. (*E*)-3-[2-(6-chloro-2*H*-chromen-3-yl)vinyl]pyridine (6b) Yield: 31 %, mp = 142-143°C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400MHz): δ (ppm) 8.69 (d, 1H, *J* = 2.4 Hz), 8.50 (dd, 1H, *J* = 4.8, 1.6 Hz), 7.87 (ddd, 1H, *J* = 8.0, 2.4, 2.0 Hz), 7.08 (dd, 1H, *J* = 8.4, 2.5 Hz), 7.03 (d, 1H, *J* = 2.5 Hz), 6.93 (d, 1H, *J* = 16.5 Hz), 6.77 (d, 1H, *J* = 8.4 Hz), 6.53 (s, 1H), 6.43 (d, 1H, *J* = 16.5 Hz), 5.09 (s, 2H,). Anal. Calcd for $C_{16}H_{12}ClNO$: C, 71.25; H, 4.48; Cl, 13.14; N, 5.19. Found: C, 71.07; H, 4.56; Cl, 13.26; N, 5.31.

5.2.3.1.1.6. (*E*)-4-[2-(2*H*-chromen-3-yl)vinyl]pyridine (7a). Yield: 41%, mp = 129-130°C. ^{1}H NMR ($CDCl_3$, 400MHz): δ (ppm) 8.55 (d, 2H, *J* = 6.0 Hz), 7.28 (d, 2H, *J* = 6.0 Hz), 7.15 (td, 1H, *J* = 8.0, 8.0, 1.6 Hz), 7.07 - 7.01 (m, 2H), 6.90 (td, 1H, *J* = 7.6, 7.6, 1.2 Hz), 6.84 (d, 1H, *J* = 8.4 Hz), 6.62 (s, 1H), 6.33 (d, 1H, *J* = 16.4 Hz), 5.08 (s, 2H). Anal. Calcd for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95. Found: C, 81.41; H, 5.72; N, 6.03.

5.2.3.1.1.7. (*Z*)-4-[2-(2*H*-chromen-3-yl)vinyl]pyridine (9a). Yield: 10%, mp = 46-48 °C. ^{1}H NMR ($CDCl_3$, 400MHz): δ (ppm) 8.53 (d, 2H, *J* = 6.0 Hz), 7.14 (d, 2H, *J* = 6.0 Hz), 7.10 (dt, 1H, *J* = 8.0, 8.0, 1.6 Hz), 6.98 (dd, 1H, *J* = 7.6, 1.6 Hz), 6.87 (dt, 1H, *J* = 7.6, 7.6, 1.2 Hz), 6.77 (d, 1H, *J* = 8.0 Hz), 6.51 (s, 1H), 6.45 (d, 1H, *J* =12.4 Hz), 6.32 (d, 1H, *J* = 12.4 Hz), 4.46 (s, 2H). Anal. Calcd for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95. Found: C, 81.50; H, 5.45; N, 5.92.

5.2.3.1.1.8. (*E*)-4-[2-(6-chloro-2*H*-chromen-3-yl)vinyl]pyridine (7b). Yield: 33 %, mp = 145-147°C from ethyl acetate. ^{1}H NMR

($CDCl_3$, 400MHz): δ (ppm) 8.56 (d, 2H, J = 6.0 Hz), 7.28 (d, 2H, J = 6.0 Hz), 7.08 (dd, 1H, J = 8.5 = 2.5 Hz), 7.03 - 6.99 (m, 2H), 6.76 (d, 1H, J = 8.5 Hz), 6.53 (s, 1H), 6.35 (d, 1H, J = 16.4 Hz), 5.07 (s, 2H). Anal. Calcd for $C_{16}H_{12}ClNO$: C, 71.25; H, 4.48; Cl, 13.14; N, 5.19. Found: C, 71.42; H, 4.53; Cl, 12.94; N, 5.03.

5.2.3.1.1.9. (*E*)-3-[2-(pyridin-2-yl)vinyl]-4*H*-chromen-4-one hydrochloride (11a). Yield: 30 %, mp = 201-202 °C from ethyl alcohol/diethyl ether. IR (KBr): 1639 cm^{-1}. ^1H NMR (DMSO D6, 400MHz): δ (ppm) 8.79 (s, 1H), 8.66 (d, 1H, J = 4.8 Hz), 8.26-8.16 (m, 2H), 8.09 (d, 1H, J = 16.2 Hz), 8.01 (d, 1H, J = 7.8 Hz), 7.86 (t, 1H, J = 8.1 Hz), 7.72 (d, 1H, J = 8.1 Hz), 7.66 (d, 1H, J = 16.2 Hz), 7.63-7.54 (m, 2H). Anal. Calcd for $C_{16}H_{12}ClNO_2$: C, 67.26; H, 4.23; Cl, 12.41; N, 4.90. Found: C, 67.50; H, 4.35; Cl, 12.38; N, 5.07.

5.2.3.1.1.10. (*E*)-6-chloro-3-[2-(pyridin-2-yl)vinyl]-4*H*-chromen-4-one hydrochloride (11b). Yield: 56 %, mp = 178 - 181 °C from ethyl alcohol/diethyl ether. IR (KBr): 1659 cm^{-1}. ^1H NMR (DMSO D6, 400MHz): δ (ppm) 8.84 (s, 1H), 8.64 (d, 1H, J = 4.8 Hz), 8.10 (d, 1H, J = 2.6 Hz), 8.02-7.80 (m, 2H), 7.90 (dd, 1H, J = 8.9, 2.6 Hz), 7.80 (d, 1H, J = 8.9 Hz), 7.71 (d, 1H, J = 7.8 Hz), 7.60 (d, 1H, J = 16.1 Hz), 7.43 (dd, 1H, J = 7.5, 4.8 Hz). Anal. Calcd for $C_{16}H_{11}Cl_2NO_2$: C, 60.02; H, 3.46; Cl, 22.15; N, 4.37. Found: C, 59.85; H, 3.49; Cl, 22.36; N, 4.20.

5.2.3.1.1.11. (*E*)-3-[2-(pyridin-4-yl)vinyl]-4*H*-chromen-4-one (12a). IR (KBr): 1644 cm^{-1}. Yield: 34 %, mp = 150-151 °C from ethyl acetate. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 8.58 (d, 2H, J = 5.8 Hz), 8.30 (dd, 1H, J = 8.0, 1.7 Hz), 8.14 (s, 1H), 7.74-7.67 (m, 2H), 7.50-7.41 (m, 2H), 7.37 (d, 2H, J = 5.8 Hz), 7.11 (d, 1H, J = 16.3 Hz). Anal. Calcd for $C_{16}H_{11}NO_2$: C, 77.10; H, 4.45; N, 5.62. Found: C, 76.95; H, 4.49; N, 5.72.

5.2.3.1.1.12. (*E*)-6-chloro-3-[2-(pyridin-4-yl)vinyl]-4*H*-chromen-4-one (12b). Yield: 37 %, mp = 221-222 °C from ethyl acetate. IR (KBr): 1643 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 8.69 (d, 2H, J = 5.7 Hz), 8.26 (d, 1H, J = 2.6 Hz), 8.15 (s, 1H), 7.72 (d, 1H, J = 16.3 Hz), 7.64 (dd, 1H, J = 8.9, 2.6 Hz), 7.46 (d, 1H, J = 8.9 Hz), 7.40 (d, 2H, J = 5.7 Hz), 7.10 (d, 1H, J = 16.3 Hz). Anal. Calcd for $C_{16}H_{10}ClNO_2$: C, 67.74; H, 3.55; Cl, 12.50; N, 4.94. Found: C, 67.50; H, 3.45; Cl, 12.32; N, 5.07.

5.2.3.1.1.13. (*Z*)-3-[2-(pyridin-3-yl)vinyl]-4*H*-chromen-4-one hydrochloride (13a). Yield: 59 %, mp = 204-209 °C from ethyl alcohol/diethyl ether. IR (KBr): 1636 cm^{-1}. ^1H NMR (DMSO D6, 400MHz): δ (ppm) 8.84 (d, 1H, J = 1.8 Hz), 8.70 (d, 1H, J = 5.6 Hz), 8.44-8.39 (m, 2H), 8.05 (dd, 1H, J = 8.0, 1.7 Hz), 7.90-7.81 (m, 2H), 7.67 (dd, 1H, J = 8.5, 1.0 Hz), 7.52 (dt, 1H, J = 8.1, 1.0 Hz), 6.90 (d, 1H, J = 12.2 Hz), 6.71 (d, 1H, J = 12.2 Hz). Anal. Calcd for $C_{16}H_{12}ClNO_2$: C, 67.26; H, 4.23; Cl, 12.41; N, 4.90. Found: C, 67.09; H, 4.35; Cl, 12.62; N, 5.02.

5.2.3.1.1.14. (*Z*)-6-chloro-3-[2-(pyridin-3-yl)vinyl]-4*H*-chromen-4-one hydrochloride (13b). Yield: 31 %, mp = 192-193 °C from ethyl alcohol/diethyl ether. IR (KBr): 1642 cm^{-1}. ^1H NMR (DMSO D6, 400MHz): δ (ppm) 8.77 (s, 1H), 8.64 (d, 1H, J = 5.2 Hz), 8.43 (s, 1H), 8.25 (d, 1H, J = 8.4 Hz), 7.98 (dd, 1H, J = 2.7, 0.9 Hz), 7.90-7.73 (m, 3H), 6.88 (d, 1H, J = 12.2 Hz), 6.65 (d, 1H, J = 12.2 Hz). Anal. Calcd for $C_{16}H_{11}Cl_2NO_2$: C, 60.02; H, 3.46; Cl, 22.15; N, 4.37. Found: C, 59.84; H, 3.51; Cl, 22.01; N, 4.25.

5.2.3.2. Virology

5.2.3.2.1. Cells

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

5.2.3.2.2. 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.

5.2.3.2.3. Viruses

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 (1966). The virus was stored at -80 °C until used.

5.2.3.2.4. XTT assay for cellular cytotoxicity

A tetrazolium-based (XTT) colorimetric assay was used to measure the cytotoxicity of compounds, as previously described.[3] Briefly, HeLa cells were seeded in 96 well tissue culture plates ($2x10^3$ cells/well) in 100 ml of growth medium with or without compounds in two-fold dilutions, starting from the maximum soluble concentration in cell culture medium. Triplicate wells were used for each drug concentration to be tested. The plates were incubated at 37 °C in 5% CO_2-air until the untreated monolayers were confluent (3 days). Then, 50 mL 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 in 100% of cells was the maximum non-cytotoxic concentration (MNTC). 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.

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

The IC_{50} values were determined as described previously.[4] Briefly, monolayers of HeLa cells in 6-well plates were infected with a virus suspension producing approximately 100 plaques per well. After 1h 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 four-fold dilutions of drugs, starting from the MNTC or alternatively from the saturation concentration in cell culture medium. 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 dose/response curves 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.

5.2.3.2.6. Virus inactivation and stabilization

For virus inactivation studies, HRV suspensions with or without **5a** (43 μM and 58 μM for serotype 1B and 14, respectively) were incubated at 33 °C for 1h. After serial ten-fold dilutions, virus titres were measured by plaque assay on HeLa cell monolayers.

For virus stabilization studies, the virus was incubated with or without **5a** (43 μM and 58 μM for serotype 1B and 14, respectively) for 1h 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. Samples were diluted ten-fold serially and titrated by plaque assay.

5.2.3.2.7. Virus yield reduction assays

Confluent monolayers of HeLa cells were infected at a multiplicity of 5 in the presence or absence of **5a** (43 μM and 58 μM for serotype 1B and 14, respectively). Infection was synchronized by allowing HRV to bind in the cold (4 °C). After 1h, the inoculum was removed by washing thrice with cold PBS. The end of virus binding was taken as 0 time. Then, MEM with or without the compound (43 μM and 58 μM for serotype 1B and 14, respectively) 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. To determine which stage of virus replication was affected by **5a**, the drug was added or removed from HRV-infected cells at various times p.i. (0, 15', 30', 45', 1 h, 2 h, 4 h, 6 h) and the cultures incubated at 33 °C up to 10 h p.i. The virus yield was determined as above.

Acknowledgements

We gratefully acknowledge the financial support from "Istituto Pasteur-Fondazione Cenci Bolognetti", Università degli Studi di Roma "La Sapienza". We also thank Dr. Ivano Pindinello for technical assistance.

References

1. Conti, C.; Desideri, N. *Bioorg. Med. Chem.* **2010**, *18*, 6480.
2. Doria, G., Passarotti, C., Sala, R., Magrini, R., Sberze, P., Tibolla, M., Ceserani, R., Castello, R.. *Farmaco*. **1986**, *41*, 417.
3. Scudiero, D. A., Shoemaker, R. H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M. J., Seniff, D., Boyd, M. R.. *Cancer Res*. **1988**, *48*, 4827.
4. Desideri, N., Conti, C., Sestili, I., Tomao, P., Stein, M.L., Orsi, N. *Antiviral Chem. Chemother*. **1992**, *3*, 195.
5. Andries, K., Dewindt, B., Snoeks, J., Wouters, L., Moereels, H., Lewi, P. J., Janssen, P. A. J.. *J. Virol*. **1990**, *64*, 1117.
6. Andries, K., Dewindt, B., Snoeks, J., Willebrords, R., Stokbroekx, R, Lewi, P. J.. *Antiviral Res*., **1991**, *16*, 213.
7. Bauer, D.J., Selway, J.W.T., Batchelor, J.F., Tisdale, M., Caldwell, J.C., Young, D.A.B.. *Nature*. **1981**, 292, 369.
8. Lonberg-Holm, K., Korant, B.D.. *J. Virol.* **1972**, *9*, 29.
9. Lonberg-Holm, K.; Yin, F.H.. *J. Virol*.. **1973**, *12*, 114.
10. Rombaut, B.; Vrijsen, R.; Boeye, A.. *Antiviral Res.,Suppl*. **1985**, *1*, 67.
11. Rombaut, B.; Andries, K.; Boeye, A.. *J. Gen. Virol*. **1991**, *72*, 2153.
12. Fuchs, R., Blaas D.. *Rev. Med. Virol*. **2010**, *20*, 281.
13. Garriga, D., Pickl-Herk, A., Luque, D., Wruss, J, Caston, J.R., Blaas, D., Verdaquer, N.. *PloS Pathog*. **2012**, *8*, e1002473.

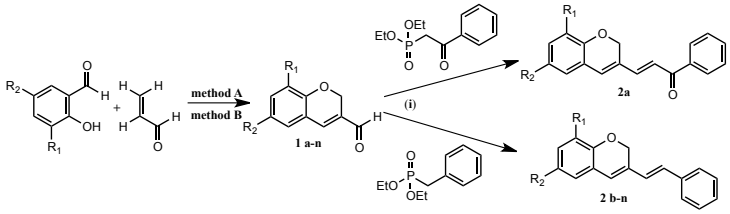
5.3. Synthesis and antiviral activity of 3-styryl-2*H*-chromenes, 3-phenylalkyl-2*H*-chromenes and 3-(3-phenylalkyl)chromans

The results of previous researches prompted us to further explore the effect of simple structural modifications on 3-styryl-2*H*-chromene scaffold.

5.3.1. Results and discussion

5.3.1.1. Chemistry

(*E*)-3-(2*H*-Chromen-3-yl)-1-phenylprop-2-en-1-one **2a** and (*E*)-3-styryl-2*H*-chromenes **2b-n** were synthesized according to the two step procedure shown in scheme 1. The 2*H*-chromene-3-carbaldehydes **1a-n** were initially prepared with some modifications of the literature procedures,[1,2] by refluxing the appropriate 2-hydroxybenzaldehyde and acrolein in the presence of potassium carbonate (method A).



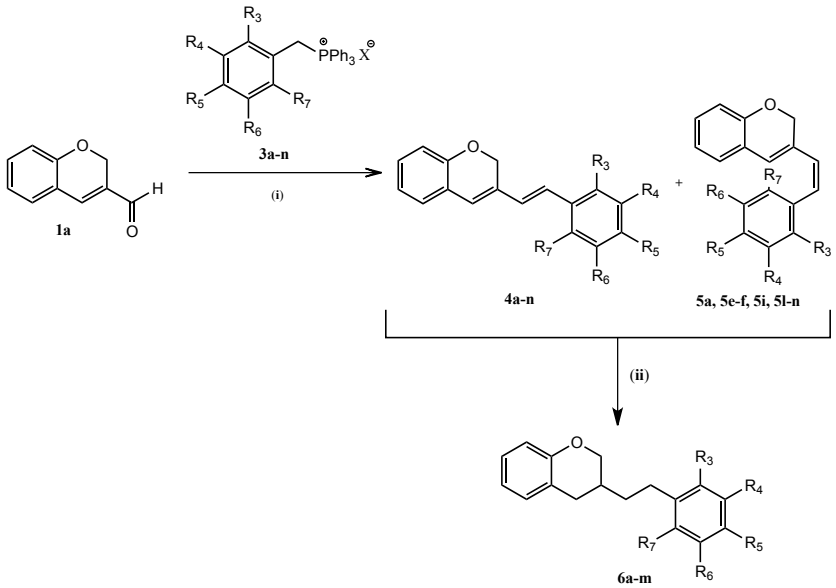
1, 2a	$R_1 = R_2 = H$	**1, 2h**	$R_1 = Cl, R_2 = F$
1, 2b	$R_1 = H, R_2 = Br$	**1, 2i**	$R_1 = Br, R_2 = H$
1, 2c	$R_1 = H, R_2 = NO_2$	**1, 2j**	$R_1 = R_2 = F$
1, 2d	$R_1 = R_2 = Br$	**1, 2k**	$R_1 = F, R_2 = Br$
1, 2e	$R_1 = Cl, R_2 = H$	**1, 2l**	$R_1 = NO_2, R_2 = Br$
1, 2f	$R_1 = R_2 = Cl$	**1, 2m**	$R_1 = Br, R_2 = NO_2$
1, 2g	$R_1 = Br, R_2 = Cl$	**1, 2n**	$R_1 = NO_2, R_2 = H$

Scheme 1. Reagents and conditions: (**method A**) K_2CO_3, dry dioxane, reflux, 5h; (**method B**) DABCO, $CHCl_3/H_2O$ (1:1), rt, 7 days; (**i**) NaH, dry THF, rt, 20 h.

With the aim to improve the yield of this reaction, a more efficient procedure was developed. The reaction was performed in the presence of 1,4–diazabicyclo-[2.2.2]-octane (DABCO) in chloroform and water (1:1), at room temperature (method B). The subsequent Horner–Wadsworth–Emmons reaction of 2*H*-chromene-3-carbaldehydes **1a-n**

with commercially available diethyl (2-oxo-2-phenylethyl)phosphonate or diethyl benzylphosphonate, carried out using sodium hydride as base in dry THF, provided only the (*E*) isomers **2a-n**. The stereochemistry of compounds was established on the basis of the coupling constant values of the protons in the chain ($J_{\alpha\text{-}\beta}$ = 16.0 Hz and ~16.5 Hz for **2a** and **2b–n**, respectively).

E and *Z* 3-styryl-2*H*-chromenes were prepared by the Wittig reaction of 2*H*-chromen-3-carbaldehyde (**1a**) with the appropriate benzylthiphenylphosphonium halide (**3a-n**). While benzyltriphenylphosponium chloride (**3a**), and 4-nitrobenzyltriphenylphosponium bromide (**3n**) are commercially available, the analogues (**3b-m**) were prepared by reaction of the corresponding benzyl alcohol with triphenylphosphine hydrobromide in dry CH_3CN.

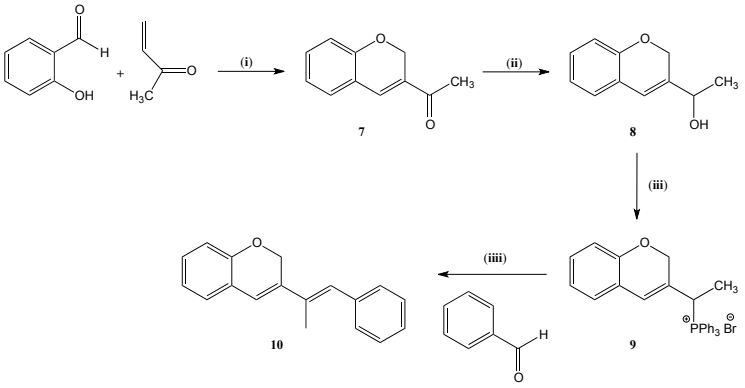


3, 4, 5, 6a $R_3 = R_4 = R_5 = R_6 = R_7 = H$ **3, 4, 6h** $R_3 = Cl$, $R_4 = R_5 = R_6 = R_7 = H$
3, 4, 6b $R_3 = CF_3$, $R_4 = R_5 = R_6 = R_7 = H$ **3, 4, 5, 6i** $R_4 = Cl$, $R_3 = R_5 = R_6 = R_7 = H$
3, 4, 6c $R_4 = CF_3$, $R_3 = R_5 = R_6 = R_7 = H$ **3, 4, 6j** $R_5 = Cl$, $R_3 = R_4 = R_6 = R_7 = H$
3, 4, 6d $R_5 = CF_3$, $R_3 = R_4 = R_6 = R_7 = H$ **3, 4, 6k** $R_3 = Br$, $R_4 = R_5 = R_6 = R_7 = H$
3, 4, 5, 6e $R_3 = R_5 = Cl$, $R_4 = R_6 = R_7 = H$ **3, 4, 5, 6l** $R_4 = Br$, $R_3 = R_5 = R_6 = R_7 = H$
3, 4, 5, 6f $R_4 = R_6 = Cl$, $R_3 = R_5 = R_7 = H$ **3, 4, 5, 6m** $R_5 = Br$, $R_3 = R_4 = R_6 = R_7 = H$
3, 4, 6g $R_3 = R_7 = Cl$, $R_4 = R_5 = R_6 = H$ **3, 4, 5n** $R_5 = NO_2$, $R_3 = R_4 = R_6 = R_7 = H$

Scheme 2. Reagents and conditions: (**i**) *tert*-BuOK, dry THF, rt, 23h; (**ii**) Pd/C 10%, dry dioxane, rt, 30 min.

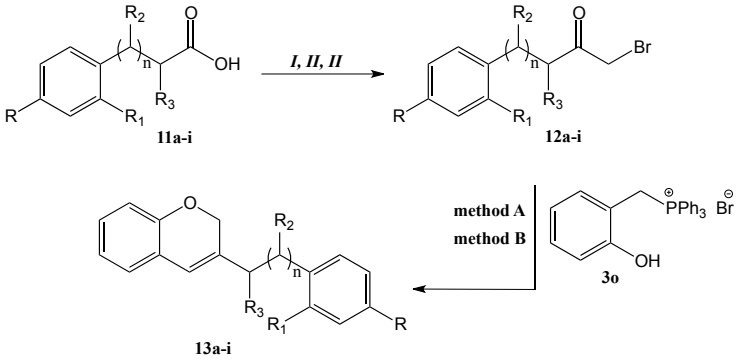
The Wittig reaction was conducted in the presence of potassium *tert*-butoxide in dry THF, at room temperature. The *E*/*Z*-mixtures

(**4/5a, 4/5e-f, 4/5i, 4/5l-n**) obtained by the Wittig reaction were separated into their individual isomers by column chromatography on silica gel. For the analogues 2'-substituted or 2',6'-disubstituted (**4g-h, 4k)** and for trifluoromethyl derivatives (**4b-d**), only the *E* isomers were obtained. The stereochemistry of compounds was established on the basis of the coupling constant values of the protons in the chain (about 16 Hz and 12 Hz for *E* and *Z* isomers, respectively). Subsequent catalytic reduction of *E/Z*-mixtures (**4/5a, 4/5e-f, 4/5i, 4/5l-m)** or (*E*)-isomers (**4b-d, 4g-h, 4j-k)** afforded the corresponding 3-phenethylchromans **6a-m** with good yields (~ 85%) (scheme 2).



Scheme 3. Reagents and conditions: (**i**) DABCO, $CHCl_3/H_2O$ (1:1), rt, 7 days; (**ii**) $NaBH_4$, MeOH, rt, 6h; (**iii**) triphenylphosphine hydrobromide, dry CH_3CN, reflux, 5h; (**iiii**) *tert*- BuOK, dry THF, rt, 23h.

The synthesis of (*E*)-3-(1-phenylpropen-2-yl)-2*H*-chromene **10** started from the preparation of 3-acetyl-2*H*-chromene (**7**) by reaction of 2-hydroxybenzaldehyde and 3-buten-2-one, according to the method B described for the synthesis of (*E*)-3-styryl-2*H*-chromenes (**2b–n**). Subsequent reduction of 3-acetyl-2*H*-chromene (**7**) with sodium borohydride in methanol provided 3-(1-hydroxyethyl)-2*H*-chromene (**8**). This intermediate was then reacted with triphenylphosphine hydrobromide in dry THF, yielding [1-(2*H*-chromen-3-yl)ethyl]triphenylphosphonium bromide (**9**) with good yield. Finally, the target compounds **10** was obtained by the Wittig reaction of benzaldehyde with the triphenylphosphonium bromide **9** (scheme 3).

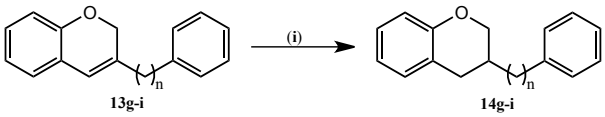


11, 12, 13a	n = 1, R = R_1 = R_2 = R_3 = H
11, 12, 13b	n = 1, R_1 = CF_3, R = R_1 = R_2 = H
11, 12, 13c	n = 1, R = CF_3, R_1 = R_2 = R_3 = H
11, 12, 13d	n = 1, R = Cl, R_1 = R_2 = R_3 = H
11, 12, 13e	n = 1, R_2 = CH_3, R = R_1 = R_3 = H
11, 12, 13f	n = 1, R_3 = CH_3, R = R_1 = R_2 = H
11, 12, 13g	n = 2, R = R_1 = R_2 = R_3 = H
11, 12, 13h	n = 3, R = R_1 = R_2 = R_3 = H
11, 12, 13i	n = 4, R = R_1 = R_2 = R_3 = H

Scheme 4. Reagents and conditions: ***I***) CO_2Cl_2, dry CH_2Cl_2, 0°C, rt, 3h; ***II***) $TMSCHN_2$, dry CH_3CN, 0°C, 2h; ***III***) HBr 48%, 0°C, rt, 18h; (**method A**) EtONa, dry EtOH, rt, 24h; (**method B**) *tert*- BuOK, dry THF, rt, 23h.

Phenylalkyl-2*H*-chromenes (**13a-i**) were synthesized as reported in scheme 4. For this purpose, the required 1-bromomethyl-phenylalkylketone (**12a-i**) was prepared in three steps starting from the treatment of the appropriate acid (**11a-i**) with oxalyl chloride in dry dichloromethane. The obtained acyl chloride was converted into the target 1-bromomethylketone (**12a-i**) modifying the method described by Gangjee.[3] In our synthetic approach, the resulting acyl chloride was treated with trimethylsilyldiazomethane ($TMSCHN_2$), used as a stable and safe substituted for hazardous diazomethane. The intermediate diazoketone was treated in situ with 48% aqueous HBr to provide the desired **12a-i** in excellent yields. In addition, (2-hydroxybenzyl)triphenylphosphonium bromide (**3o**) was obtained, in almost quantitative yield, by reaction of 2-hydroxybenzyl alcohol with triphenylphosphine hydrobromide in refluxing acetonitrile. Subsequent reaction of **12a-i** with **3o** in the presence of a strong base, allowed alkylation of the phenolic hydroxyl group and cyclization by an intramolecular Wittig reaction. The reaction was carried out at room temperature using a base such as sodium ethoxide in dry ethanol (compounds **13a, d)** or *tert*-BuOK in dry THF (compounds **13b-c,**

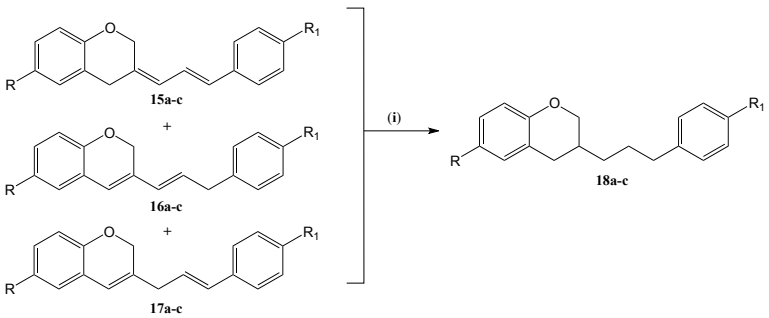
13e-i) (scheme 4).



13, 14g n = 3 **13, 14h** n = 4 **13, 14i** n = 5

Scheme 5. Reagents and conditions: (**i**) Pd/C 10%, dry dioxane, rt, 30 min.

Subsequent catalytic hydrogenation of phenylalkyl-2*H*-chromenes **13g-i** afforded the corresponding 3-(3-phenylalkyl)chromans **14g-i** with high yields (scheme 5).
As shown in scheme 6, the chloro substituted 3-(3-phenylpropyl)chromans (**18a-c**) were prepared by catalytic hydrogenation of the appropriate mixture of (*Z*)-3-[(*E*)-3-phenylallylidene]-chroman, (*E*)-3-(3-phenyl) prop-1-en-1-yl)-2*H*-chromene, and 3-cinnamyl-2*H*-chromene (**15, 16, 17a**; **15, 16, 17b** or **15, 16, 17c**). As previously reported,[4] these mixture were obtained by reduction of the appropriate (*E*)-3-[(*E*)-3-phenylallylidene]chroman-4-one with lithium aluminum hydride.



15, 16, 17, 18a R = Cl, R_1 = H
15, 16, 17, 18b R = R_1 = Cl
15, 16, 17, 18c R = H, R_1 =Cl

Scheme 6. Reagents and conditions: (**i**) Pd/C 10%, dry dioxane, rt, 15 min.

5.3.1.2. Anti-HRV assays

In preliminary studies, the cytotoxicity of all the new

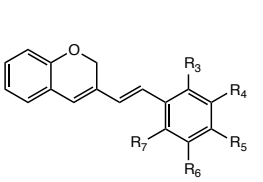
synthesized compounds (**4a-m, 5e-f, 5i, 5l-m, 6a-m, 10, 13a-i, 14g-i, 18a-c**) was determined by evaluating the effects on morphology, viability and growth of HeLa (Ohio) cells, a human cell line suitable for the replication of HRVs. Morphological alterations were scored microscopically, and the action of the compounds on logarithmic cell growth was determined by the XTT colorimetric method.[5] 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.

The inhibitory activity of all the compounds on HRV replication was evaluated in a plaque reduction assay, starting from the MNTC, as already described.[6]

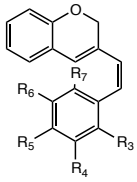
The results of cytotoxicity and anti-HRV activity of (*E*) and (*Z*) 3-styryl-2*H*-chromenes (**4a-m, 5e-f, 5i, 5l-m**) and (*E*)-3-(1-phenylprop-1-en-2-yl)-2*H*-chromene (**10**) are reported in table 1. When the IC_{50} value was higher than the MNTC, the percentage inhibition at this dose is reported in parentheses. For comparison, table 1 includes previously reported data of (*E*)-3-styryl-2*H*-chromene (**4a**).

Within the 3-styrylchromene series, all compounds presented low cytotoxicity against HeLa cells showing TC_{50} ranging from 12.5 μM to > 200 μM (the highest concentration tested). Moreover, the new synthesize analogues exhibited antiviral activity against HRV 1B in the micromolar or submicromolar range (table 1).

Although the introduction of electron withdrawing substituents on phenyl ring did not provide improvements in potency with respect to unsubstituted 3-styrylchromene **4a**, several analogues (**4e, 5f, 4g, 4j** and **5m**) presented antiviral potency in the submicromolar range. The high inhibitory activity on HRV 1B coupled with the low cytotoxicity resulted in compounds as selective as **4a** (**4j**, TI = 250.00) or more selective than **4a** (**4e,** TI = 333.33; **5f,** TI = 344.83; **5m,** TI = 375.00). A comparison among the activity of *E* and *Z* isomers (**4f**/**5f, 4i/5i, 4l/5l, 4m/5m**) showed that the *Z* isomers exhibited a higher potency than corresponding *E* isomers against HRV 1B. Only the *Z* isomer **4e** was about 8-fold more active than *E* isomer **5e** against this serotype.

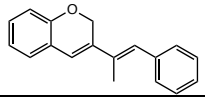


4a-m



5a, 5e-f, 5i, 5l-m

Table 1. Cytotoxicity and anti-HRV activity of (*E*)-3-styryl-2*H*-chromenes **4a-m**, (*Z*)-3-styryl-2*H*-chromenes **5e-f, 5i, 5l-m,** and (*E*)-3-(1-phenylpropen-2-yl)-2*H*-chromene **10.**

Comp.	R_3	R_4	R_5	R_6	R_7	MNTC (μM)[a]	TC_{50} (μM)[b]	IC_{50} (μM)[c] HRV 1B	TI[d]	IC_{50} (μM)[c] HRV 14	TI[d]
4a	H	H	H	H	H	25.00	50.00	0.20	250.00	1.38	36.23
4b	CF_3	H	H	H	H	25.00	100.00	5.76	17.40	2.06	48.54
4c	H	CF_3	H	H	H	100.00	>200.00[e]	4.69	>42.6	>100.00 (30%)	-
4d	H	H	CF_3	H	H	100.00	>200.00[e]	20.19	>9.90	>100.00 (27%)	-
4e	Cl	H	Cl	H	H	25.00	100.00	0.30	333.33	NT[h]	-
5e	Cl	H	Cl	H	H	25.00	100.00	2.59	38.61	NT[h]	-
4f	H	Cl	H	Cl	H	3.12	12.50	> 3.12 (33%)	-	NT[h]	-
5f	H	Cl	H	Cl	H	25.00	100.00	0.29	344.83	NT[h]	-
4g	Cl	H	H	H	Cl	50.00	75.00	0.70	107.14	NT[h]	-
4h	Cl	H	H	H	H	50.00	150.00	12.50[f]	12.00	2.92	51.37
4i	H	Cl	H	H	H	50.00	75.00	50.00	1.50	2.53	29.64
5i	H	Cl	H	H	H	25.00	36.50	4.28	8.53	25.00	1.46
4j	H	H	Cl	H	H	50.00	100.00	0.40	250.00	18.39	5.44
4k	Br	H	H	H	H	12.50	75.00	8.28[f]	9.06	NT[h]	-
4l	H	Br	H	H	H	50.00	75.00	5.84	12.84	NT[h]	-
5l	H	Br	H	H	H	12.50	37.50	4.45	8.43	NT[h]	-
4m	H	H	Br	H	H	50.00	100.00	1.42[f]	70.42	NT[h]	-
5m	H	H	Br	H	H	50.00	150.00	0.40	375.00	NT[h]	-
10						12.50	25.00	IN[g]	-	IN[g]	-

[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.

[b]The TC_{50} value was the concentration of compound which reduced the Hela cell viability by 50%, as compared with the control.

[c] The IC_{50} value was the dose of compound reducing the plaque number by 50% and was calculated by plotting the log of 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] Compounds producing a reduction in the mean viral plaque size, beside an effect on plaque number.

[g] Not active up to the highest concentration tested (MNTC).

[h] Not tested.

It is noteworthy that some analogues (**4b, 4h** and **4i**) were more effective against HRV14 than HRV1B. Moreover the higher potency were coupled with a good selectivity.

On the contrary, the addition of a methyl group in the linker chain between the two cycles (**10**) cancel the efficacy against both serotypes.

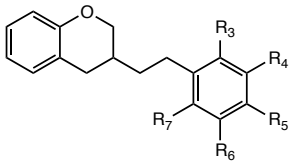
In order to evaluate the impact on activity and cytotoxicity of the reduction of both double bounds, the 3-phenethylchromans **6a-m** were synthesized as racemates, and tested. The results of the biological evaluation are reported in table 2.

Generally, the 3-phenethylchromans **(6a-m)** showed low cytotoxicity against HeLa cells, with four analogues **(6h, 6i, 6l, 6m)** exhibiting TC_{50} higher than the saturation concentration (200 μM) in cell culture medium. However, in this series of compounds, the more active analogues against HRV 1B (**6a** and **6e**) were also the more cytotoxic. In particular, the reduction of both double bounds of **4a** to give 3-phenethylchroman **(6a)** caused a 2-fold decrease in the inhibitory effect against HRV1B and a dramatic loss of activity against HRV14. Moreover, despite the anti-HRV1B effect within the submicromolar range (IC_{50} = 0.49 μM), **6a** showed a modest therapeutic index (TI = 12.75) due its significant cytotoxicity (TC_{50} = 6.25 μM).

Also the 2,4-dichloro derivative **6e**, exhibiting an anti-HRV1B effect in the submicromolar range, were about 2-fold less potent (IC_{50} = 0.58 μM) and about 8-fold less selective (TI = 43.10) than the corresponding 3-styrylchromene **4e**.

Generally, the tested compounds exhibited higher potency against HRV1B than HRV14. Only the 2-trifluoromethyl derivative **6b** showed comparable potency against both HRV serotypes (IC_{50} = 6.27 μM and 6.20 μM against HRV 1B and 14, respectively). However, the broader spectrum of anti-HRV activity was coupled with modest therapeutic indexes (TI = 11.96 and 12.09 against HRV 1B and 14, respectively).

On the contrary, the 3-chloro analogue **6i** displayed higher efficacy against HRV14 than HRV1B (IC_{50} = 4.84 μM and 2.94 μM against HRV 1B and 14, respectively) coupled with remarkable selectivity (TI > 41.32 and > 68.03 against HRV 1B and 14, respectively).



6a-m

Table 2. Cytotoxicity and anti-HRV activity of 3-phenylethylchromans **6a-m.**

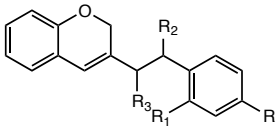
Comp.	R_3	R_4	R_5	R_6	R_7	MNTC (μM)[a]	TC_{50} (μM)[b]	IC_{50} (μM)[c] HRV 1B	TI[d]	IC_{50} (μM)[c] HRV 14	TI[d]
6a	H	H	H	H	H	3.12	6.25	0.49	12.75	>3.12 (16%)	-
6b	CF_3	H	H	H	H	50.00	75.00	6.27	11.96	6.20	12.09
6c	H	CF_3	H	H	H	25.00	75.00	6.49	11.55	>25.00 (26%)	-
6d	H	H	CF_3	H	H	25.00	100.00	2.50	40.00	10.52	9.51
6e	Cl	H	Cl	H	H	6.25	25.00	0.58	43.10	NT[g]	-
6f	H	Cl	H	Cl	H	50.00	100.00	21.16	4.73	NT[g]	-
6g	Cl	H	H	H	Cl	50.00	75.00	7.74	9.69	NT[g]	-
6h	Cl	H	H	H	H	100.00	>200.00[e]	6.54	>30.58	20.28	>9.86
6i	H	Cl	H	H	H	100.00	>200.00[e]	4.84[f]	>41.32	2.94	>68.03
6j	H	H	Cl	H	H	50.00	200.00	1.66[f]	120.48	18.27	10.95
6k	Br	H	H	H	H	100.00	150.00	5.08[f]	29.53	NT[g]	-
6l	H	Br	H	H	H	200.00	>200.00[e]	3.83[f]	>52.22	63.95	>3.13
6m	H	H	Br	H	H	200.00	>200.00[e]	2.12[f]	>94.34	34.27	>5.84

[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.
[b]The TC_{50} value was the concentration of compound which reduced the Hela cell viability by 50%, as compared with the control.
[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] Compounds producing a reduction in the mean viral plaque size, beside an effect on plaque number.
[g] Not tested.

A small series of 3-phenylalkyl-2*H*-chromenes (**13a-f**) were also prepared. The results of the biological evaluation are reported in table 3. When compared to the corresponding 3-phenethylchroman **(6a**), the unsubstituted 3-phenylethyl-2*H*-chromene (**13a**) exhibited a similar potency against HRV 1B (IC_{50} = 0.48 μM) coupled with a better selectivity (TI = 103.09). The efficacy towards the serotype 14 remained modest up to the MNTC. The introduction of a chlorine atom at the 4'-position resulted in compound **13d** with the same potency than **13a** against HRV 1B and 3-fold more selective. However, **13d** were totally inactive against HRV 14. The

corresponding 4'-trifluoromethyl analogue **13c** presented good and comparable efficacy against both HRV serotypes (IC_{50} = 1.56 μM and 2.03 μM against HRV1B and 14, respectively), however **13c** showed modest therapeutic indexes (TI = 12.00 and 9.23 against HRV1B and 14, respectively).

Also within the phenylethyl-2*H*-chromene series, the addition of a methyl group in the α-position of the linker chain between the two cycles (**13f**) resulted in a loss of activity against HRV 1B. In contrast, the shift of the methyl group to the β-position led to the isomer **13e** endowed with good activity against HRV 1B (IC_{50} = 1.17 μM) but low selectivity (TI = 10.68). Both analogues exhibited a modest inhibitory effect on HRV 14 replication. For these reasons, branched chains into the spacer were not further explored.



13a-f

Table 3. Cytotoxicity and anti-HRV activity of phenylalkyl-2*H*-chromenes **13a-f.**

Comp.	R	R_1	R_2	R_3	MNTC (μM)[a]	TC_{50} (μM)[b]	IC_{50} (μM)[c] HRV 1B	TI[d]	IC_{50} (μM)[c] HRV 14	TI[d]
13a	H	H	H	H	25.00	50.00	0.48	103.09	>25.00 (26%)	-
13b	H	CF_3	H	H	12.50	37.50	>12.50 (14%)	-	4.57	8.21
13c	CF_3	H	H	H	6.25	18.75	1.56	12.00	2.03	9.23
13d	Cl	H	H	H	50.00	150.00	0.48	309.28	IN[e]	-
13e	H	H	CH_3	H	6.25	12.50	1.17	10.68	>6.25 (5%)	-
13f	H	H	H	CH_3	12.50	25.00	IN[e]	-	>12.50 (31%)	-

[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.

[b]The TC_{50} value was the concentration of compound which reduced the Hela cell viability by 50%, as compared with the control.

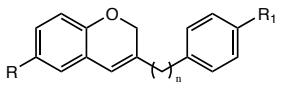
[c] The IC_{50} value was the dose of compound reducing the plaque number by 50% and was calculated by plotting the drug concentration versus the percentage of plaque reduction. When a 50% reduction was not achieved, the percent of inhibition obtained at the MNTC was reported in parentheses.

[d] The therapeutic index (TI) value was equal to TC_{50}/IC_{50}.

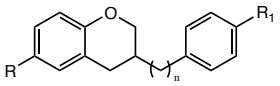
[e] Not active up to the highest concentration tested (MNTC).

In order to evaluate the impact on cytotoxicity and antiviral activity of the linker elongation, two new series of 3-phenylalkyl-2*H*-chromenes (**13g-i**) and 3-phenylalkylchromanes (**14g-i** and **18a-c**) were prepared and tested (table 4).

The elongation up to 5 carbon atoms of the linear, polymethylene spacer connecting 2*H*-chromene with phenyl ring furnished analogues **13g-i** showing very low cytotoxicity, potent inhibitory activity and high selectivity against HRV 1B. The corresponding chromane analogues **14g-i** maintained the low cytotoxicity but exhibited a lower efficacy than chromenes **13g-i** against serotype 1B. Both series of compounds were endowed with a weak activity against HRV14.



13g-i



14g-i, 18a-c

Table 4. Cytotoxicity and anti-HRV activity of phenylalkyl-2*H*-chromenes **13g-i**, 3-(phenylalkyl) chromans **14g-i**, and **18a-c**.

Comp.	n	R	R_1	MNTC (μM)[a]	TC50 (μM)[b]	IC_{50} (μM)[c] HRV 1B	TI[d]	IC_{50} (μM)[c] HRV 14	TI[d]
13g	3	H	H	100.00	200.00	2.37	84.21	40.47	4.94
13h	4	H	H	50.00	100.00	0.54	183.82	12.70	7.87
13i	5	H	H	50.00	100.00	0.56	178.57	>50.00 (37%)	-
14g	3	H	H	100.00	200.00	9.59	20.85	>100.00 (37%)	-
14h	4	H	H	50.00	100.00	0.71	140.45	>50.00 (25%)	-
14i	5	H	H	100.00	200.00	4.74	42.18	33.08	6.05
18a	3	Cl	H	100.00	200.00	1.79	111.73	25.00	8.00
18b	3	Cl	Cl	100.00	200.00	0.56	357.78	30.00	6.66
18c	3	H	Cl	50.00	100.00	0.48	206.18	1.36	73.26

[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.

[b]The TC_{50} value was the concentration of compound which reduced the Hela cell viability by 50%, as compared with the control.

[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}.

The introduction of chlorine at the 4' and/or 6 position of the 3-(3-phenylpropyl)chroman **14g** led to derivatives **18a-c** showing improved potency and selectivity against both serotypes, with respect to **14g**. In particular, 3-[3-(4-chlorophenyl)propyl]chroman **18c** showed a broad spectrum of anti-HRV activity and displayed the highest level of activity against both serotypes (IC_{50} of 0.48 μM and 1.36 μM towards HRV 1B and 14, respectively) coupled with significant selectivity (TI = 206.18 and 73.26, respectively). Therefore, this chroman is a good candidate for further studies.

5.3.1.3. Antiviral assays against selected RNA and DNA viruses

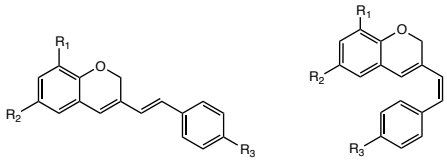
To evaluate the selectivity of the new compounds as anti-HRV inhibitors, selected derivatives were evaluated against a large panel of RNA and DNA viruses. Among single-stranded, positive RNA viruses ($ssRNA^+$), we considered two Picornaviruses (Coxsackie Virus type-5, CVB-5 and Poliovirus type-1, Sabin strain, Sb-1), a Flavivirus (Yellow Fever Virus, YFV) and a Pestivirus (Bovine Viral Diarrhea Virus, BVDV). Among single-stranded, negative RNA viruses ($ssRNA^-$) a Paramyxoviridae (Respiratory Syncytial Virus, RSV) and a Rhabdoviridae (Vesicular Stomatitis Virus, VSV) were selected as representatives. Among double-stranded RNA (dsRNA) viruses, a Reoviridae family member (Reo-1) was included. Finally, two representatives of DNA virus families were also included: Herpes Simplex Virus type-1, HSV-1 (Herpesviridae) and Vaccinia Virus, VV (Poxviridae). Pleconaril, ribavirin, 6-azauridine, acyclovir and mycophenolic acid were used as reference inhibitors.

Cytotoxicity and antiviral activities of all the new synthesized and reference compounds are summarized in tables 5-7.

All the tested compounds showed a low or no toxicity against the cellular lines utilized (MDBK, BHK-21, Vero 76). Many analogues showed TC_{50} values higher than the maximum tested concentration (100 μM).

With the exception of (*E*)-8-chloro-3-styryl-2*H*-chromene (**2e**) and 3-phenethyl-2*H*-chromene (**13a)**, the chromene and chroman derivatives were unable to interfere with the virus replication. (*E*)-8-Chloro-3-styryl-2*H*-chromene (**2e**) showed a moderate activity against RSV (IC_{50} = 37 μM). The introduction of an additional halogen at the 6 position (**2f, 2h**) or the replacement of the chlorine with a bromine atom (**2i**) abolished the activity.

Furthermore, 3-(2-phenylethyl)-2*H*-chromene (**13a**) interfered with the replication of BVDV and YFV, both belonging to the *Flaviviridae* family. Although **13a** exhibited a modest potency, this chromene displayed comparable efficacy than the reference inhibitor ribavirin against YFV (IC_{50} = 60 μM and 50 μM, respectively). Concerning its cytotoxicity, **13a** showed no toxicity up to the highest concentration tested (100 μM) towards the cellular lines suitable for the replication of BVDV and YFV (MDBK and BHK21, respectively), while ribavirin presented a higher toxicity against MDBK (TC_{50} = 47 μM) than **13a.**



2b-n, 4n **5n**

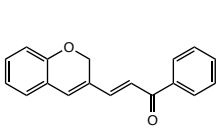
2b: R_1 = H, R_2 = Br, R_3 = H
2c: R_1 = H, R_2 = NO_2, R_3 = H
2d: R_1 = Br, R_2 = Br, R_3 = H
2e: R_1 = Cl, R_2 = H, R_3 = H
2f: R_1 = Cl, R_2 = Cl, R_3 = H
2g: R_1 = Br, R_2 = Cl, R_3 = H
2h: R_1 = Cl, R_2 = F, R_3 = H
2i: R_1 = Br, R_2 = H, R_3 = H
2j: R_1 = F, R_2 = F, R_3 = H
2k: R_1 = F, R_2 = Br, R_3 = H
2l: R_1 = NO_2, R_2 = Br, R_3 = H
2m: R_1 = Br, R_2 = NO_2, R_3= H
2n: R_1 = NO_2, R_2 = H, R_3 = H
4n: R_1 = H, R_2 = H, R_3 = NO_2
5n: R_1 = H, R_2 = H, R_3 = NO_2

Table 5. Cytotoxicity and antiviral activity of (*E*)-3-styryl-2*H*-chromenes **2b-n, 4n**, and (*Z*)-3-styryl-2*H*-chromene **5n**.

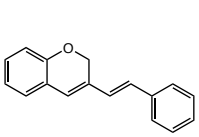
Comp.	MDBK	BVDV	BHK21	YFV	Reo1	Vero76	HSV1	VV	VSV	CVB5	Sb1	RSV
	TC_{50}[a]	IC_{50}[b]	TC_{50}[c]	IC_{50}[d]	IC_{50}[d]	TC_{50}[e]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]
2b	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2c	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2d	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2e	>100	>100	>100	>100	>100	80	>80	>80	>80	>80	>80	37
2f	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2g	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2h	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2i	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2j	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2k	>100	>100	>100	>100	>100	90	>90	>90	>90	>90	>90	>90
2l	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2m	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2n	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
4n	>100	>100	>100	>100	>100	12	>12	>12	>12	>12	>12	>12
5n	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
A	-	-	-	-	-	60	-	-	-	0.004	3	-
B	-	-	-	-	-	>100	0.3	-	-	-	-	-
C	-	-	-	-	-	20	-	0.8	-	-	-	
D	-	-	>100	-	30	6	-	-	-	-	-	0.4
E	47	15	>100	50	-	-	-	-	-	-	-	-

[a]Concentration of the compound (μM) that reduces the viability of MDBK cells by 50% compared to control, determined using the MTT method.
[b]Concentration of the compound (μM) required to produce a 50% protection of MDBK cells by the effect of cytopathic BVDV, determined using the MTT method.
[c]Concentration of the compound (μM) that reduces the viability of the monolayer of BHK cells by 50% compared to control, determined using the MTT method.

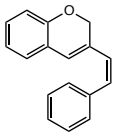
[d]Concentration of the compound (μM) required to produce a 50% protection of BHK cells cytopathic effect induced by YFV, Reo-1, determined using the MTT method.
[e]Concentration of the compound (μM) that reduces the viability of the cells VERO-76 by 50%, compared to the control, as determined by the MTT method.
[f]Concentration of the compound (μM) required to produce a 50% protection of VERO-76 cells by the effect of cytopathic HSV-1, VV, VSV, CVB-5 Sb-1, RSV, determined using the MTT method.
A = Pleconaril, **B** = Acyclovir, **C** = Mycophenolic acid, **D** = 6-Azauridine, **E** = Ribavirin.



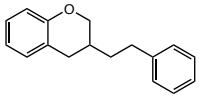
2a



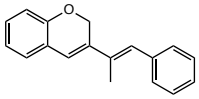
4a



5a



6a

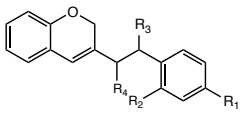


10

Table 6. Cytotoxicity and antiviral activity of (*E*)-3-(2*H*-chromen-3-yl)-1-phenylprop-2-en-1-one **2a**, (*E*)-3-styryl-2*H*-chromene hromene **5a**, 3-phenylethylchromans **6a** and (*E*)-3-(1-phenylpropen-2-yl)-2*H*-chromene **10**.

Comp.	MDBK	BVDV	BHK21	YFV	Reo1	Vero76	HSV1	VV	VSV	CVB5	Sb1	RSV
	TC_{50}[a]	IC_{50}[b]	TC_{50}[c]	IC_{50}[d]	IC_{50}[d]	TC_{50}[e]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]
2a	>100	>100	39	>39	>39	80	>80	>80	>80	>80	>80	>80
4a	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
5a	>100	>100	>100	>100	>100	85	>85	>85	>85	>85	>85	>85
6a	>100	>100	>100	>100	>100	80	>80	>80	>80	>80	>80	>80
10	>100	>100	>100	>100	>100	90	>90	>90	>90	>90	>90	>90
A	-	-	-	-	-	60	-	-	-	0.004	3	-
B	-	-	-	-	-	>100	0.3	-	-	-	-	-
C	-	-	-	-	-	20	-	0.8	-	-	-	-
D	-	-	>100	-	30	6	-	-	-	-	-	0.4
E	47	15	>100	50	-	-	-	-	-	-	-	-

[a]Concentration of the compound (μM) that reduces the viability of MDBK cells by 50% compared to control, determined using the MTT method.
[b]Concentration of the compound (μM) required to produce a 50% protection of MDBK cells by the effect of cytopathic BVDV, determined using the MTT method.
[c]Concentration of the compound (μM) that reduces the viability of the monolayer of BHK cells by 50% compared to control, determined using the MTT method.
[d]Concentration of the compound (μM) required to produce a 50% protection of BHK cells cytopathic effect induced by YFV, Reo-1, determined using the MTT method.
[e]Concentration of the compound (μM) that reduces the viability of the cells VERO-76 by 50%, compared to the control, as determined by the MTT method.
[f]Concentration of the compound (μM) required to produce a 50% protection of VERO-76 cells by the effect of cytopathic HSV-1, VV, VSV, CVB-5 Sb-1, RSV, determined using the MTT method.
A = Pleconaril, **B** = Acyclovir, **C** = Mycophenolic acid, **D** = 6-Azauridine, **E** = Ribavirin



13a: $R_1 = R_2 = R_3 = R_4 =$ H,
13d: $R_1 =$ Cl, $R_2 = R_3 = R_4 =$ H
13b: $R_1 = R_3 = R_4 =$ H, $R_2 = CF_3$
13e: $R_1 = R_2 = R_4 =$ H, $R_3 = CH_3$
13c: $R_1 = CF_3$, $R_2 = R_3 = R_4 =$ H
13f: $R_1 = R_2 = R_3 =$ H, $R_4 = CH_3$

Table 7. Cytotoxicity and antiviral activity of phenylalkyl-2*H*-chromenes **13a-f.**

Comp.	MDBK	BVDV	BHK21	YFV	Reo1	Vero76	HSV1	VV	VSV	CVB5	Sb1	RSV
	TC_{50}[a]	IC_{50}[b]	TC_{50}[c]	IC_{50}[d]	IC_{50}[d]	TC_{50}[e]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]	IC_{50}[f]
13a	>100	79	>100	60	>100	80	>80	>80	>80	>80	>80	>80
13b	31	>31	>100	>100	>100	55	>55	>55	>55	>55	>55	>55
13c	76	>76	80	>80	>80	75	>75	>75	>75	>75	>75	>75
13d	>100	>100	81	>81	>81	65	>65	>65	>65	>65	>65	>65
13e	>100	>100	>100	>100	>100	65	>65	>65	>65	>65	>65	>65
13f	>100	>100	>100	>100	>100	70	>70	>70	>70	>70	>70	>70
A	-	-	-	-	-	60	-	-	-	0.004	3	-
B	-	-	-	-	-	>100	0.3	-	-	-	-	-
C	-	-	-	-	-	20	-	0.8	-	-	-	-
D	-	-	>100	-	30	6	-	-	-	-	-	0.4
E	47	15	>100	50	-	-	-	-	-	-	-	-

[a]Concentration of the compound (μM) that reduces the viability of MDBK cells by 50% compared to control, determined using the MTT method.
[b]Concentration of the compound (μM) required to produce a 50% protection of MDBK cells by the effect of cytopathic BVDV, determined using the MTT method.
[c]Concentration of the compound (μM) that reduces the viability of the monolayer of BHK cells by 50% compared to control, determined using the MTT method.
[d]Concentration of the compound (μM) required to produce a 50% protection of BHK cells cytopathic effect induced by YFV, Reo-1, determined using the MTT method.
[e]Concentration of the compound (μM) that reduces the viability of the cells VERO-76 by 50%, compared to the control, as determined by the MTT method.
[f]Concentration of the compound (μM) required to produce a 50% protection of VERO-76 cells by the effect of cytopathic HSV-1, VV, VSV, CVB-5 Sb-1, RSV, determined using the MTT method.
A = Pleconaril, **B** = Acyclovir, **C** = Mycophenolic acid, **D** = 6-Azauridine, **E** = Ribavirin

5.3.2. Experimental

5.3.2.1. Chemistry

Chemicals were purchased from Sigma-Aldrich and used without further purification. Melting points were determined on a Stanford Research Systems OptiMelt (MPA-100) apparatus and are uncorrected. 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 (Merck, Kieselgel or Aluminium oxide 60 F254). Components were visualised by UV light. Elemental analyses (C, H, N, Cl, Br) of all new compounds were within ±0.4% of theoretical values.

5.3.2.1.1. General procedure for the synthesis of the 2*H*-chromene-3-carbaldehydes (1 a-n) methods A and B.

Method A

Acrolein (75 mmol) was added dropwise to a suspension of the appropriate 2-hydroxybenzaldehyde (50 mmol) and K_2CO_3 (79 mmol) in dry dioxane (115 mL). The mixture was refluxed for 4 h. After cooling, the solvent was removed under reduced pressure and the residue was distributed between CH_2Cl_2 and 2N NaOH. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was purified by column chromatography on silica gel eluting with a mixture of dichloromethane and light petroleum (1:1).

Method B

Acrolein (15 mmol) was added dropwise to a suspension of the appropriate 2-hydroxybenzaldehyde (10 mmol) and 1,4–diazabicyclo-[2.2.2]-octane (DABCO) (8 mmol) in $CHCl_3$ (7 mL) and water (7 mL) at room temperature. The mixture was stirred at room temperature and under nitrogen atmosphere for a week. After this period, the separated solid was filtered and washed with water. The organic layer was washed with 2N NaOH, 2N HCl, and brine, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified as described in method A.

5.3.2.1.1.1. 2*H*-Chromene-3-carbaldehyde (1a). Yield: 27% (method B), mp = 40-41 °C (lit 42-43 °C).[7] The compound exhibited spectroscopic data identical to those previously reported.[7]

5.3.2.1.1.2. 6-Bromo-2*H*-chromene-3-carbaldehyde (1b). Yield: 44% (method A), 27% (method B), mp = 110-111 °C. IR (KBr): 2811, 2708, 1665 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.59 (s, 1H, CHO), 7.37 (dd, 1H, H7, $J_{7\text{-}8}$ = 8.4 Hz, $J_{7\text{-}5}$ = 2.4 Hz), 7.33 (d, 1H, H5,

$J_{7\text{-}5}$ = 2.4 Hz), 7.17 (s, 1H, H4), 6.78 – 6.75 (m, 1H, H8), 5.18 (s, 2H, H2).

5.3.2.1.1.3. 6-Nitro-2*H*-chromene-3-carbaldehyde (1c). Yield: 1% (method A), 18% (method B), mp = 194-195 °C. IR (KBr): 2844, 2741, 1673, 1505, 1338 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.65 (s, 1H, CHO), 8.17 (dd, 1H, H7, $J_{7\text{-}8}$ = 9.2 Hz, $J_{7\text{-}5}$ = 2.8 Hz), 8.13 (d, 1H, H5, $J_{7\text{-}5}$ = 2.8 Hz), 7.81 (s, 1H, H4), 6.95 (d, 1H, H8, $J_{7\text{-}8}$ = 9.2 Hz), 5.38 (s, 2H, H2).

5.3.2.1.1.4. 6,8-Dibromo-2*H*-chromene-3-carbaldehyde (1d). Yield: 3% (method A), 30% (method B), mp = 143-144 °C. IR (KBr): 2848, 2736, 1676 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.62 (s, 1H, CHO), 7.63 (d, 1H, H5, $J_{7\text{-}5}$ = 2.4 Hz), 7.28 (d, 1H, H7, $J_{7\text{-}5}$ = 2.4 Hz), 7.14 (d, 1H, H4, $J_{2\text{-}4}$ = 1.2 Hz), 5.14 (d, 2H, H2, $J_{2\text{-}4}$ = 1.2 Hz).

5.3.2.1.1.5. 8-Chloro-2*H*-chromene-3-carbaldehyde (1e). Yield: 6% (method A), 20% (method B), mp = 97-98 °C. IR (KBr): 2812, 2723, 1668 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.61 (s, 1H, CHO), 7.35 (dd, 1H, H5, $J_{5\text{-}6}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 7.23 (d, 1H, H4, $J_{2\text{-}4}$ = 1.2 Hz), 7.12 (dd, 1H, H7, $J_{7\text{-}6}$ = 7.6 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.90 (dd, 1H, H6, $J_{5\text{-}6}$ = 8.0 Hz, $J_{7\text{-}6}$ = 7.6 Hz), 5.15 (d, 2H, H2, $J_{2\text{-}4}$ = 1.2 Hz).

5.3.2.1.1.6. 6,8-Dichloro-2*H*-chromene-3-carbaldehyde (1f). Yield: 1% (method A), 25% (method B), mp = 124-126 °C. IR (KBr): 2851, 2723, 1678 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.62 (s, 1H, CHO), 7.34 (d, 1H, H7, $J_{7\text{-}5}$ = 2.4 Hz), 7.16 (d, 1H, H4, $J_{2\text{-}4}$ = 1.6 Hz), 7.11 (d, 1H, H5, $J_{7\text{-}5}$ = 2.4 Hz), 5.14 (d, 2H, H2, $J_{2\text{-}4}$ = 1.6 Hz).

5.3.2.1.1.7. 8-Bromo-6-chloro-2*H*-chromene-3-carbaldehyde (1g). Yield: 2% (method A), 40% (method B), mp = 151-153 °C. IR (KBr): 2846, 2738 1677 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.62 (s, 1H, CHO), 7.50 (d, 1H, $J_{7\text{-}5}$ = 2.8 Hz), 7.15 (d, 1H, H5, $J_{7\text{-}5}$ = 2.8 Hz), 7.12 (s, 1H, H4), 5.15 (s, 2H, H2).

5.3.2.1.1.8. 8-Chloro-6-fluoro-2*H*-chromene-3-carbaldehyde (1h). Yield: 13% (method A), 44% (method B), mp = 119-121 °C. IR (KBr): 2853, 2721, 1661 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.63 (s, 1H, CHO), 7.17 (d, 1H, H4, $J_{2\text{-}4}$ = 1.2 Hz), 7.12 (d, 1H, H7, $J_{7\text{-}5}$ = 2.8 Hz), 6.87 (d, 1H, H5, $J_{7\text{-}5}$ = 2.8 Hz), 5.11 (s, 2H, H2, $J_{2\text{-}4}$ = 1.2 Hz).

5.3.2.1.1.9. 8-Bromo-2*H*-chromene-3-carbaldehyde (1i). Yield: 17% (method A), 20% (method B), mp = 110-101 °C. IR (KBr): 2811, 2715, 1665 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.61 (s, 1H, CHO), 7.51 (dd, 1H, H5, J_{5-6} = 8.0 Hz, J_{7-5} = 1.2 Hz), 7.22 (d, 1H, H4, J_{2-4} = 1.2 Hz), 7.15 (dd, 1H, H7, J_{7-6} = 7.6 Hz, J_{7-5} = 1.2 Hz), 6.85 (dd, 1H, H6, J_{5-6} = 8.0, Hz, J_{7-6} = 7.6 Hz), 5.11 (d, 2H, H2, J_{2-4} = 1.2 Hz).

5.3.2.1.1.10. 6,8-Difluoro-2*H*-chromene-3-carbaldehyde (1j). Yield: 17% (method A), 31% (method B), mp = 91-92 °C. IR (KBr): 2853, 2730, 1688 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.62 (s, 1H, CHO), 7.20 (d, 1H, H4, J_{2-4} = 1.2 Hz), 6.90 (s, 1H, H5), 6.76 (s, 1H, H7), 5.07 (s, 2H, H2, J_{2-4} = 1.2 Hz).

5.3.2.1.1.11. 6-Bromo-8-fluoro-2*H*-chromene-3-carbaldehyde (1k). Yield: 4% (method A), 22% (method B), mp = 120-121 °C. IR (KBr): 2841, 2730, 1684 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.61 (s, 1H, CHO), 7.25 (dd, 1H, H5, J_{7-5} = 2.4 Hz), 7.18 – 7.14 (m, 2H, H4, H7), 5.10 (d, 2H, H2, J_{2-4} = 1.2 Hz).

5.3.2.1.1.12. 6-Bromo-8-nitro-2*H*-chromene-3-carbaldehyde (1l). Yield: 6% (method B), mp = 222-223 °C. IR (KBr): 2848, 2739, 1664, 1526, 1335 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.64 (s, 1H, CHO), 8.00 (d, 1H, H5, J_{7-5} = 2.4 Hz), 7.54 (d, 1H, H7, J_{7-5} = 2.4 Hz), 7.21 (d, 1H, H4, J_{2-4} = 1.2 Hz), 5.20 (d, 2H, H2, J_{2-4} = 1.2 Hz).

5.3.2.1.1.13. 8-Bromo-6-nitro-2*H*-chromene-3-carbaldehyde (1m). Yield: 17% (method A), 5% (method B), mp = 227-228 °C. IR (KBr): 2840, 2737, 1668, 1524, 1330 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.67 (s, 1H, CHO), 8.42 (d, 1H, H7, J_{7-5} = 2.4 Hz), 8.07 (d, 1H, H5, J_{7-5} = 2.4 Hz), 7.26 (d, 1H, H4, J_{2-4} = 1.2 Hz), 5.30 (s, 2H, H2, J_{2-4} = 1.2 Hz).

5.3.2.1.1.14. 8-Nitro-2*H*-chromene-3-carbaldehyde (1n). Yield: 4% (method A), 20% (method B), mp = 118 - 119 °C. IR (KBr): 2822, 2720, 1683, 1519, 1337 cm^{-1}. ^1H NMR ($CDCl_3$, 400MHz): δ (ppm) 9.64 (s, 1H, CHO), 7.88 (dd, 1H, H5, J_{5-6} = 8.0 Hz, J_{7-5} = 1.6 Hz), 7.43 (dd, 1H, H7, J_{7-6} = 7.6 Hz, J_{7-5} = 1.6 Hz), 7.28 (d, 1H, H4, J_{2-4} = 1.2 Hz), 7.04 (dd, 1H, H6, J_{5-6} = 8.0 Hz, J_{7-6} = 7.6 Hz), 5.20 (s, 2H, H2, J_{2-4} = 1.2 Hz).

5.3.2.1.2. General procedure for the synthesis of the (*E*)-3-(2*H*-chromen-3-yl)-1-phenylprop-2-en-1-one (2a) and (*E*)-styryl-2*H*-chromenes (2 b-n).

A solution of diethyl (2-oxo-2-phenethyl)phosphonate or diethyl benzylphosphonate (11 mmol) in dry THF (30 mL) was added dropwise to a stirred suspension of NaH (11 mmol) in dry THF (15 mL) under nitrogen. The mixture was stirred for 10 min at room temperature, then a solution of the appropriate 2*H*-chromene-3-carbaldehyde (**1 a-n**) (10 mmol) in dry THF (15 mL) was added dropwise. After stirring for 20 h at room temperature, water was added to the mixture and THF was removed under reduced pressure. The solid was collected by filtration, washed with water, and chromatographed on silica gel, eluting with a mixture of dichloromethane and light petroleum (1:1). The product was further purified by crystallization from n-hexane.

5.3.2.1.2.1. (*E*)-3-(2*H*-Chromen-3-yl)-1-phenylprop-2-en-1-one (2a). Yield: 72%, mp = 150-151 °C from n-hexane. IR (KBr): 1651 cm^{-1}. ^1H NMR (CD_3OD, 400 MHz): δ (ppm) 8.04 – 8.01 (m, 2H, H2', H6'), 7.65 – 7.52 (m, 4H, H3', H4', H5', Hα), 7.22 – 7.16 (m, 2H, H5, H7), 7.10 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 16.0 Hz), 7.00 (s, 1H, H4), 6.93 (t, 1H, H6, $J_{7\text{-}6} = J_{5\text{-}6}$ = 7.2 Hz), 6.84 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 5.13 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 190.09, 154.66, 141.69, 135.70, 132.80, 132.25, 131.03, 129.01, 128.65, 128.41, 128.13, 121.96, 121.83, 120.63, 115.88, 65.28.

5.3.2.1.2.2. (*E*)-6-Bromo-3-styryl-2*H*-chromene (2b). Yield: 22%, mp = 172-173 °C from n-hexane. IR (KBr): 1477 cm^{-1}.^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.36 (d, 2H, H2', H6', $J_{2'\text{–}3'}$ = 7.2 Hz), 7.27 (t, 2H, H3', H5', $J_{2'\text{–}3'} = J_{3'\text{–}4'}$ = 7.2 Hz), 7.25 (t, 1H, H4', $J_{3'\text{–}4'}$ = 7.2 Hz), 7.10 (dd, 1H, H7, $J_{7\text{-}8}$ = 8.4 Hz, $J_{7\text{–}5}$ = 2.0 Hz), 7.05 (d, 1H, H5, $J_{7\text{–}5}$ = 2.0 Hz), 6.76 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 16.8 Hz), 6.62 (d, 1H, H8, $J_{7\text{-}8}$ = 8.4 Hz), 6.38 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 16.8 Hz), 6.34 (s, 1H, H4), 5.01 (s, 2H, H2).

5.3.2.1.2.3. (*E*)-6-Nitro-3-styryl-2*H*-chromene (2c). Yield: 18%, mp = 176-177 °C from n-hexane. IR (KBr): 1488, 1336 cm^{-1}.^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.00 (dd, 1H, H7, $J_{7\text{-}8}$ = 8.8 Hz, $J_{7\text{-}5}$ = 2.4 Hz), 7.91 (d, 1H, H5, $J_{7\text{-}5}$ = 2.4 Hz), 7.47–7.45 (m, 2H, H2', H6'), 7.39–7.35 (m, 2H, H3', H5'), 7.31–7.27 (m, 1H, H4'), 6.88 (d, 1H,

H8, $J_{7–8}$ = 8.8 Hz), 6.83 (d, 1H, Hα, $J_{α–β}$ = 16.6 Hz), 6.52–6.47 (m, 2H, H4, Hβ), 5.25 (s, 2H, H2).

5.3.2.1.2.4. (*E*)-6,8-Dibromo-3-styryl-2*H*-chromene (2d). Yield: 20%, mp = 145-146 °C from n-hexane. IR (KBr): 1465 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.43 (d, 2H, H2', H6', $J_{2'–3'}$ = 7.6 Hz), 7.41 (d, 1H, H5, J_{7-5} = 2.4 Hz), 7.35 (t, 2H, H3', H5', $J_{2'–3'} = J_{3'–4'}$ = 7.6 Hz), 7.27 (t, 1H, H4', $J_{3'–4'}$ = 7.6 Hz), 7.06 (d, 1H, H7, J_{7-5} = 2.4 Hz), 6.81 (d, 1H, Hα, $J_{α-β}$ = 16.4 Hz), 6.46 (d, 1H, Hβ, $J_{α-β}$ = 16.8 Hz), 6.37 (s, 1H, H4), 5.15 (s, 2H, H2).

5.3.2.1.2.5. (*E*)-8-Chloro-3-styryl-2*H*-chromene (2e). Yield: 34%, mp = 130-132 °C from n-hexane. IR (KBr): 1444 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.44 (d, 2H, H2', H6', $J_{2'–3'}$ = 7.2 Hz), 7.34 (t, 2H, H3', H5', $J_{2'–3'} = J_{3'–4'}$ = 7.2 Hz), 7.29–7.25 (m, 1H, H4'), 7.16 (dd, 1H, H5, J_{5-6} = 8.0 Hz, J_{7-5} = 1.6 Hz), 6.93 (dd, 1H, H7, $J_{7–6}$ = 7.6 Hz, J_{7-5} = 1.6 Hz), 6.85 (d, 1H, Hα, $J_{α-β}$ = 17.6 Hz), 6.81 (dd, 1H, H6, J_{5-6} = 8.0 Hz, J_{7-6} = 7.6 Hz), 6.49 (s, 1H, H4), 6.46 (d, 1H, Hβ, $J_{α–β}$= 17.6 Hz), 5.21 (s, 2H, H2).

5.3.2.1.2.6. (*E*)-6,8-Dichloro-3-styryl-2*H*-chromene (2f). Yield: 19%, mp = 157-158 °C from n-hexane. IR (KBr): 1471 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.45 (d, 2H, H2', H6', $J_{2'–3'}$ = 7.2 Hz), 7.38 – 7.34 (m 2H, H3', H5'), 7.30–7.26 (m, 1H, H4'), 7.15 (d, 1H, H7, $J_{7–5}$ = 2.4 Hz), 6.91 (d, 1H, H5, J_{7-5} = 2.4 Hz), 6.84 (d, 1H, Hα, $J_{α-β}$ = 16.4 Hz), 6.49 (d, 1H, Hβ, $J_{α-β}$ = 16.4 Hz), 6.41 (s, 1H, H4), 5.19 (s, 2H, H2).

5.3.2.1.2.7. (*E*)-8-Bromo-6-chloro-3-styryl-2*H*-chromene (2g). Yield: 23%, mp = 159-160 °C from n-hexane. IR (KBr): 1467 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.45 (dd, 2H, H2', H6', $J_{2'–3'}$ = 7.2 Hz, $J_{2'-4'}$ = 1.6 Hz), 7.36 (t, 2H, H3', H5', $J_{2'–3'} = J_{3'-4'}$ = 7.2 Hz), 7.29 (m, 2H, H4', H7), 6.95 (d, 1H, H5, J_{7-5} = 2.4 Hz), 6.84 (d, 1H, Hα, $J_{α-β}$ = 16.4 Hz), 6.49 (d, 1H, Hβ, $J_{α-β}$ = 16.4 Hz), 6.40 (s, 1H, H4), 5.20 (s, 2H, H2).

5.3.2.1.2.8. (*E*)-8-Chloro-6-fluoro-3-styryl-2*H*-chromene (2h). Yield: 24%, mp = 151-152 °C from n-hexane. IR (KBr): 1475 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.42 (d, 2H, H2', H6', $J_{2'–3'}$ = 7.6 Hz), 7.36–7.32 (m, 2H, H3', H5'), 7.26 (s, 1H, H4'), 6.88 (m, 1H,

H7), 6.81 (d, 1H, Hα, $J_{α-β}$ = 16.4 Hz), 6.64 (m, 1H, H5), 6.47 (d, 1H, Hβ, $J_{α-β}$ = 16.4 Hz), 6.39 (s, 1H, H4), 5.13 (s, 2H, H2).

5.3.2.1.2.9. (*E*)-8-Bromo-3-styryl-2*H*-chromene (2i). Yield: 27%, mp = 126-127 °C from n-hexane. IR (KBr): 1443 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.46–7.43 (m, 2H, H2', H6'), 7.37–7.35 (m, 2H, H3', H5'), 7.31 (dd, 1H, H5 , J_{5-6} = 7.4 Hz, J_{7-5} = 1.6 Hz), 7.29–7.24 (m, 1H, H4'), 7.05 (s, 1H, H4), 6.97 (dd, 1H, H7, $J_{7–6}$ = 7.6 Hz, J_{7-5} = 1.6 Hz), 6.86 (d, 1H, Hα, $J_{α-β}$ = 16.4 Hz), 6.78–6.74 (m, 1H, H6), 6.48 (d, 1H, Hβ, $J_{α–β}$ = 16.4 Hz), 5.21 (s, 2H, H2).

5.3.2.1.2.10. (*E*)-6,8-Difluoro-3-styryl-2*H*-chromene (2j). Yield: 20%, mp = 148-149 °C from n-hexane. IR (KBr): 1488 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.47–7.44 (m, 2H, H2', H6'), 7.38 – 7.34 (m, 2H, H3', H5'), 7.30–7.26 (m, 1H, H4'), 6.84 (d, 1H, Hα, $J_{α–β}$ = 16.4 Hz), 6.72 – 6.66 (m, 1H, H5), 6.59–6.56 (m, 1H, H7), 6.51 (d, 1H, Hβ, $J_{α-β}$ = 16.4 Hz), 6.45 (s, 1H, H4), 5.12 (d, 2H, H2).

5.3.2.1.2.11. (*E*)-6-Bromo-8-fluoro-3-styryl-2*H*-chromene (2k). Yield: 24%, mp = 162-163 °C from n-hexane. IR (KBr): 1481 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.45 – 7.43 (m, 2H, H2', H6'), 7.38–7.34 (m, 2H, H3', H5'), 7.30–7.26 (m, 1H, H4'), 7.07 (m, 1H, H7), 6.95 (d, 1H, H5, J_{7-5} = 1.6 Hz), 6.83 (d, 1H, Hα, $J_{α-β}$ = 16.8 Hz), 6.49 (d, 1H, Hβ, $J_{α-β}$ = 16.8 Hz), 6.43 (d, 1H, H4), 5.15 (s, 2H, H2).

5.3.2.1.2.12. (*E*)-6-Bromo-8-nitro-3-styryl-2*H*-chromene (2l). Yield: 11%, mp = 180-181 °C from n-hexane. IR (KBr): 1515, 1336 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.81 (d, 1H, H5, J_{7-5} = 2.4 Hz), 7.47–7.44 (m, 2H, H2', H6'), 7.39–7.34 (m, 2H, H3', H5'), 7.32 (d, 1H, H7, J_{7-5} = 2.4 Hz), 7.30–7.28 (m, 1H, H4'), 6.84 (d, 1H, Hα, $J_{α-β}$ = 16.4 Hz), 6.53 (d, 1H, Hβ, $J_{α-β}$ = 16.4 Hz), 6.46 (s, 1H, H4), 5.27 (s, 2H, H2).

5.3.2.1.2.13. (*E*)-8-Bromo-6-nitro-3-styryl-2*H*-chromene (2m). Yield: 8%, mp = 172-173 °C from n-hexane. IR (KBr): 1511, 1338 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.16 (d, 1H, H7, J_{7-5} = 2.8 Hz), 7.78 (d, 1H, H5, J_{7-5} = 2.8 Hz), 7-40-7.37 (m, 2H, H2', H6'), 7.31–7.27 (m, 2H, H3', H5'), 7.25–7.21 (m, 1H, H4'), 6.78 (d, 1H, Hα, $J_{α-β}$ = 16.0 Hz), 6.44 (d, 1H, Hβ, $J_{α-β}$ = 16.0 Hz), 6.42 (s, 1H, H4), 5.29 (s, 2H, H2).

5.3.2.1.2.14. (*E*)-8-Nitro-3-styryl-2*H*-chromene (2n). Yield: 7%, mp = 185-186 °C from n-hexane. IR (KBr): 1547, 1337 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.68 (dd, 1H, H5, $J_{5\text{-}6}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 7.46–7.43 (m, 2H, H2', H6'), 7.38 – 7.33 (m, 2H, H3', H5'), 7.30–7.26 (m, 1H, H4'), 7.21 (dd, 1H, H7, $J_{7\text{-}6}$ = 7.6 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.93 (dd, 1H, H6, $J_{7\text{-}6}$ = 7.6 Hz, $J_{5\text{-}6}$ = 8.0 Hz), 6.84 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 16.4 Hz), 6.53 (d, 1H, H4), 6.50 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 16.4 Hz), 5.25 (s, 2H, H2).

5.3.2.1.3. General procedure for the synthesis of the benzyltriphenylphosphonium bromides (3 b-m and 3o).

Triphenylphosphine hydrobromide (20 mmol) was added to a solution of the appropriate benzyl alcohol (20 mmol) in dry CH_3CN (47 mL). The obtained solution was refluxed for 5 h, while stirring. After this period, the mixture was cooled and Et_2O was added. The white solid was filtered and used for the next reaction without further purification.

5.3.2.1.3. (2-Trifluoromethylbenzyl)triphenylphosphonium bromide (3b). Yield: 73%, mp > 200 °C. ^1H NMR (DMSO D6, 400 MHz): δ (ppm) 7.96 - 7.92 (m, 1H, H3), 7.81-7.70 (m, 3H, H4, H5, H6), 7.68-7.54 (m, 15H, 3H2' - H6'), 7.43-7.41 (m, 1H, H2), 5.17 (d, 2H, CH_2, $J_{C\text{-}P}$ = 15.2 Hz).

5.3.2.1.3.2. (3-Trifluoromethylbenzyl)triphenylphosphonium bromide (3c). Yield: 75%, mp > 200 °C. ^1H NMR (DMSO D6, 400 MHz): δ (ppm) 7.94-7.91 (m, 3H, H4, H5, H6), 7.79 - 7.51 (m, 15H, 3H2' - H6'), 7.43-7.41 (m, 1H, H2), 5.40 (d, 2H, CH_2, $J_{C\text{-}P}$ = 16.4 Hz).

5.3.2.1.3.3. (4-Trifluoromethylbenzyl)triphenylphosphonium bromide (3d). Yield: 58%, mp > 200 °C. ^1H NMR (DMSO D6, 400 MHz): δ (ppm) 7.94-7.91 (m, 4H, H2, H3, H5, H6), 7.77-7.71 (m, 15H, 3H2' -H6'), 5.40 (d, 2H, CH_2, $J_{C\text{-}P}$ = 16.4 Hz).

5.3.2.1.3.4. (2,4-Dichlorobenzyl)triphenylphosphonium bromide (3e). Yield: 45%, mp > 200 °C. ^1H NMR (DMSO D6, 400 MHz): δ (ppm) 7.93 (m, 1H, H3), 7.78-755 (m, 15H, 3H2'- H6'), 7.40-7.37 (m, 2H, H5, H6), 5.22 (d, CH_2, $J_{C\text{–}P}$ = 14.5 Hz).

5.3.2.1.3.5. (3,5-Dichlorobenzyl)triphenylphosphonium bromide (3f). Yield: 55%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ (ppm) 7.94 (s, 1H, H4) 7.81-7.72 (m, 15H, 3H2' - H6'), 7.65–7.62 (m, 2H, H2', H6'), 5.35 (d, CH_2, J_{C-P} = 14.5 Hz).

5.3.2.1.3.6. (2,6-Dichlorobenzyl)triphenylphosphonium bromide (3g). Yield: 80%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ (ppm) 7.76-7.74 (m, 3H, H3, H4, H5), 7.73-7.64 (m, 15H, 3H2' - H6'), 5.17 (d, CH_2, J_{C-P} = 14.4 Hz).

5.3.2.1.3.7. (2-Chlorobenzyl)triphenylphosphonium bromide (3h). Yield: 84%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ (ppm) 7.93 (m, 3H, 3H4'), 7.78-7.65 (m, 12H, 3H2', 3H3', 3H5', 3H6'), 7.63–7.54 (m, 4H, H3, H4, H5, H6), 5.23 (d, CH_2, J_{C-P} = 14.8 Hz).

5.3.2.1.3.8. (3-Chlorobenzyl)triphenylphosphonium bromide (3i). Yield: 68%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ (ppm) 7.9 –7.91 (m, 3H, 3H4'), 7.79-7.69 (m, 12H, 3H2', 3H3', 3H5', 3H6'), 7.39-7.29 (m, 3H, H4, H5, H6), 6.90 (s, 1H, H2), 5.26 (d, CH_2, J_{C-P} = 16.0 Hz).

5.3.2.1.3.9. (4-Chlorobenzyl)triphenylphosphonium bromide (3j). Yield: 73%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ (ppm) 7.92–7.89 (m, 3H, 3H4'), 7.77-7.67 (m, 12H, 3H2', 3H3', 3H5', 3H6'), 7.41-7.33 (m, 3H, H4, H5, H6), 6.88 (s, 1H, H2), 5.28 (d, CH_2, J_{C-P} = 16.2 Hz).

5.3.2.1.3.10. (2-Bromobenzyl)triphenylphosphonium bromide (3k). Yield: 86%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ (ppm) 7.92 (s, 3H, 3H4'), 7.73-7.63 (m, 12H, 3H2', 3H3', 3H5', 3H6'), 7.56 (d, 1H, H3, J_{2-3} = 7.6 Hz), 7.31-7.19 (m, 3H, H4, H5, H6), 5.23 (d, CH_2, J_{C-P} = 14.8 Hz).

5.3.2.1.3.11. (3-Bromobenzyl)triphenylphosphonium bromide (3l). Yield: 60%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ (ppm) 7.94–7.90 (m, 3H, 3H4'), 7.76-7.68 (m, 12H, 3H2', 3H3', 3H5', 3H6'), 7.51 (t, 1H, H5, J_{4-5} = J_{5-6} = 7.6 Hz), 7.31-7.19 (m, 3H, H4, H5, H6), 5.25 (d, CH_2, J_{C-P} = 15.6 Hz).

5.3.2.1.3.12. (4-Bromobenzyl)triphenylphosphonium bromide (3m). Yield: 80%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ

(ppm) 7.94–7.90 (m, 3H, 3H4'), 7.77-7.70 (m, 12H, 3H2', 3H3', 3H5', 3H6'), 7.46 (d, 2H, H3, H5, $J_{2\text{-}3} = J_{5\text{-}6} = 8.0$ Hz), 6.93 (d, 2H, H2, H6, $J_{2\text{-}3} = J_{5\text{-}6} = 8.0$ Hz), 4.92 (d, CH_2, $J_{C-P} = 14.8$ Hz).

5.3.2.1.3.14. (2-Hydroxybenzyl)triphenylphosphonium bromide (3o). Yield: 80%, mp > 200 °C. ^{1}H NMR (DMSO D6, 400 MHz): δ (ppm) 9.74 (s, 1H, OH), 7.91-7.87 (m, 4H, H3, H4, H5, H6), 7.74-7.64 (m, 15H, 3H2' - H6'), 4.92 (d, CH_2, $J_{C-P} = 14.8$ Hz).

5.3.2.1.4. General procedure for the synthesis of the 3-styryl-2*H*-chromenes (4a-n, 5a, 5e-f, 5i, 5l-n).

tert-BuOK (40 mmol) was added to a solution of the appropriate benzyltriphenylphosphonium halide (**3a-n**) (20 mmol) in dry THF (86 mL) at room temperature. After 10 minutes, a solution of *2H*-chromen-3-carbaldehyde (20 mmol) (**1a**) in dry THF (87 mL) was added dropwise. The mixture was stirred for 24 h at room temperature, under nitrogen. After this period, ice and water was added and the mixture was neutralized with 2N HCl. The obtained suspension was extracted with $CHCl_3$. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The obtained *E*/*Z*-mixtures (**4/5a, 4/5e-f, 4/5i, 4/5l-n**) were separated by column chromatography on silica gel eluting with $CHCl_3$ and light petroleum (1:3). The (*Z*) isomer was eluted at first. For compounds (**4b-d, 4g-h, 4j-k)** only the *E* isomers were purified by column chromatography on silica gel eluting with a mixture of dichloromethane and light petroleum (1:1).

5.3.2.1.4.1. (*E*)-3-Styryl-2*H*-chromene (4a). Yield: 47%, mp = 122-124 °C from n-hexane. IR (KBr): 1670 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.44 (d, 2H, H2', H6', $J_{2'-3'} = 7.6$ Hz), 7.34 (t, 2H, H3', H5', $J_{2'-3'} = J_{3'-4'} = 7.6$ Hz), 7.25 (t, 1H, H4', $J_{3'-4'} = 7.6$ Hz), 7.11 (dt, 1H, H7, $J_{7-6} = J_{7-8} = 7.5$ Hz, $J_{7\text{-}5} = 1.5$ Hz), 7.03 (dd, 1H, H5, $J_{5-6} = 7.5$ Hz, $J_{7\text{-}5} = 1.5$ Hz), 6.91–6.81 (m, 3H, H6, H8, Hβ), 6.52 (s, 1H, H4), 6.44 (d, 1H, Hα, $J_{\alpha-\beta} = 16.5$ Hz), 4.83 (d, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.8, 137.0, 130.7, 129.1, 128.8, 128.0, 127.9, 127.0, 126.6, 126.4, 124.0, 122.8, 121.5, 115.5, 65.6.

5.3.2.1.4.2. (*Z*)-3-Styryl-2*H*-chromene (5a). Resa: 0.60%. thick oil.

IR (KBr): 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.32-7.22 (m, 5H, H2', H3', H4', H5', H6'), 7.08 (dt, 1H, H7, $J_{7\text{-}6}$ = $J_{7\text{-}8}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 7.00 (dd, 1H, H5, $J_{5\text{-}6}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.86 (t, 1H, H6, $J_{7\text{-}6}$ = $J_{5\text{-}6}$ = 8.0 Hz), 6.75 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 6.6 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 12.0 Hz), 6.49 (s, 1H, H4), 6.23 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 12.0 Hz), 5.08 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm): 153.77, 138.25, 131.43, 130.96, 129.06, 128.66, 128.21, 128.16, 127.48, 126.74, 125.56, 123.29, 121.55, 115.49, 67.00.

5.3.2.1.4.3. (*E*)-3-[2-(Trifluoromethyl)styryl]-2*H*-chromene (4b). Yield: 24%, mp = 84–86 °C from n-hexane. IR (KBr): 1620 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.87 (d, 1H, H3', $J_{3'\text{-}4'}$ = 7.6 Hz), 7.70 (d, 1H, H6', $J_{6'\text{-}5'}$ = 8.0 Hz), 7.62 (t, 1H, H4', $J_{3'\text{-}4'}$ = $J_{4'\text{-}5'}$ = 7.6 Hz), 7.44 (dd, 1H, H5', $J_{6'\text{-}5'}$ = 8.0 Hz, $J_{4'\text{-}5'}$ = 7.6 Hz), 7.15-7.09 (m, 2H, H7, H5), 7.03 (d, Hβ, $J_{\alpha\text{-}\beta}$ = 16.0 Hz), 6.88 (t, 1H, H6, $J_{6\text{-}7}$ = $J_{6\text{-}5}$ = 7.6 Hz), 6.84-6.80 (m, 2H, H8, Hα), 6.71 (s, 1H, H4), 5.09 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.98, 136.02, 131.93, 130.42, 130.37, 129.61, 127.36, 127.21 (q, J = 28 Hz), 127.20, 126.69, 126.06 (q, J = 5.7 Hz), 125.66, 124.46 (q, J = 272 Hz), 123.36, 122.49, 121.58, 115.67, 65.47.

5.3.2.1.4.4. (*E*)-3-[3-(Trifluoromethyl)styryl]-2*H*-chromene (4c). Yield: 17%, mp = 103–105 °C from n-hexane. IR (KBr): 1596 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.64 (s, 1H, H2'), 7.53 (d, 1H, H6', $J_{6'\text{-}5'}$ = 7.6 Hz), 7.46 (d, 1H, H4', $J_{4'\text{-}5'}$ = 7.6 Hz), 7.39 (t, 1H, H5', $J_{4'\text{-}5'}$ = $J_{5'\text{-}6'}$ = 7.6 Hz), 7.11 (m, 1H, H7, $J_{6\text{-}7}$ = $J_{7\text{-}8}$ = 8.0 Hz, $J_{5\text{-}7}$ = 1.2 Hz), 7.03 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.6 Hz, $J_{5\text{-}7}$ = 1.2 Hz), 6.88-6.80 (m, 3H, H6, H8, Hα), 6.52 (s, 1H, H4), 6.37 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 16.4 Hz), 5.04 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.87, 137.80, 131.19 (q, J = 31.7 Hz), 130.13, 129.53, 129.41, 129.17, 128.31, 127.16, 126.23, 125.31, 124.18 (q, J = 3.6 Hz), 124.14 (q, J = 271 Hz), 122.99 (q, J = 3.7 Hz), 122.52, 121.62, 115.58, 65.47.

5.3.2.1.4.5. (*E*)-3-[4-(Trifluoromethyl)styryl]-2*H*-chromene (4d). Yield: 26%, mp = 156–157 °C from n-hexane. IR (KBr): 1607 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.53 (m, 4H, H2', H3', H5', H6'), 7.10–7.04 (m, 1H, H7, $J_{6\text{-}7}$ = 7.2 Hz, $J_{7\text{-}8}$ = 8.0 Hz, $J_{5\text{-}7}$ = 0.8 Hz), 7.03 (dd, 1H, H5, $J_{5\text{-}6}$ = 6.4 Hz, $J_{5\text{-}7}$ = 0.8 Hz), 6.88 (m, 2H, H6, Hα), 6.83 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 6.56 (s, 1H, H4), 6.4 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 16.4 Hz), 5.07 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm)

153.91, 140.47, 130.14, 129.59, 129.44 (q, J = 32 Hz), 128.98, 127.16, 126.46, 126.26, 125.69, 125.62 (q, J = 6.6 Hz), 124.86 (q, J = 280 Hz), 122.48, 121.61, 115.58, 65.49.

5.3.2.1.4.6. (*E*)-3-(2,4-Dichlorostyryl)-2*H*-chromene (4e). Yield: 47%, mp = 129–135 °C from n-hexane. IR (KBr): 1595 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.49 (d, 1H, H6', $J_{5'-6'}$ = 8.4 Hz), 7.35 (d, 1H, H3', $J_{3'-5'}$ = 2.0 Hz), 7.19 (dd, 1H, H5', $J_{5'-6'}$ = 8.4 Hz, $J_{3'-5'}$ = 2.0 Hz), 7.11 (t, 1H, H7, J_{7-6} = 7.6 Hz), 7.01 (d, 1H, H5, J_{5-6} = 7.6 Hz), 6.87 (d, 1H, H6, J_{5-6} = J_{7-6} = 7.6 Hz), 6.82 (d, 1H, H8, J_{7-8} = 7.6 Hz), 6.77 (d, 1H, H_α, $J_{\alpha-\beta}$ = 16.4 Hz), 6.52 (s, 1H, H4), 6.69 (d, 1H, H_β, $J_{\alpha-\beta}$ = 16.4 Hz), 5.07 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.95, 133.86, 133.78, 130.40, 129.65, 129.60, 129.39, 128.71, 127.36, 127.16, 126.82, 125.58, 122.58, 122.47, 121.58, 115.64, 65.47.

5.3.2.1.4.7. (*Z*)-3-(2,4-Dichlorostyryl)-2*H*-chromene (5e). Yield: 31%, thick oil. IR (KBr): 1609 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.41 (d, 1H, H3', $J_{3'-5'}$ = 2.0 Hz), 7.19–7.07 (m, 3H, H5', H6', H7), 6.98 (d, 1H, H5, J_{5-6} = 7.6 Hz), 6.87 (t, 1H, H6, J_{5-6} = J_{7-6} = 7.6 Hz), 6.74 (d, 1H, H8, J_{7-8} = 8.0 Hz), 6.50 (s, 1H, H4), 6.46 (d, 1H, Hβ, $J_{\alpha-\beta}$ = 12.0 Hz), 6.36 (d, 1H, H_β, $J_{\alpha-\beta}$ = 12.0 Hz), 4.37 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.69, 135.26, 134.17, 134.14, 131.42, 130.41, 130.32, 129.50, 129.13, 126.96, 126.81, 126.31, 125.57, 122.91, 121.68, 115.55, 66.78.

5.3.2.1.4.8. (*E*)-3-(3,5-Dichlorostyryl)-2*H*-chromene (4f). Yield: 75%, mp = 131–134 °C from n-hexane. IR (KBr): 1597 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.24 – 7.22 (m, 2H, H2', H6'), 7.18 (s, 1H, H4'), 711 (t, 1H, H7, J_{7-6} = J_{7-8} = 7.2 Hz), 7.01 (d, 1H, H5, J_{5-6} = 7.2 Hz), 6.87 (t, 1H, H6, J_{5-6} = J_{7-6} = 7.2 Hz), 6.82–6.76 (m, 2H, H8, Hβ), 6.51 (s, 1H, H4), 6.21 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.4 Hz), 5.01 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.89, 140.06, 135.29, 129.82, 129.73, 129.19, 127.36, 127.26,125.96, 124.97, 124.61, 122.37, 121.65, 115.62, 65.35.

5.3.2.1.4.9. (*Z*)-3-(3,5-Dichlorostyryl)-2*H*-chromene (5f). Yield: 6%, mp = 45–47 °C from n-hexane. IR (KBr): 1596 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.62 (s, 1H, H5'), 7.13–7.09 (m, 3H, H2', H6', H7), 7.01 (d, 1H, H5, J_{5-6} = 8.0 Hz), 6.88 (t, 1H, H6, J_{7-6} = J_{5-6} = 8.0 Hz), 6.77 (d, 1H, H8, J_{7-8} = 8.0 Hz), 6.53 (s, 1H, H4), 6.42 (d, 1H,

Hα, $J_{\alpha\text{-}\beta}$ = 12.0 Hz), 6.28 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 12.0 Hz), 4.41 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.76, 141.18, 134.75, 130.43, 130.69, 129.58, 128.57, 128.44, 127.41, 127.23, 126.99, 126.93, 121.72, 115.60, 66.84.

5.3.2.1.4.10. (*E*)-3-(2,6-Dichlorostyryl)-2*H*-chromene (4g). Yield: 23%, mp = 107–108 °C from n-hexane. IR (KBr): 1597 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.28 (d, 2H, H3', H5', $J_{2'\text{-}3'}$ = 8.0 Hz), 7.10 (t, 1H, H7, $J_{7\text{-}6}$ = $J_{7\text{-}8}$ = 7.6 Hz), 7.05–6.98 (m 2H, H4', H5'), 6.91 – 6.81 (m, 3H, H6, H8, Hα), 6.49 (s, 1H, H4), 6.40 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 16.8 Hz), 5.09 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.97, 134.83, 134.41, 134.16, 130.12, 129.61, 128.71, 128.21, 127.25, 125.73, 122.47, 121.81, 121.58, 115.62, 65.30.

5.3.2.1.4.11. (*E*)-3-(2-Chlorostyryl)-2*H*-chromene (4h). Yield: 30%, mp = 101–102 °C from n-hexane. IR (KBr): 1610 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.54 (d, 1H, H3', $J_{3'\text{-}4'}$ = 7.6 Hz), 7.32 (d, 1H, H6', $J_{6'\text{-}5'}$ = 8.0 Hz), 7.18 (t, 1H, H4', $J_{3'\text{-}4'}$ = $J_{4'\text{-}5'}$ = 7.6 Hz), 7.13 (m, 2H, H7, H5'), 6.98 (d, 1H, H5, $J_{5\text{-}6}$ = 6.8 Hz), 6.86-6.80 (m, 4H, H6, H8, Hα, Hβ), 6.46 (s, 1H, H4), 5.05 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.83, 134.90, 133.30, 130.56, 129.82, 129.34, 128.85, 128.58, 127.02, 126.86, 126.07, 124.99, 123.63, 122.50, 121.44, 115.52, 65.47.

5.3.2.1.4.12. (*E*)-3-(3-Chlorostyryl)-2*H*-chromene (4i). Yield: 21%, mp = 61–63 °C from n-hexane. IR (KBr): 1681 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.36 (s, 1H, H2'), 7.18 (m, 3H, H6', H5', H4'), 7.08 (t, 1H, H7, $J_{7\text{-}6}$ = $J_{7\text{-}8}$ = 8.0 Hz), 7.03 (d, 1H, H5, $J_{5\text{-}6}$ = 7.2 Hz), 6.87–6.73 (m, 3H, H6, H8, Hβ), 6.45 (s, 1H, H4), 6.26 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 16.4 Hz), 5.00 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.85, 138.90, 134.74, 130.27, 129.96, 129.47, 127.94, 127.70, 127.16, 126.38, 126.23, 125.03, 124.71, 122.61, 121.63, 115.60, 65.50.

5.3.2.1.4.13. (*Z*)-3-(3-Chlorostyryl)-2*H*-chromene (5i). Yield: 4%, thick oil. IR (KBr): 1681 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.53 (m, 3H, H2', H4', H5'), 7.09 (m, 2H, H7, H6'), 6.98 (d, 1H, H5, $J_{5\text{-}6}$ = 7.6 Hz), 6.86 (t, 1H, H6, $J_{5\text{-}6}$ = $J_{7\text{-}6}$ = 7.6 Hz), 6.75 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 6.48 (m, 2H, H4, Hα), 6.4 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 12.0 Hz), 4.40 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.71,

140.01, 134.05, 130.68, 129.35, 129.30, 129.01, 128.59, 127.48, 126.84, 126.72, 126.40, 123.02, 121.61, 115.51, 66.85.

5.3.2.1.4.14. (*E*)-3-(4-Chlorostyryl)-2*H*-chromene (4j). Yield: 40%, mp = 166–167 °C from ethyl acetate (lit 167-168 °C).[8] The compound exhibited spectroscopic data identical to those previously reported.[8]

5.3.2.1.4.15. (*E*)-3-(2-Bromostyryl)-2*H*-chromene (4k). Yield: 11%, mp = 83–84 °C from n-hexane. IR (KBr): 1596 cm^{-1}. ^1H NMR (acetoneD, 400 MHz): δ (ppm) 7.77 (d, 1H, H3', $J_{3'-4'}$ = 7.6 Hz), 7.62 (d, 1H, H6', $J_{5'-6'}$ = 7.6 Hz), 7.38 (t, 1H, H5', $J_{4'-5'} = J_{5'-6'}$ = 7.6 Hz), 7.19 (t, 1H, H4', $J_{4'-5'} = J_{3'-4'}$ = 7.6 Hz), 7.14–7.11 (m, 2H, H7, H5), 7.02 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.8 Hz), 6.91 – 6.80 (m, 3H, H6, H8, Hβ), 6.72 (s, 1H, H4), 5.13 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.91, 136.69, 133.19, 130.65, 129.45, 129.14, 128.97, 127.61, 127.09, 126.54, 126.40, 125.08, 124.18, 122.60, 121.56, 115.62, 65.57.

5.3.2.1.4.16. (*E*)-3-(3-Bromostyryl)-2*H*-chromene (4l). Yield: 24%, mp = 138–139 °C from n-hexane. IR (KBr): 1594 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.55 (s, 1H, H2'), 7.35 – 7.29 (m, 2H, H4', H6'), 7.16 (t, 1H, H5', $J_{4'-5'} = J_{5'-6'}$ = 8.0 Hz), 7.10 (t, 1H, H7, $J_{7-6} = J_{7-8}$ = 7.6 Hz), 7.01 (d, 1H, H5, J_{5-6} = 7.6 Hz), 6.87 (t, 1H, H6, , $J_{5-6} = J_{7-6}$ = 7.6 Hz), 6.88–6.77 (m, 2H, H8, Hβ), 6.49 (s, 1H, H4), 6.29 (d, 1H, Hα $J_{\alpha-\beta}$ = 16.4 Hz), 5.03 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.79, 139.15, 130.53, 130.19, 130.16, 129.40, 129.11, 127.93, 127.06, 126.22, 125.04, 124.98, 122.94, 122.53, 121.55, 115.52, 65.44.

5.3.2.1.4.17. (*Z*)-3-(3-Bromostyryl)-2*H*-chromene (5l). Yield: 5%, thick oil. IR (KBr): 1681 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.35 (s, 1H, H2'), 7.16–7.13 (m, 3H, H4', H5', H6'), 7.11 (t, 1H, H7, $J_{7-6} = J_{7-8}$ = 7.6 Hz), 7.00 (d, 1H, H5, J_{5-6} = 7.6 Hz), 6.87 (t, 1H, H6, $J_{5-6} = J_{7-6}$ = 7.6 Hz), 6.76 (d, 1H, H8, J_{7-8} = 7.6 Hz), 6.51–6.48 (m, 2H, H4, Hα), 6.4 (d, 1H, Hβ, $J_{\alpha-\beta}$ = 11.6 Hz), 4.41 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.73, 140.34, 131.51, 130.69, 130.42, 129.63, 129.41, 129.32, 128.94, 127.16, 126.86, 126.45, 123.04, 122.27, 121.63, 115.53, 66.91.

5.3.2.1.4.18. (*E*)-3-(4-Bromostyryl)-2*H*-chromene (4m). Yield: 30%, mp = 185–186 °C from n-hexane. IR (KBr): 1609 cm^{-1}. ^1H

NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.45 (d, 2H, H3', H5', $J_{2'-3'}$ = 8.4 Hz), 7.29 (d, 2H, H2', H6', $J_{2'-3'}$ = 8.4 Hz), 7.11 (t, 1H, H7, $J_{7-6} = J_{7-8}$ = 7.6 Hz), 7.03 (d, 1H, H5, J_{5-6} = 7.2 Hz), 6.90–6.81 (m, 3H, H6, H8, Hα), 6.52 (s, 1H, H4), 6.35 (d, 1H, Hβ, $J_{\alpha-\beta}$ = 16.4 Hz), 5.06 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.78, 135.93, 131.87, 130.39, 129.33, 127.84, 127.28, 127.00, 126.62, 124.60, 122.64, 121.58, 115.53, 65.49.

5.3.2.1.4.19. (*Z*)-3-(4-Bromostyryl)-2*H*-chromene (5m). Yield: 18%, mp = 76–77 °C from n-hexane. IR (KBr): 11591 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.43 (d, 2H, H3', H5', $J_{2'-3'}$ = 8.4 Hz), 7.10–7.05 (m, 3H, H2', H6', H7), 6.97 (d, 1H, H5, J_{5-6} = 7.2 Hz), 6.88 (t, 1H, H6, $J_{7-6} = J_{5-6}$ = 7.2 Hz), 6.75 (d, 1H, H8, J_{7-8} = 8.0 Hz), 6.48 – 6.44 (m, 2H, H4, Hα), 6.21 (d, 1H, Hβ, $J_{\alpha-\beta}$ = 12.0 Hz), 4.42 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.65, 136.96, 131.28, 130.77, 130.22, 129.42, 129.24, 128.74, 126.79, 125.98, 123.03, 121.60, 121.48, 115.51, 66.83.

5.3.2.1.4.20. (*E*)-3-(4-Nitrostyryl)-2*H*-chromene (4n). Yield: 36%, mp = 174-175 °C from n-hexane. IR (KBr): 1504, 1332 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.20 (d, 2H, H3', H5', $J_{2'-3'}$ = 8.8 Hz), 7.55 (d, 2H, H2', H6', $J_{2'-3'}$ = 8.8 Hz), 7.17 – 7.13 (m, 1H, H7), 7.00 (dd, 1H, H5, J_{5-6} = 7.6 Hz, J_{7-5} = 1.6 Hz), 6.97 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.4 Hz), 6.92–6.88 (m, 1H, H6, $J_{7-6} = J_{5-6}$ = 7.6 Hz, J_{6-8} = 1.2 Hz), 6.84 (dd, 1H, H8, J_{7-8} = 7.6 Hz, J_{6-8} = 1.2 Hz), 6.63 (s, 1H, H4), 6.50 (d, 1H, Hβ, $J_{\alpha-\beta}$ = 16.4 Hz), 5.09 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.06, 146.87, 143.49, 130.95, 130.04, 129.89, 127.41, 127.03, 126.74, 125.40, 124.20, 122.32, 121.74, 115.70, 65.34.

5.3.2.1.4.21. (*Z*)-3-(4-Nitrostyryl)-2*H*-chromene (5n). Yield: 8%, mp = 120-121 °C from n-hexane. IR (KBr): 1506, 1343 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.17 (d, 2H, H3', H5', $J_{2'-3'}$ = 8.8 Hz), 7.40 (d, 2H, H2', H6', $J_{2'-3'}$ = 8.8 Hz), 7.13–7.11 (m, 1H, H7), 6.99 (dd, 1H, H5, J_{5-6} = 7.6 Hz, J_{7-5} = 1.6 Hz), 6.90–6.86 (m, 1H, H6, $J_{7-6} = J_{5-6}$ = 7.6 Hz, J_{6-8} = 1.2 Hz), 6.76 (dd, 1H, H8, J_{7-8} = 7.6 Hz, J_{6-8} = 1.2 Hz), 6.55-6.52 (m, 2H, H4, Hα), 6.36 (d, 1H, Hβ, $J_{\alpha-\beta}$ = 12.0 Hz), 4.43 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.74, 146.90, 144.85, 130.94, 129.76, 129.44, 128.24, 127.25, 127.09, 124.17, 123.51, 122.75, 121.81, 115.66, 66.84.

5.3.2.1.5. General procedure for the synthesis of the 3-phenethylchromans (6a-m).

10% Pd/C (15.2 mmol) was added to a solution of the appropriate (*E*)-3-styryl-2*H*-chromene (**4b-d, 4g-h, 4j-k**) or to a mixture of *E, Z* isomers (**4/5a, 4/5e-f, 4/5i, 4/5l-m** (10 mmol) in dry dioxane (80 mL). The suspension was hydrogenated at room temperature for 30 minutes, under 40 psi pressure. After this period, the catalyst was filtered off and the solvent was removed under reduced pressure. The residue was chromatographed on silica gel eluting with a mixture of $CHCl_3$/light petroleum (1:1). The obtained solid was further purified by crystallization from n-hexane (**6a-m**).

5.3.2.1.5.1. 3-Phenethylchroman (6a). Yield: 69%, mp = 42–43 °C from n-hexane. IR (KBr): 1607 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.28 - 7.24 (m, 2H, H3', H5'), 7.19-7.15 (m, 3H, H2', H6', H4'), 7.08-7.00 (m, 2H, H6, H5), 6.84 - 6.77 (m, 2H, H8, H7), 4.20 (m, 1H, H2, J_{gem} = 10.8 Hz, J_{2-3} = 9.2 Hz), 3.76 (dd, 1H, H2, J_{gem} = 10.8 Hz, J_{2-3} = 9.2 Hz), 2.88 (dd, 1H, H4, J_{gem} = 16.2 Hz, J_{3-4} = 5.2 Hz), 2.78-2.66 (m, 2H, Hβ), 2.49 (m, 1H, H4), 2.07-1.97 (m, 1H, H3), 1.75-1.56 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.67, 141.84, 129.88, 128.45, 128.32, 127.23, 125.96, 121.57, 120.25, 116.44, 70.38, 33.50, 33.03, 31.67, 31.49.

5.3.2.1.5.2. 3-[2-(Trifluoromethyl)phenethyl]chroman (6b). Yield: 76%, mp = 42–44 °C from n-hexane. IR (KBr): 1608 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.58 (d, 1H, H3', $J_{3'-4'}$ = 7.6 Hz), 7.40 (t, 1H, H5', $J_{4'-5'} = J_{5'-6'}$ = 7.6 Hz), 7.27 (d, 1H, H6', $J_{5'-6'}$ = 7.6 Hz), 7.23 (t, 1H. H4', $J_{4'-5'} = J_{5'-6'}$ = 7.6 Hz), 7.07–7.00 (m, 2H, H6, H5), 6.83-6.78 (m, 2H, H8, H7), 4.20 (d, 1H, H2, J_{gem} = 10.8 Hz), 3.74 (d, 1H, H2, J_{gem} = 10.8 Hz), 2.93-2.78 (m, 3H, H4, 2Hβ), 2.48 (dd, 1H, H4, J_{gem} = 16 Hz, J_{3-4} = 9.6 Hz), 2.07 -2.0 (m, 1H, H3), 1.71-1.57 (m, 2H, 2Hα).^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.69, 140.85, 131.85, 130.93, 129.94, 128.39 (q, J = 29.5 Hz), 127.29, 126.10, 126.02 (q, J = 5.7 Hz), 124.76 (q, J = 272.2 Hz), 121.46, 120.34, 116.50, 70.21, 33.91, 32.35, 31.41, 30.04.

5.3.2.1.5.3. 3-[3-(Trifluoromethyl)phenethyl]chroman (6c). Yield: 65%, mp = 43–45 °C from n-hexane. IR (KBr): 1610 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.44-7.43 (m, 2H, H2', H5'), 7.40-7.36 (m, 2H, H6', H4'), 7.08 (t, 1H, H7, $J_{7-8} = J_{7-6}$ = 7.2 Hz), 7.03 (d, 1H,

H5, $J_{5\text{-}6}$ = 7.6 Hz), 6.85-6.79 (m, 2H, H6, H8), 4.21 (m, 1H, H2, J_{gem} = 10.4 Hz, $J_{2\text{-}3}$ = 2.8 Hz), 3.79 (m, 1H, H2, J_{gem} = 10.4 Hz, $J_{2\text{-}3}$ = 2.8 Hz), 2.90 (dd, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 4.8 Hz), 2.84-2.72 (m, 2H, 2Hβ), 2.51 (dd, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 9.2 Hz), 2.07-1.98 (m, 1H, H3), 1.78-1.66 (m, 2H, 2Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.61, 142.79, 131.75, 130.84 (q, J = 31.7 Hz), 129.93, 128.89, 127.34, 124.97 (q, J = 3.9 Hz), 124.21 (q, J = 271.7 Hz), 122.92 (q, J = 3.4 Hz), 121.33, 120.40, 116.51, 70.17, 33.27, 32.93, 31.81, 31.40.

5.3.2.1.5.4. 3-[4-(Trifluoromethyl)phenethyl]chroman (6d). Yield: 67%, mp = 43–45 °C from n-hexane. IR (KBr): 1617 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.51 (d, 2H, H3', H5', $J_{2'\text{-}3'}$ = 8.0 Hz), 7.27 (d, 2H, H2', H6', $J_{2'\text{-}3'}$ = 8.0 Hz), 7.07 (t, 1H, H7, $J_{7\text{-}8}$ = $J_{7\text{-}6}$ = 7.6 Hz), 7.02 (d, 1H, H5, $J_{5\text{-}6}$ = 7.2 Hz), 6.85-6.79 (m, 2H, H6, H8), 4.21 (m, 1H, H2, J_{gem} = 10.4 Hz, $J_{2\text{-}3}$ = 3.2 Hz), 3.76 (d, 1H, H2, J_{gem} = 10.4 Hz), 2.89 (dd, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 4.8 Hz), 2.81-2.69 (m, 2H, Hβ), 2.48 (dd, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 9.2 Hz), 2.03-1.95 (m, 1H, H3), 1.74 - 1.63 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.62, 146.03, 129.95, 128.67, 128.39 (q, J = 32.2 Hz), 127.35, 125.39 (q, J = 3.6 Hz), 124.45 (q, J = 270.1 Hz), 121.35, 120.40, 116.51, 70.15, 33.14, 32.98, 31.68, 31.38.

5.3.2.1.5.5. 3-(2,4-Dichlorophenethyl)chroman (6e). Yield: 84%, mp = 53–54 °C from n-hexane. IR (KBr): 1605 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.31 (d, 1H, H3', $J_{3'\text{-}5'}$ = 1.6 Hz), 7.16–6.99 (m, 4H, H5', H6', H5, H7), 6.83 – 6.77 (m, 2H, H6, H8), 4.20 (d, 1H, H2, J_{gem} = 10.8 Hz), 3.75 (t, 1H, H2, J_{gem} = $J_{2\text{-}3}$ = 9.2 Hz), 2.87 (dd, 1H, H4, J_{gem} = 16.4 Hz, $J_{3\text{-}4}$ = 4.8 Hz), 2.83 - 2.69 (m, 2H, Hβ), 2.49 (m, 1H, H4, J_{gem} = 16.4 Hz, $J_{3\text{-}4}$ = 9.2 Hz), 2.03-1.98 (m, 1H, H3), 1.68 - 1.55 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.68, 138.16, 134.58, 132.47, 131.01, 129.97, 129.39, 127.36, 127.22, 121.43, 120.42, 116.56, 70.24, 32.01, 31.81, 31.48, 30.43.

5.3.2.1.5.6. 3-(3,5-Dichlorophenethyl)chroman (6f). Yield: 75%, mp = 51–52 °C from n-hexane. IR (KBr): 1583 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.17 (s, 1H, H4'), 7.09-7.01 (m, 4H, H2', H6', H5, H7), 6.85–6.78 (m, 2H, H6, H8), 4.20 (d, 1H, H2, J_{gem} = 10.8 Hz), 3.77 (d, 1H, H2, J_{gem} = 10.8 Hz, $J_{2\text{-}3}$ = 8.8 Hz), 2.88 (dd, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 4.8 Hz), 2.72-2.60 (m, 2H, Hβ), 2.49 (m, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 8.8 Hz), 2.02-1.98 (m, 1H, H3), 1.73-1.55 (m,

2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.57, 145.23, 134.91, 129.94, 127.39, 126.88, 126.31, 121.19, 120.45, 116.54, 70.05, 32.91, 32.62, 31.70, 31.35.

5.3.2.1.5.7. 3-(2,6-Dichlorophenethyl)chroman (6g). Yield: 91%, mp = 51–52 °C from n-hexane. IR (KBr): 1609 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.22 (d, 2H, H3', H5', $J_{3'-4'}$ = 8.0 Hz), 7.07-6.97 (m, 3H, H5, H7, H4'), 6.83 – 6.78 (m, 2H, H6, H8), 4.27–4.24 (m, 1H, H2, J_{gem} = 10.8 Hz, J_{2-3} = 9.2 Hz), 3.75 (t, 1H, H2, J_{gem} = J_{2-3} = 10.0 Hz), 3.06-2.95 (m, 2H, Hβ), 2.90 (dd, 1H, H4, J_{gem} = 17.2 Hz, J_{3-4} = 4.8 Hz), 2.49 (m, 1H, H4, J_{gem} = 16.0 Hz, J_{3-4} = 9.6 Hz), 2.09–2.05 (m, 1H, H3), 1.65-1.53 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.58, 137.80, 135.10, 129.81, 128.14, 127.61, 127.17, 121.47, 120.20, 116.39, 70.22, 32.30, 31.34, 30.16, 28.68.

5.3.2.1.5.8. 3-(2-Chlorophenethyl)chroman (6h). Yield: 58%, mp = 48–49 °C from n-hexane. IR (KBr): 1609 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.31 (d, 1H, H3', $J_{2'-3'}$ = 7.6 Hz), 7.19- 7.00 (m, 5H, H4', H5', H6', H5, H7), 6.83 – 6.78 (m, 2H, H6, H8), 4.22 (m, 1H, H2, J_{gem} = 10.0 Hz, J_{2-3} = 9.0 Hz), 3.76 (t, 1H, H2, J_{gem} = J_{2-3} = 10.0 Hz), 2.92-277 (m, 3H, H4, 2Hβ), 2.50 (m, 1H, H4, J_{gem} = 16.0 Hz, J_{3-4} = 9.6 Hz), 2.05-2.14 (m, 1H, H3), 1.71 - 1.59 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.72, 139.55, 133.94, 130.30, 129.97, 129.65, 127.55, 127.31, 126.95, 121.60, 120.35, 116.52, 70.39, 32.01, 31.98, 31.53, 30.93.

5.3.2.1.5.9. 3-(3-Chlorophenethyl)chroman (6i). Yield: 63%, mp = 52–53 °C from n-hexane. IR (KBr): 1598 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.17–7.13 (m, 3H, H6', H5, H7), 7.07– 6.98 (m, 3H, H4', H5', H2'), 6.82–6.77 (m, 2H, H6, H8), 4.17 (d, 1H, H2, J_{gem} = 10.4 Hz), 3.72 (t, 1H, H2, J_{gem} = 10.8 Hz, J_{2-3} = 9.2 Hz), 2.88 (dd, 1H, H4, J_{gem} = 16.0 Hz, J_{3-4} = 4.4 Hz), 2.70-2.58 (m, 2H, Hβ), 2.49 (m, 1H, H4, J_{gem} = 16.0 Hz, J_{3-4} = 9.2 Hz), 1.98-1.96 (m, 1H, H3), 1.69-1.57 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.72, 144.02, 134.32, 130.02, 129.81, 128.54, 127.40, 126.65, 126.29, 121.48, 120.44, 116.59, 70.26, 33.27, 32.81, 31.75, 31.48.

5.3.2.1.5.10. 3-(4-Chlorophenethyl)chroman (6j). Yield: 83%, mp = 41–42 °C from n-hexane. IR (KBr): 1581 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.20 (d, 2H, H3', H5', $J_{2'-3'}$ = 8.4 Hz), 7.07–6.99 (m, 4H, H2', H6', H5, H7), 6.82–6.77 (m, 2H, H6, H8), 4.17–4.14 (m, 1H,

H2), 3.76 (dd, 1H, H2, J_{gem} = 10.4 Hz, J_{2-3} = 9.2 Hz), 2.88 (dd, 1H, H4, J_{gem} = 16.4 Hz, J_{3-4} = 4.8 Hz), 2.70-2.58 (m, 2H, Hβ), 2.49 (m, 1H, H4, J_{gem} = 16.4 Hz, J_{3-4} = 9.2 Hz), 2.01-1.91 (m, 1H, H3), 1.68-1.58 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.53, 140.21, 131.58, 129.84, 129.60, 128.46, 127.22, 121.34, 120.26, 116.40, 70.12, 33.25, 32.29, 31.52, 31.32.

5.3.2.1.5.11. 3-(2-Bromophenethyl)chroman (6k). Yield: 30%, mp = 53–54 °C from n-hexane. IR (KBr): 1590 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.41 - 7.36 (m, 3H, H3', H5', H7'), 7.19-7.15 (m, 3H, H4', H6', H5), 7.12-7.08 (m, 2H, H6, H8), 4.20 (m, 1H, H2, J_{gem} = 10.8 Hz, J_{2-3} = 9.2 Hz), 3.79 (dd, 1H, H2, J_{gem} = 10.8 Hz, J_{2-3} = 9.2 Hz), 2.89 (dd, 1H, H4, J_{gem} = 16.2 Hz, J_{3-4} = 5.2 Hz), 2.81-2.69 (m, 2H, Hβ), 2.51 (m, 1H, H4, J_{gem} = 16.0 Hz, J_{3-4} = 8.8 Hz), 2.04-1.99 (m, 1H, H3), 1.77-1.59 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.66, 141.22, 132.94, 130.23, 129.93, 128.48, 128.35, 127.77, 127.57, 127.28, 120.32, 116.48, 70.38, 33.50, 32.12, 31.99, 31.50.

5.3.2.1.5.12. 3-(3-Bromophenethyl)chroman (6l). Yield: 13%, mp = 54–55 °C from n-hexane. IR (KBr): 1582 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.35-7.31 (m, 2H, H4', H5'), 7.14-7.02 (m, 4H, H2', H6', H5, H7), 6.85–6.78 (m, 2H, H6, H8), 4.20 (m, 1H, H2, J_{gem} = 10.4 Hz), 3.79 (t, 1H, H2, J_{gem} = J_{2-3} = 9.2 Hz), 2.89 (dd, 1H, H4, J_{gem} = 16.4 Hz, J_{3-4} = 4.8 Hz), 2.76-2.64 (m, 2H, Hβ), 2.50 (m, 1H, H4, J_{gem} = 16.0 Hz, J_{3-4} = 9.2 Hz), 2.04-2.02 (m, 1H, H3), 1.74-1.62 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.59, 144.20, 131.37, 130.01, 129.91, 129.14, 128.46, 128.33, 127.31, 127.01, 120.36, 116.48, 70.20, 33.22, 32.73, 31.66, 31.40.

5.3.2.1.5.13. 3-(4-Bromophenethyl)chroman (6m). Yield: 30%, mp = 59–60 °C from n-hexane. IR (KBr): 1605 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.38 (d, 2H, H3', H5', $J_{2',3'}$ = 8.4 Hz), 7.09-7.01 (m, 4H, H2', H6', H4', H5, H7), 6.84–6.77 (m, 2H, H6, H8), 4.20 (d, 1H, H2, J_{gem} = 10.4 Hz), 3.77 (t, 1H, H2, J_{gem} = 10.8 Hz, J_{2-3} = 9.2 Hz), 2.89 (dd, 1H, H4, J_{gem} = 16.0 Hz, J_{3-4} = 4.8 Hz), 2.74 - 2.62 (m, 2H, Hβ), 2.49 (m, 1H, H4, J_{gem} = 16.0 Hz, J_{3-4} = 8.8 Hz), 2.02–2.01 (m, 1H, H3), 1.73-1.55 (m, 2H, Hα). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.59, 140.77, 131.50, 130.06, 129.87, 127.28, 121.37, 120.32, 119.70, 116.46, 70.21, 33.26, 32.47, 31.63, 31.41.

5.3.2.1.6. 1-(2*H*-Chromen-3-yl)ethanone (7).

3-buten-2-one (40 mmol) was added dropwise to a suspension of 2-hydroxybenzaldehyde (20 mmol) and 1,4–diazabicyclo-[2.2.2]-octane (DABCO) (20 mmol) in $CHCl_3$ (14 mL) and H_2O (14 mL) at room temperature. The mixture was stirred at room temperature and under nitrogen atmosphere for a week. After this period, the separated solid was filtered and washed with water. The organic layer was washed with 2N NaOH, 2N HCl and brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The obtained solid was purified by column chromatography on silica gel eluting with a mixture of chloroform/light petroleum (1:1). Yield: 46%. mp = 44–45 °C IR (KBr): 1637 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.30 (d, 1H, H4, $J_{2\text{-}4}$ = 1.6 Hz), 7.27-7.23 (m, 1H, H5), 7.16 (m, 1H, H7, $J_{7\text{-}8}$ = 8.0 Hz, $J_{7\text{-}6}$ = 7.6 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.93 (7, 1H, H6, $J_{5\text{-}6}$ = $J_{7\text{-}6}$ = 7.6 Hz), 6.85 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 5.00 (s, 2H, H2, $J_{2\text{-}4}$ = 1.6 Hz), 2.40 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 194.65, 154.43, 132.72, 131.29, 129.56, 128.10, 120.65, 119.63, 115.13, 63.10, 23.80.

5.3.2.1.7. 1-(2*H*-Chromen-3-yl)ethanol (8).

A solution of $NaBH_4$ (10 mmol) in 2N NaOH (5 mL) is added, in a single portion, to a stirred solution of 3-acetyl-2*H*-chromene in dry EtOH (50 mL). After stirring at room temperature for 2 hours, the solvent was evaporated to dryness under vacuum and the residue was taken up with H_2O and extracted with Et_2O. The organic phase was washed with brine, dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The white oil was used for the next reaction without further purification. Yield: 89%, oil. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.08 (m, 1H, H7, $J_{7\text{-}8}$ = $J_{7\text{-}6}$ = 8.0 Hz, $J_{5\text{-}7}$ = 1.6 Hz), 6.96 (dd, 1H, H5, $J_{6\text{-}5}$ = 7.6 Hz, $J_{5\text{-}7}$ = 1.6 Hz) , 6.85 (m, 1H, H6, $J_{6\text{-}5}$ = 7.6 Hz, $J_{7\text{-}6}$ = 8.0 Hz, $J_{6\text{-}8}$ = 1.2 Hz), 6.78 (dd, 1H, H8 $J_{7\text{-}8}$ = 8.0 Hz, $J_{6\text{-}8}$ = 1.2 Hz), 6.32 (s, 1H, H4), 4.76 (s, 2H, H2), 4.36 (q, 1H, CH, $J_{CH–CH3}$ = 6.4 Hz), 2.12 (s, 1H, OH), 1.34 (d, 3H, CH_3, $J_{CH\text{-}CH3}$ = 6.4 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.55, 137.48, 128.98, 126.75, 122.49, 121.49, 118.46, 115.54, 68.62, 65.51, 21.30.

5.3.2.1.8. [1-(2*H*-Chromen-3-yl)ethyl]triphenylphosphonium bromide (9).

Triphenylphosphine hydrobromide (10 mmol) was added at room temperature to a solution of the 1-(2*H*-chromen-3-yl)ethanol (**8**) (10 mmol) in dry CH_3CN (24 mL). The reaction mixture was refluxed for 5 h. After cooling to room temperature, Et_2O was added. The white precipitate was filtered and used for the next reaction without further purification. Yield: 45%. Mp > 200 °C ^{1}H-NMR (CD_3OD, 400 MHz): δ (ppm) 7.96-7.88 (m, 3H, 3H4'), 7.81-7.7 (m, 6H, 3H3', 3H5'), 7.61-7.54 (m, 6H, 3H2', 3H6'), 7.17-7.13 (m, 1H, H5), 6.89 - 6.84 (m, 1H, H7), 6.80-6.75 (m, 1H, H6), 6.24 (m, 1H, H8), 4.91-4.83 (m, 1H, H4), 4.44-4.26 (m, 2H, H2), 3.32-3.30 (m, 1H, CH), 1.75 - 169 (m, 3H, CH_3).

5.3.2.1.9. (*E*)-3-(1-Phenylprop-1-en-2-yl)-2*H*chromene (10).

tert-BuOK (10 mmol) was added to a suspension of [1-(2*H*-chromen-3-yl)ethyl]triphenylphosphonium bromide (**9**) (10 mmol) in dry THF (43 mL) at room temperature. After 30 minutes, a solution of benzaldehyde (10 mmol) in dry THF (45 mL) was added dropwise. The mixture was stirred for 24 h at room temperature under nitrogen. After this period, ice and water was added and the mixture was neutralized with 2N HCl. The obtained suspension was extracted with $CHCl_3$ and the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The obtained solid was chromatographed on silica gel eluting with a mixture of $CHCl_3$/light petroleum (1:1), and further purified by crystallization from n-hexane. Yield: 24%, mp = 56–57 °C from n-hexane. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.36-7.33 (m, 2H, H2', H6'), 7.29-7.21 (m, 3H, H3', H5', H4'), 7.11 (m, 1H, H7, $J_{7\text{-}6} = J_{7\text{-}8} = 8.0$ Hz, $J_{7\text{-}5} = 1.6$ Hz), 7.04 (dd, 1H, H5, $J_{5\text{-}6} = 8.0$ Hz, $J_{7\text{-}5} = 1.6$ Hz), 6.88 (t, 1H, H6, $J_{7\text{-}6} = J_{5\text{-}6} = 8.0$ Hz), 6.83 (d, 1H, H8, $J_{7\text{-}8} = 8.0$ Hz) 6.61 (s, 1H, H4), 6.45 (s, 1H, Hβ), 5.10 (s, 2H, H2), 2.19 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm): 153.54, 137.71, 133.44, 133.24, 129.40, 128.96, 128.17, 127.13, 126.79, 126.47, 122.91, 121.46, 120.41, 115.35, 66.48, 14.88.

5.3.2.1.10. General procedure for the synthesis of the 1-bromomethylphenylalkyl-2-ones (12a-i).

Oxalyl chloride (23 mmol) was added dropwise to a solution of the appropriate phenylalkyl acid (**11 a-i**) (10 mmol) in dry CH_2Cl_2 (18.5 mL) cooled at 0 °C. After complete addition, the mixture was stirred at room temperature for 3 h. After this period, the mixture was evaporated to dryness and the obtained phenylalkyl acyl chloride was used for the next reaction without further purification. 2N trimethylsilyldiazomethane in dry Et_2O (27.3 mL) was added dropwise to a solution of the appropriate phenylalkyl acyl chloride (10 mmol) in dry CH_3CN (13.5 mL) cooled at 0 °C. After stirring at 0 °C for 2 h, 48% aqueous HBr (15.3 mL) was added dropwise to the mixture cooled at 0°C. After complete addition, the mixture was stirred at room temperature overnight, then diluted with water and extracted with ethyl acetate. The organic layer was washed with brine and $NaHCO_3$ solution, then dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by column chromatography on silica gel eluting with a mixture of ethyl acetate/light petroleum (1:2).

5.3.2.1.10.1. 1-Bromo-4-phenylbutan-2-one (12a). Yield: 72%, mp = 43–44 °C. IR (KBr): 1718 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.32-7.27 (m, 2H, H3', H5'), 7.22-7.17 (m, 3H, H2', H4', H6'), 3.84 (s, 2H, 2H1), 2.98-2.94 (m, 4H, 2H3, 2H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm): 200.63, 136.23, 130.29, 128.96, 128.60, 40.82, 33.85, 27.10.

5.3.2.1.10.2. 1-Bromo-4-[2-(trifluoromethyl)phenyl]butan-2-one (12b). Yield: 69%, mp = 42–43 °C IR (KBr): 1718 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.60 (d, 1H, H3', $J_{3'\text{-}4'}$ = 7.6 Hz), 7.46 (t, 1H, H5', $J_{4'\text{-}5'} = J_{5'\text{-}6'}$ = 7.6 Hz), 7.34-7.27 (m, 2H, H4', H6'), 3.90 (s, 2H, 2H1), 3.10 (t, 2H, 2H3, $J_{3\text{-}4}$ = 7.6 Hz), 2.97 (m, 2H, 2H4, $J_{3\text{–}4}$ = 7.6 Hz, $J_{4\text{–}6'}$ = 1.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 199.34, 138.06, 130.98, 130.04, 127.30 (q, J = 29.5 Hz), 125.45, 124.50 (q, J = 5.7 Hz), 123.50 (q, J = 272.0 Hz), 40.21, 33.02, 25.59.

5.3.2.1.10.3. 1-Bromo-4-[4-(trifluoromethyl)phenyl]butan-2-one (12c). Yield: 88%, mp = 43–44 °C. IR (KBr): 1717 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.53 (d, 2H, H3', H5', $J_{2'\text{-}3'}$ = 8.0 Hz), 7.30 (d, 2H, H2', H6' $J_{2'\text{-}3'}$ = 8.0 Hz), 3.86 (s, 2H, 2H1), 3.01-2.99 (m, 4H, 2H3, 2H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm): 200.70,

144.80, 128.87, 128.68 (q, J = 32.1 Hz), 125.55 (q, J = 3.8 Hz), 124.44 (q, J = 270.2 Hz), 40.81, 34.30, 29.55.

5.3.2.1.10.4. 1-Bromo-4-(4-chlorophenyl)butan-2-one (12d). Yield: 82%, mp = 47–48 °C. IR (KBr): 1719 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.26-7.24 (m, 2H, H3', H5'), 7.13-7.11 (m, 2H, H2', H6'), 3.84 (s, 2H, 2H1), 2.99-2.89 (m, 4H, 2H3, 2H4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm): 200.86, 138.75, 132.13, 129.68, 128.66, 41.10, 34.03, 29.10.

5.3.2.1.10.5. 1-Bromo-3-methyl-4-phenylbutan-2-one (12e). Yield: 75%, mp = 48–49 °C. IR (KBr): 1713 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.24 (t, 2H, H3', H5', $J_{2'\text{-}3'} = J_{3'\text{-}4'}$ = 7.6 Hz), 7.16 (t, 1H, H4', $J_{4'\text{-}5'} = J_{3'\text{-}4'}$ = 7.6 Hz), 7.10 (d, 2H, H2', H6', $J_{2'\text{-}3'}$ = 7.6 Hz), 3.77-3.68 (m, 2H, 2H1), 3.14-3.07 (m, 1H, H4), 2.95-2.90 (m, 1H, H3), 2.61-2.56 (m, 1H, H4), 1.09 (d, 3H, CH_3, $J_{CH3\text{-}H}$ = 6.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 204.95, 139.01, 128.89, 128.63, 126.60, 45.68, 39.73, 34.31, 16.94.

5.3.2.1.10.6. 1-Bromo-4-methyl-4-phenylbutan-2-one (12f). Yield: 76%, mp = 46–47 °C. IR (KBr): 1718 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.26 (m, 2H, H3', H5', $J_{2'\text{-}3'} = J_{3'\text{-}4'}$ = 7.6 Hz $J_{3'\text{-}5'}$ = 1.6 Hz), 7.19-7.14 (m, 3H, H2', H4', H6'), 3.70 (m, 2H, 2H1), 3.32-3.27 (m, 1H, H4), 2.93-2.79 (m, 2H, 2H3), 1.25 (d, 3H, CH_3, $J_{CH3\text{–}H}$ = 6.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 200.50, 145.44, 128.55, 126.66, 126.45, 47.93, 35.46, 34.95, 21.79.

5.3.2.1.10.7. 1-Bromo-5-phenylpentan-2-one (12g). Yield: 30%, mp = 51–50 °C. IR (KBr): 1715 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.30–7.26 (m, 2H, H3', H5'), 7.10–7.14 (m, 3H, H2', H6', H4'), 3.83 (s 2H, 2H1), 2.66–2.61 (m, 4H, 2H3, 2H5), 1.98–1.91 (m, 2H, 2H4). ^{13}C NMR ($CDCl_3$, 100 MHz) : δ (ppm) 201.74, 141.19, 128.46, 126.08, 125.97, 51.46, 35.11, 34.24, 25.23.

5.3.2.1.10.8. 1-Bromo-6-phenylhexan-2-one (12h). Yield: 58%, mp = 48–49 °C. IR (KBr): 1715 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.20–7.18 (m, 2H, H3', H5'), 7.12–7.08 (m, 3H, H2', H6', H4'), 3.73 (s, 2H, 2H'), 2.54–2.45 (m, 4H, 2H3, 2H6), 1.57–1.48 (m, 4H, 2H4, 2H5). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 201.25, 141.93, 128.63, 125.69, 48.23, 39.28, 35.47, 30.55, 23.27.

5.3.2.1.10.9. 1-Bromo-7-phenylheptan-2-one (12i). Yield: 47%, mp = 47–48 °C. IR (KBr): 1717 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.23-7.17 (m, 2H, H3', H5'), 7.15–7.13 (m, 3H, H2', H6', H4'), 3.82 (s, 2H, 2H1), 2.61–2.57 (m, 4H, 2H3, 2H7), 1.66–1.57 (m, 4H, 2H4, 2H6), 1.36–1.28 (m, 2H, 2H5). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 201.95, 142.35, 128.34, 128.24, 125.66, 39.61, 35.64, 34.35, 31.09, 28.54, 23.61.

5.3.2.1.11. General procedure for the synthesis of the phenylalkyl-2*H*-chromenes (13a-i).

Method A

A solution of EtONa prepared from Na (20 mmol) in dry EtOH (27 mL) was added dropwise to a solution of (2-hydroxybenzyl)triphenylphosphonium bromide (**3o**) (10 mmol) in dry EtOH (30 mL) at room temperature. After stirring for 30 minutes, a solution of the appropriate bromomethyl-phenylketone (**12a, d**) (5 mmol) in dry EtOH (23 mL) was added dropwise. The mixture was stirred at room temperature overnight, water was added and EtOH was removed under reduced pressure. The remaining mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The solid products was chromatographed on silica gel eluting with a mixture of $CHCl_3$/light petroleum (1:1) and further purified by crystallization from n-hexane.

Method B

tert-BuOK (10 mmol) was added to a solution of (2-hydroxybenzyl)triphenylphosphonium bromide (**3o**) (10 mmol) in dry THF (43 mL) at room temperature. After 10 minutes, a solution of the appropriate bromomethylphenylketone (**12b-c, 12e-i**) (10 mmol) in dry THF (44 mL) was added dropwise. After 2 h, a new portion of *tert*-BuOK (10 mmol) was added and the mixture was stirred for 24 h at room temperature under nitrogen. After this period, ice and water were added and the mixture was neutralized and extracted with $CHCl_3$. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The solid products was chromatographed on silica gel eluting with a mixture of

$CHCl_3$/light petroleum (1:1) and further purified by crystallization by n-hexane.

5.3.2.1.11.1. 3-Phenethyl-2*H*-chromene (13a). Yield: (Method A) 22%, mp = 39–40 °C from n-hexane. IR (KBr): 1604 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.29-7.16 (m, 2H, H3', H5'), 7.21-7.17 (m, 3H, H2', H6', H4'), 7.06 (m, 1H, H7, $J_{7\text{-}6} = J_{7\text{-}8} = 7.4$ Hz, $J_{5\text{-}7} = 1.6$ Hz), 7.03 (m, 1H, H5, $J_{5\text{-}6} = 7.4$ Hz, $J_{5\text{-}7} = 1.6$ Hz), 6.90 (t, 1H, H6, $J_{5\text{-}6} = J_{6\text{-}7} = 7.4$ Hz,), 6.83 (m, 1H, H8), 6.15 (m, 1H, H4), 4.66 (s, 2H, H2), 2.80 (t, 2H, Hβ, $J_{\alpha\text{-}\beta} = 7.6$ Hz), 2.36 (t, 2H, Hα, $J_{\alpha\text{-}\beta} = 7.6$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.98, 141.24, 134.44, 128.48, 128.31, 128.25, 126.14, 126.03, 122.87, 121.31, 119.22, 115.29, 68.31, 33.52, 29.71.

5.3.2.1.11.2. 3-(2-Trifluoromethyl)phenethyl-2*H*-chromene (13b). Yield: (Method B) 37%, mp = 58–59 °C from n-hexane. IR (KBr): 1607 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.59 (d, 1H, H3', $J_{3'\text{-}4'} = 8.0$ Hz), 7.41 (t, 1H, H5', $J_{6'\text{-}5'} = J_{5'\text{-}4'} = 8.0$ Hz) 7.28-7.21 (m, 2H, H4', H6'), 7.03 (m, 1H, H7, $J_{7\text{-}6} = J_{7\text{-}8} = 7.6$ Hz, $J_{7\text{-}5} = 1.6$ Hz), 6.89 (dd, 1H, H5, $J_{5\text{-}6} = 7.6$ Hz, $J_{7\text{-}5} = 1.6$ Hz), 6.80 (m, 1H, H6, $J_{5\text{-}6} = J_{7\text{-}6} = 7.6$ Hz, $J_{6\text{-}8} = 1.2$ Hz), 6.76 (dd, 1H, H8, $J_{7\text{-}8} = 7.6$ Hz, $J_{6\text{-}8} = 1.2$ Hz), 6.15 (s, 1H, H4), 4.66 (s, 2H, H2), 2.94 (t, 2H, Hβ, $J_{\alpha\text{-}\beta} = 8.0$ Hz), 2.36 (t, 2H, Hα, $J_{\alpha\text{-}\beta} = 8.0$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.20, 140.02, 133.97, 131.89 (q, $J = 1.1$ Hz), 131.07, 128.44 (q, $J = 29.6$ Hz), 128.43. 126.34, 126.19, 126.12 (q, $J = 5.7$ Hz), 124.77 (q, $J = 272$ Hz), 122.81, 121.40, 119.51, 115.40, 68.16, 35.09, 30.51.

5.3.2.1.11.3. 3-(4-Trifluoromethyl)phenethyl-2*H*-chromene (13c). Yield: (Method B) 39%, mp = 59–61 °C from n-hexane. IR (KBr): 1615 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.52 (d, 2H, H3', H5', $J_{2'\text{-}3'} = 8.0$ Hz), 7.27 (d, 2H, H2', H6', $J_{2'\text{-}3'} = 8.0$ Hz), 7.04 (m, 1H, H7, $J_{7\text{-}6} = 7.6$ Hz, $J_{7\text{-}8} = 8.0$ Hz, $J_{7\text{-}5} = 1.6$ Hz), 6.89 (dd, 1H, H5, $J_{5\text{-}6} = 7.6$ Hz, $J_{7\text{-}5} = 1.6$ Hz), 6.83 (t, 1H, H6, $J_{5\text{-}6} = J_{7\text{-}6} = 7.6$ Hz), 6.77 (d, 1H, H8, $J_{7\text{-}8} = 8.0$ Hz,), 6.14 (s, 1H, H4), 4.65 (s, 2H, H2), 2.84 (t, 2H, Hβ, $J_{\alpha\text{-}\beta} = 8.0$ Hz), 2.36 (t, 2H, Hα, $J_{\alpha\text{-}\beta} = 8.0$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.98, 145.33, 133.74, 128.68, 128.58 (q, $J = 21.7$ Hz), 128.47, 126.15, 125.42 (q, $J = 3.7$ Hz), 124.39 (q, $J = 270.1$ Hz), 122.72, 121.45, 119.55, 115.39, 114.94, 68.22, 34.57, 33.20.

5.3.2.1.11.4. 3-(4-Chlorophenethyl)-2*H*-chromene (13d). Yield: (Method A) 5%, mp = 46–48 °C from n-hexane. IR (KBr): 1731 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.24–7.20 (m, 2H, H3', H5'), 7.11–7.08 (2H, H2', H6'), 7.06 (m, 1H, H7, $J_{7\text{-}6}$ = 7.6 Hz, $J_{7\text{-}8}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.91 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.6 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.84 (m, 1H, H6, $J_{5\text{-}6}$ = $J_{7\text{-}6}$ = 7.6 Hz), 6.77 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 6.15 (s, 1H, H4), 4.66 (s, 2H, H2), 2.80 (t, 2H, Hβ, $J_{\alpha\text{-}\beta}$ = 8.0 Hz), 2,36 (t, 2H, Hα, $J_{\alpha\text{-}\beta}$ = 8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.93, 139.60, 133.92, 131.91 129.67, 128.58, 128.36, 126.07, 122.73, 121.37, 119.49, 115.32, 68.26, 34.89, 32.83.

5.3.2.1.11.5. 3-(2-phenylpropyl)-2*H*-chromene (13e). Yield: (Method B) 58%, mp = 63–64 °C from n-hexane. IR (KBr): 1604 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.49 (m, 2H, H3', H5'), 7.39–7.36 (m, 3H, H2', H6', H4'), 7.24–7.21 (m, 1H, H7, $J_{7\text{-}6}$ = $J_{7\text{-}8}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 7.10 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.2 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 7.03 (m, 1H, H6, $J_{5\text{-}6}$ = 7.2 Hz, $J_{7\text{-}6}$ = 8.0 Hz), 6.99 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 6.31 (s, 1H, H4), 4.76 (dd, 2H, H2), 3.16-3.07 (m, 1H, Hβ), 2,63-2.49 (m, 2H, Hα), 1.50 (d, 3H, CH_3, $J_{CH3\text{-}\beta}$ = 6.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.89, 146.32, 133.35, 128.40, 128.13, 126.70, 126.21, 125.91, 122.84, 121.17, 120.61, 115.20, 68.22, 42.58, 37.99, 21.73.

5.3.2.1.11.6. 3-(1-phenylpropan-yl)-2*H*-chromene (13f). Yield: (Method B) 38%, mp = 65–68 °C from n-hexane. IR (KBr): 1604 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.23 (t, 2H, H3', H5', $J_{2'\text{-}3'}$ = 8.0 Hz), 7.16-7.10 (m, 3H, H2', H6', H4'), 7.01–6.98 (m, 1H, H7, $J_{7\text{-}6}$ = $J_{7\text{-}8}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.89 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.6 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.81 (m, 1H, H6, $J_{5\text{-}6}$ = 7.6 Hz, $J_{7\text{-}6}$ = 8.0 Hz), 6.76 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 6.11 (s, 1H, H4), 4.68–4.66 (m, 2H, H2), 2,84-2.79 (m, 1H, Hβ), 2,57-2.52 (m, 1H, Hα), 2.47-2.38 (m, 1H, CH), 1.05 (d, 3H, CH_3, $J_{CH3\text{-}H}$ = 7.2 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.18, 140.01, 138.97, 129.00, 128.24, 128.18, 126.15, 126.08, 123.09, 121.29, 118.18, 115.27, 67.21, 41.37, 39.41, 18.04.

5.3.2.1.11.7. 3-(3-Phenylpropyl)-2*H*-chromene (13g). Yield: (Method B) 43%, mp = 43–45 °C from n-hexane. IR (KBr): 1604 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.23–7.22 (m, 2H, H3', H5'), 7.17–7.12 (m, 3H, H2', H6', H4'), 7.05 (m, 1H, H7, $J_{7\text{-}8}$ = $J_{7\text{-}6}$ = 8.0 Hz, $J_{7\text{-}5}$

= 1.6 Hz), 6.87 (dd, 1H, H5, $J_{5\text{-}6}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.79 (m, 1H, H6, $J_{7\text{-}6}$ = $J_{5\text{-}6}$ = 8.0 Hz, $J_{6\text{-}8}$ =1.2 Hz), 6.74 (dd, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz, $J_{6\text{-}8}$ = 1.2 Hz), 6.10 (s, 1H, H4), 4.6 (s, 1H, H2), 2.59 (t, 2H, 2Hα, $J_{\alpha\text{-}\beta}$ = 7.2 Hz), 2.02 (t, 1H, 2Hγ, $J_{\beta\text{-}\gamma}$ = 8.0 Hz), 1.80–1.73 (m, 2H, 2Hβ). ^{13}C NMR ($CDCl_3$, 100 MHz) : δ (ppm) 153.16, 142.02, 134.97, 128.62, 128.58, 128.35, 126.14, 126.10, 123.12, 121.47, 119.04, 115.48, 68.51, 35.59, 32.81, 28.60.

5.3.2.1.11.8. 3-(4-Phenylbutyl)-2*H*-chromene (13h). Yield: (Method B) 42%, mp = 45–46 °C from n-hexane. IR (KBr): 1604 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.29–7.45 (m, 2H, H3', H5'), 7.19–7.15 (m, 3H, H2', H6', H4'), 7.04–6.99 (m, 1H, H7, $J_{7\text{-}6}$ = $J_{7\text{-}8}$ = 7.6 Hz, $J_{7\text{-}5}$ = 2.0 Hz), 6.90 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.6 Hz, $J_{7\text{-}5}$ = 2.0 Hz), 6.84–6.80 (m, 1H, H6, $J_{5\text{-}6}$ = $J_{7\text{-}6}$ = 7.6 Hz, $J_{6\text{-}8}$ = 1.2 Hz), 6.74 (dd, 1H, H8, $J_{7\text{-}8}$ = 7.6 Hz, $J_{6\text{-}8}$ = 1.2 Hz), 6.10 (s, 1H, H4), 4.6 (s, 1H, H2), 2,63 (t, 2H, 2Hα, $J_{\alpha\text{-}\beta}$ = 7.2 Hz), 2.08 (t, 2H, 2Hδ, $J_{\gamma\text{-}\delta}$ = 7.6 Hz), 1.68–1.62 (m, 2H, 2Hγ), 1.56-1.49 (m, 2H, 2Hβ). ^{13}C NMR ($CDCl_3$, 100 MHz) : δ (ppm) 152.88, 142.26, 135.01, 128.38, 128.31, 128.07, 125.87, 125.75, 122.94, 121.25, 118.69, 115.22, 68.32, 35.69, 33.07, 30.99, 26.36.

5.3.2.1.11.9. 3-(5-Phenylpentyl)-2*H*-chromene (13i). Yield: (Method B) 55%, mp = 45–46 °C from n-hexane. IR (KBr): 1604 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.27-7.23 (m, 2H, H3', H5'), 7.16-7.14 (m, 3H, H2', H6', H4'), 7.04–6.99 (m, 1H, H7, $J_{7\text{-}6}$ = $J_{7\text{-}8}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.88 (dd, 1H, H5, $J_{5\text{-}6}$ = 8.0 Hz, $J_{7\text{-}5}$ = 1.6 Hz), 6.81 (t, 1H, H6, $J_{5\text{-}6}$ = $J_{7\text{-}6}$ = 8.0 Hz), 6.75 (d, 1H, H8, $J_{7\text{-}8}$ = 8.0 Hz), 6.10 (s, 1H, H4), 4.63 (s, 2H, H2), 2.59 (t, 2H, 2Hα, $J_{\alpha\text{-}\beta}$ = 7.6 Hz), 2.03 (t, 2H, 2Hδ, $J_{\delta\text{-}\varepsilon}$ = 7.6 Hz), 1.67–1.59 (m, 2H, 2Hε), 1.53–1.46 (m, 2H, 2Hβ), 1.42–1.33 (m, 2H, 2Hγ). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.87, 142.52, 135.19, 128.35, 128.25, 128.03, 125.86, 125.45, 122.96, 121.23, 118.56, 115.21, 68.32, 35.82, 33.11, 31.23, 28.88, 26.68.

5.3.2.1.12. General procedure for the synthesis of 3-phenylalkylchromans (14g-i, 18a-c).

A solution of the appropriate phenylalkyl-2*H*-chromene (**13g-i**) or the appropriate mixture of (*Z*)-3-[(*E*)-3-phenylallylidene]chroman, (*E*)-3-(3-phenyl)prop-1-en-1-yl)-2*H*-chromene, and 3-cinnamyl-2*H*-chromene (**15, 16, 17a**; **15, 16, 17b** or **15, 16, 17c**)[4] **(**10 mmol) in dry

dioxane (80 mL) was hydrogenated over 10% Pd/C (15.2 mmol) at room temperature for 30 minutes, under 40 psi pressure. After this period, the catalyst was filtered off and the solvent was removed under reduced pressure. The residue was chromatographed on silica gel eluting with a mixture of chloroform/light petroleum (1:1). The product was further purified by crystallization from n-hexane.

5.3.2.1.12.1. 3-(3-Phenylpropyl)chroman (14g). Yield: 34%, mp = 41–42 °C from n-hexane. IR (KBr): 1606 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.28–7.24 (m, 2H, H3', H5'), 7.18-7.15 (m, 3H, H2', H6', H4'), 7.07–7.03 (t, 1H, H7, $J_{7\text{-}6} = J_{7\text{-}8} = 8.0$ Hz), 6.99 (d, 1H, H5, $J_{5\text{-}6} = 7.6$ Hz), 6.82 (dd, 1H, H6, $J_{5\text{-}6} = 7.2$ Hz, $J_{6\text{-}8} = 1.2$ Hz), 6.78 (d, 1H, H8, $J_{7\text{-}8} = 8.0$ Hz, $J_{6\text{-}8} = 1.2$ Hz), 4.19–4.15 (m, 1H, H2), 3.72–3.67 (m, 1H, H2), 2.82 (dd, 1H, H4, $J_{gem} = 16$ Hz, $J_{3\text{-}4} = 4.0$ Hz), 2.64-2.58 (m, 2H, 2Hγ), 2.45–2.38 (m, 1H, H4, $J_{gem} = 16$ Hz, $J_{3\text{-}4} = 9.6$ Hz), 2.03–1.97 (m, 1H, H3), 1.77–1.66 (m, 2H, 2Hα), 1.42–1.20 (m, 2H, 2Hβ). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.65, 142.14, 129.83, 128.34, 128.32, 127.16, 125.79, 121.67, 120.18, 116.39, 70.49, 35.95, 32.03, 31.48, 31.36, 28.58.

5.3.2.1.12.2. 3-(4-Phenylbutyl)chroman (14h). Yield: 86%, mp = 43–44 °C from n-hexane. IR (KBr): 1607 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.26–7.23 (m, 2H, H3', H5'), 7.16-7.13 (m, 3H, H2', H6', H4'), 7.04 (t, 1H, H7, $J_{7\text{-}6} = J_{7\text{-}8} = 7.6$ Hz), 6.97 (d, 1H, H5, $J_{5\text{-}6} = 7.2$ Hz), 6.81–6.77 (m, 2H, H6, H8), 4.16–4.12 (m, 1H, H2), 3.69–3.65 (m, 1H, H2), 2.78 (dd, 1H, H4, $J_{gem} = 16.0$ Hz, $J_{3\text{-}4} = 4.8$ Hz), 2.59 (t, 2H, 2Hδ, $J_{\gamma\text{-}\delta} = 7.2$ Hz) 2.41–2.35 (m, 1H, H2, $J_{gem} = 16.0$ Hz), 1.99–1.88 (m, 1H, H3), 1.65–1.58 (m, 2H, CH_3, 2Hγ), 1.46-1.22 (m, 4H, 2Hα, 2Hβ) . ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.61, 142.38, 129.81, 128.32, 128.24, 127.10, 125.65, 121.68, 120.11, 116.34, 70.48, 35.77, 31.98, 31.55, 31.48, 31.46, 26.31.

5.3.2.1.12.3. 3-(5-Phenylpentyl)chroman (14i). Yield: 81%, mp = 42–43 °C from n-hexane. IR (KBr): 1607 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.26–7.23 (m, 2H, H3', H5'), 7.15-7.13 (m, 3H, H2', H6', H4'), 7.04 (t, 1H, H7, $J_{7\text{-}6} = J_{7\text{-}8} = 8.0$ Hz), 6.98 (d, 1H, H5, $J_{5\text{-}6} = 7.2$ Hz), 6.82–6.77 (m, 2H, H6, H8), 4.17–4.13 (m, 1H, H2), 3.69–3.65 (m, 1H, H2), 2.79 (dd, 1H, H4, $J_{gem} = 16.4$ Hz, $J_{3\text{-}4} = 4.8$ Hz), 2.58 (t, 2H, Hδ, $J_{\gamma\text{-}\delta} = 7.6$ Hz), 2,41-2.35 (m, 1H, H4, $J_{gem} = 16.4$ Hz, $J_{3\text{-}4} = 9.6$ Hz), 1.96–1.91 (m, 1H, H3), 1.65–1.57 (m, 2H, 2Hγ), 1.46–

1.18 (m, 6H, 2Hε, 2Hα, 2Hβ). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.62, 142.56, 129.81, 128.32, 128.20, 127.00, 125.59, 121.72, 120.10, 116.33, 70.52, 32.00, 31.66, 31.49, 31.35, 29.33, 26.57.

5.3.2.1.12.4. 6-chloro-3-(3-phenylpropyl)chroman (18a). Yield: 79%, mp = 42–43 °C from n-hexane. IR (KBr): 1484 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.29–7.26 (m, 2H, H3', H5'), 7.20-7.16 (m, 3H, H2', H6', H4'), 7.00 (dd, 1H, H7, $J_{7\text{-}6}$ = 8.4 Hz, $J_{7\text{-}5}$ = 2.4 Hz), 6.97 (d, 1H, H5, $J_{7\text{-}5}$ = 2.4 Hz), 6.70 (d, 1H, H8, $J_{7\text{-}8}$ = 8.4 Hz), 4.18–4.15 (m, 1H, H2), 3.71–3.67 (m, 1H, H2), 2.80 (dd, 1H, H2, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 4.0 Hz), 2,61 (t, 2H, 2Hγ, $J_{\beta\text{-}\gamma}$ = 7.6 Hz), 2.42-2.36 (m, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 9.2 Hz), 2.03–1.95 (m, 1H, H3), 1.76–1.68 (m, 2H, 2Hα), 1.45-1.30 (m, 2H, 2Hβ). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.29, 142.05, 129.36, 128.37, 127.14, 127.13, 125.87, 124.83, 123.29, 117.70, 70.57, 35.94, 31.75, 31.34, 31.19, 28.55.

5.3.2.1.12.5. 6-chloro-3-[3-(4-chlorophenyl)propyl]chroman (18b). Yield: 85%, mp = 41–42 °C from n-hexane. IR (KBr): 1484 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.20 (d, 2H, H3', H5', $J_{2'\text{-}3'}$ = 8.0 Hz), 6.96 (m, 1H, H7, $J_{7\text{-}8}$ = 8.8 Hz, $J_{7\text{-}5}$ = 2.4 Hz), 6.92 (d, 1H, H5, $J_{7\text{-}5}$ = 2.4 Hz), 6.67 (d, 1H, H8, $J_{7\text{-}8}$ = 8.8 Hz), 4.12–4.09 (m, 1H, H2), 3.65–3.60 (d, 1H, H2), 2.71 (s, 1H, H4, J_{gem} = 16.4 Hz, $J_{3\text{-}4}$ = 4.8 Hz), 2.54 (t, 2H, Hγ, $J_{\beta\text{-}\gamma}$ = 7.6 Hz), 2,34-2.28 (m, 1H, H4, J_{gem} = 16.4 Hz, $J_{3\text{-}4}$ = 9.6 Hz), 1.91–1.87 (m, 1H, H3), 1.70 (m, 2H, Hα), 1.37–1.19 (m, 2H, 2Hβ). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.19, 140.41, 131.44, 129.64, 128.28, 128.36, 127.05, 124.69, 123.15, 117.64, 70.34, 35.16, 31.58, 31.18, 30.98, 28.34.

5.3.2.1.12.6. 3-[3-(4-chlorophenyl)propyl]chroman (18c). Yield: 16%, mp = 42–43 °C from n-hexane. IR (KBr): 1489 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.23 (d, 2H, H3', H5', $J_{2'\text{-}3'}$ = 8.4 Hz), 7.10-7.04 (m, 3H, H2', H6', H7), 7.00 (d, 1H, H5, $J_{5\text{-}6}$ = 7.2 Hz), 6.84–6.77 (m, 2H, H6, H8), 4.19–4.16 (m, 1H, H2), 3.74–3.69 (d, 1H, H2), 2.83 (dd, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 4.4 Hz), 2.59 (t, 2H, Hγ, $J_{\beta\text{-}\gamma}$ = 8.0 Hz), 2,46-2.39 (m, 1H, H4, J_{gem} = 16.0 Hz, $J_{3\text{-}4}$ = 9.6 Hz), 2,04–1.95 (m, 1H, H3), 1.76–1.63 (m, 2H, 2Hα), 1,44–1.27 (m, 2H, 2Hβ). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 154.62, 140.56, 131.54, 129.86, 129.71, 128.44, 127.21, 121.58, 120.24, 116.41, 70.43, 35.28, 32.03, 31.47, 31.23, 28.51.

5.3.2.2.1. Anti-HRV assays

The cytotoxicity and anti-HRV activity assays were performed by Prof. Cinzia Conti, Department of Public Health Sciences, Section of Microbiology, Sapienza University of Rome. The new compounds were evaluated following the experimental protocol previously described.[4]

5.3.2.2.2. Antiviral assays against selected RNA and DNA viruses

Tests of cytotoxicity and antiviral activity *in vitro* were performed by Dr. Roberta Loddo at the Department of Biomedical Sciences and Technologies, Section of General Microbiology and Virology & Microbial Biotechnologies, University of Cagliari.

5.3.2.2.2.1 Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Cell lines supporting the multiplication of RNA and DNA viruses were the following: Madin Darby Bovine Kidney (MDBK) [ATCC CCL 22 (NBL-1) Bos Taurus]; Baby Hamster Kidney (BHK-21) [ATCC CCL 10 (C-13) Mesocricetus auratus] and Monkey kidney (Vero 76) [ATCC CRL 1587 Cercopithecus Aethiops]. Viruses were purchased from American Type Culture Collection (ATCC) except Yellow Fever Virus (YFV). Viruses representative of positive-sense single strand RNA ($ssRNA^+$) group used were: (**ii**) Flaviviridae family: YFV [strain 17-D vaccine (Stamaril Pasteur J07B01)] and Bovine Viral Diarrhea Virus (BVDV) [strain NADL (ATCC VR-534)] (**iii**) Picornaviridae family: Human Coxsackievirus type B5 (CVB-5) strain Ohio-1 (ATCC VR-29) and Human Poliovirus type-1 Sabin (Sb-1) [strain Chat (ATCC VR-1562)]. Virus representative of a negative-sense single-strand RNA ($ssRNA^-$) group used were: Vesicular Stomatitis Virus (VSV) [strain Indiana Lab (ATCC VR-158)] and Human Respiratory Syncytial Virus (RSV) [strain A2 (ATCC VR-1540)]. A virus representative of a double strand RNA (dsRNA) group used was: Reovirus type-1 [strain 3651 (SV-12, simian virus 12) (ATCC VR-214)]. Viruses representatives of DNA group used were: (**i**) Poxviridae family: Vaccinia Virus (VV) [strain Elstree-Lister Vaccine

(ATCC VR-1549)]; (**ii**) Herpesviridae family: Human Herpesvirus 1 (HSV-1) [strain KOS (ATCC VR-1493)]. As reference inhibitors were used: Pleconaril for picornaviruses, Ribavirin for $ssRNA^+$ and dsRNA viruses; 6-Azauridine, Ribavirin and Mycophenolic acid for the $ssRNA^-$ viruses; Mycophenolic acid and Acyclovir for DNA viruses.

5.3.2.2.2.2. Cytotoxicity assays

MDBK and BHK cells were seeded at an initial density of $6x10^5$ and $1x10^6$ cells/mL in 96-well plates, respectively, in culture medium [Minimum Essential Medium with Earle's salts (MEM-E) with L-glutamine, supplemented with 10% horse serum and 1 mM sodium pyruvate (for MDBK cells) or with 10% fetal bovine serum (FBS) (for BHK cells), 0.025 g/L kanamycin]. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48–96 h at 37 °C by the MTT method. Vero-76 cells were seeded at an initial density of $4x10^5$ cells/mL in 24-well plates, in culture medium [Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine, supplemented with fetal bovine serum (FBS), 0.025 g/L kanamycin]. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48–96 h at 37 °C by the crystal violet staining method.

5.3.2.2.2.3. Antiviral assays

Compounds activity against YFV and Reo-1 was based on inhibition of virus-induced cytopathogenicity in BHK-21 cells acutely infected with a m.o.i. of 0.01. Compounds activity against BVDV was based on inhibition of virus-induced cytopathogenicity in MDBK cells acutely infected with a m.o.i. of 0.01. Briefly, BHK and MDBK cells were seeded in 96-well plates at a density of $5x10^4$ and $3x10^4$ cells/well, respectively, and were allowed to form confluent monolayers by incubating overnight in growth medium at 37 °C in a humidified CO_2 (5%) atmosphere. Cell monolayers were then infected with 50 µL of a proper virus dilution in maintenance medium (MEM-E with L-glutamine, supplemented with 0.5% inactivated FBS, 1 mM sodium pyruvate and 0.025 g/L kanamycin) to give a m.o.i of 0.01. After 1 h, 50 µL of maintenance medium, without or with serial dilutions of test compounds, were added. After a 3–4 days incubation

at 37 °C, cell viability was determined by the MTT method. Compounds activity against CVB-5, Sb-1, VSV, VV, HSV-1 and RSV was determined by plaque reduction assays in infected Vero 76 cell monolayers. To this end, Vero 76 cells were seeded in 24-well plates at a density of $2x10^5$ cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium [Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and 4500 mg/L D-glucose, supplemented with 10% FBS and 0.025 g/L Kanamycin] at 37 °C in a humidified CO_2 (5%) atmosphere. Then, monolayers were infected for 2 h with 250 µL of proper virus dilutions to give 50–100 PFU/well. Following removal of unadsorbed virus, 500 µL of maintenance medium (DMEM medium with L-glutamine and 4500 mg/L D-glucose supplemented with 1% inactivated FBS and 0.75% methyl-cellulose), without or with serial dilutions of test compounds, were added. Cultures were incubated at 37 °C for 2 (Sb-1 and VSV), 3 (CVB-5, VV and HSV-1) or 5 days (RSV) and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. Plaques were then counted and IC_{50} (50% effective concentration) was calculated by linear regression technique. The cytotoxicity of test compounds was determined in parallel on the same 24-well plate used for the IC_{50} determination. Linear regression analysis: viral and cell growth at each drug concentration was expressed as percentage of untreated controls and concentrations resulting in 50% (IC_{50} and TC_{50}) growth inhibition were determined by linear regression analysis.

References

1. Loiodice, F.; Longo, A.; Bianco, P.; Tortorella, V. Tetrahedron: *Asymmetry* **1995**, *6*, 1001.
2. Silva, V. L. M.; Silva, A. M. S.; Pinto, D. C. G. A.; Cavaliero, J. A. S.; Patanay, T. *Synlett* **2004**, 2717.
3. Gangjee A, Yang J, Ihnat MA, Kamat S. Bioorg Med Chem. **2003**, *11*, 5155–5170.
4. Conti C., Proietti Monaco L., Desideri N., *Bioorg & Med. Chem.* **2011**, *19*, 7357.
5. Scudiero, D. A.; Shoemaker, R. H.; Paull, K.D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827.
6. Desideri N., Conti C., Sestili I., Tomao P., Stein M.L, Orsi N., *Antiviral Chem. Chemother.* **1992**, *3***,** 195.
7. Alami, M.; Peyrat, J. F.; Belachmi, L.; Brion, J. D. *Eur. J. Org. Chem*. **2001**, *14*, 4207.
8. Conti, C.; Desideri, N. *Bioorg. Med. Chem.* **2010**, *18*, 6480.

5.4. Conclusions

New series of 3-arylalkyl 4*H*-chromen-4-ones, chromans and 2*H*-chromenes have been synthesized and tested in vitro against HRV 1B and 14, two representative serotypes for HRV group B and A, respectively.

By anti-HRV data we can draw the following structure-activity relationships:

- electron-withdrawing substituents, in particular a chlorine atom at the 6 and/or 4' position, have the best impact on the activity;
- the oxidation of chromene ring causes decrease in activity, while the reduction of the ring maintains or improves the potency;
- the branching of the chain between the two rings usually leads to an increase in cytotoxity and reduction or loss of anti-HRV activity. Compounds containing an unsaturated chain with four carbon atoms are usually less powerful than the analogues with shorter or saturated chains. The substitution with a saturated or monounsaturated chain from two to five carbon atoms leads to analogues endowed with potency in the submicromolar range, in particular against serotype 1B.
- The bioisosteric replacement of phenyl ring with a pyridine ring produce analogues active at the micromolar or submicromolar concentrations.

Several 2*H*-chromene and chroman derivatives displayed a wide spectrum of anti-HRV activity. The efficacy in the micromolar or submicromolar range coupled with the low cytotoxicity resulted in compounds with high therapeutic index. The antiviral evaluation against a large panel of RNA and DNA viruses demonstrated the selectivity of the new compounds as anti-HRV inhibitors.

Mechanism of action studies of selected derivatives suggest that these compounds act as capsid-binders and interfere with early stages of virus replication. However, while (*E*)-3-styryl-2*H*-chromene was able to interfere also with virus binding to cellular receptor, both (*E*)-6-chloro-3-(3-phenylprop-1-en-1-yl)-2*H*-chromene and (*E*)-2-[2-(2*H*-chromen-3-yl)vinyl]pyridine did not appear to affect the absorption of virus suggesting an interference during the uncoating process.

CHAPTER 2

FLAVONOID ANALOGUES AS POTENT AND SELECTIVE MAO-B INHIBITORS

1. INTRODUCTION

Monoamine oxidase (MAO, EC 1.4.3.4) is a flavoenzyme that catalyzes the deamination of a broad spectrum of biogenic and xenobiotic amines. Two isoforms of MAOs have been identified and designated as MAO-A and MAO-B. The MAO isozymes are products of separate genes and they share approximately 70% amino acid sequence identity.[1] Studies on X-ray crystallographic structures of human MAO-A and MAO-B indicate that 6 of the 16 amino acid residues comprising the active sites differ between the two enzymes.[2,3] For these reasons, MAO-A and MAO-B have different substrate and inhibitor specificities. MAO-A preferentially metabolizes the neurotransmitters, serotonin, epinephrine and norepinephrine, and it is selectively inhibited by clorgyline. MAO-B are predominantly involved in the metabolism of benzylamine and β−phenylethylamine, and it is selectively inhibited by selegiline and rasagiline. Dopamine and tyramine are considered to be a substrate for both isozymes.[4] Although in humans both isozymes are expressed in most peripheral tissues and organs, MAO-A is preponderant in placenta, lung and small intestine, while MAO-B is the only isoform in platelets and lymphocytes. In brain MAO-B (present in glial cells) predominates over MAO-A.[1] These properties determine the therapeutic potential of MAO inhibitors. MAO-A inhibitors have therapeutic utility for the treatment of depression[5] whereas MAO-B inhibitors are used with L-DOPA and/or dopamine agonists in the symptomatic treatment of Parkinson's disease[6,7], and are potential drugs in the therapy of Alzheimer's disease (AD).[8,9] However, the first generation of non selective and irreversible MAO inhibitors was characterized by adverse side-effects such as serious hypertensive crisis following the ingestion of dietary tyramine. The efforts devoted to the discovery of safer inhibitors led to the new generation of MAO inhibitors characterized by the selectivity against MAO isoforms and in some cases by the reversibility of action. Various structural classes of potent MAO inhibitors, including hydrazide, amide, thiazole, imidazole, oxazolidinone, oxadiazolone, diacylurea derivatives, etc. have been identified.[10-23] Figure 1 shows the chemical structures of some representative MAO inhibitors used in research or clinical practice.

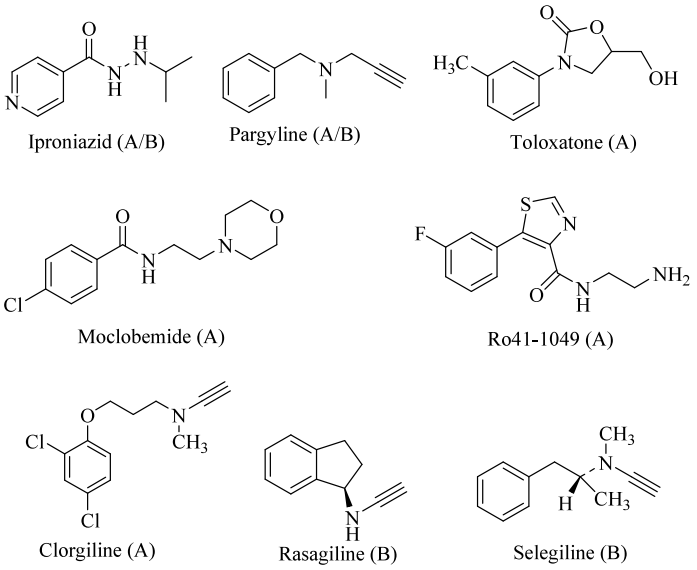


Figure 1. Some representative structures of non selective and selective MAO inhibitors

As the ideal drug candidate has not been achieved, researchers continued to explore this field.

References

1. Shih J.C., Chen K., Ridd M.J., *Annu. Rev. Neurosci*., **1999**, *22* ,197.
2. Son S.Y., Ma J., Kondou Y., Yoshimura M., Yamashita E., Sukihara T. T., *Proc. Natl. Acad. Sci. U.S.A*., **2008**, *105*, 5739.
3. C. Binda C., Wang J., Pisani L., Caccia C., Carotti A., Salvati P., Edmondson D.E., Mattevi A., *J. Med. Chem*. **2007**, *50*, 5848.
4. Youdim M.B., Edmondson D.E., Tipton K.F., *Nat. Rev. Neurosci*. **2006**, *7*, 295.
5. Yamada M., Yasuhara H., *Neurotoxicology*, **2004**, *25*, 215.
6. Drukarch B., Van Muiswinkel F.L, *Biochem. Pharmacol*. **2000**, *59*, 1023.
7. Schapira A.H., *Arch. Neurol*. **2007**, *64*, 1083.
8. Riederer P., Danielczyk W., Grunblatt E., *Neurotoxicology*, **2004**, *25*, 271.
9. Thomas T., *Neurobiol. Aging*, **2000**, *21*, 343.
10. Da Prada M., Kettler R., Keller H.H., Burkard W.P., Muggli-Maniglio D., Haefely W.E.. *J. Pharmacol. Exp. Ther*. **1989**, *248*, 400.
11. Da Prada M., Kettler R., Keller H.H., Cesura A.M., Richards J.G., Saura M.J., Muggli-Maniglio D., Wyss P.C., Kyburz E., Imhof R.J., *Neural Transm*., Suppl. **1990**, *29*, 279.
12. Moureau F., Wouters J., Vercauteren D.P., Collin S., Evrard G., Durant F., Ducrey F., Koenig J.J., Jarreau F.X., *Eur. J. Med. Chem*. **1992**, *27*, 939.
13. Mazouz F., Gueddari S., Burstein C., Mansuy D., Milcent R., *J. Med. Chem*. **1993**, *36*, 1157.

14. Loscher W., Lehmann H., Teschendorf H.J., Traut M., Gross G.J., *Pharmacol. Exp. Ther*., **1999**, *288*, 984.
15. Chen J.F., Xu K., Petzer J.P., Staal R., Xu Y.H., Beilstein M., Sonsalla P.K., Castagnoli K., Castagnoli N. Jr., Schwarzschild M.A., *J. Neurosci*. **2001**, *21*, RC143.
16. Khan K.M., Rahat S., Choudhary M.I., Atta-ur-Rahman Ghani U., Perveen S., Khatoon S. Dar A., Malik A., *Helv. Chim. Acta*, **2002**, *85*, 559.
17. Gokhan N., Yesilada A., Ucar G., Erol K., Bilgin A.A., *Arch. Pharm*., **2003**, *336*, 362.
18. Chimenti F., Maccioni E., Secci D., Bolasco A., Chimenti P., Granese A., Turini P., Alcaro, S., Ortuso F., Cirilli R., La Torre F., Cardia M.C., Distinto S.,. *J. Med. Chem*., **2005**, *48*, 7113.
19. Dar A., Khan K.M., Ateeq H.S., Khan S., Rahat S., Perveen S., Supuran C.T.J., *Enzyme Inhib. Med. Chem*., **2005**, *20*, 269.
20. Chimenti F., Secci D., Bolasco A., Chimenti P., Granese A., Carradori S., Befani O., Turini P., Alcaro S., Ortuso F., *Bioorg. Med. Chem. Lett*., **2006**, *16*, 4135.
21. Vlok N., Malan S.F., Castagnoli N., Bergh J.J., Petzer J.P., *Bioorg. Med. Chem*., **2006**, *14*, 3512.
22. Chimenti F., Maccioni E., Secci D., Bolasco A., Chimenti P., Granese A., Befani O., Turini P., Alcaro S., Ortuso F., Cardia M.C., Distinto S., *J. Med. Chem*., **2007**, *50*, 707.
23. Van den Berg D., Zoellner K.R., Ogunrombi M.O., Malan S.F., Terre'Blanche G., Castagnoli N., Bergh J.J., Petzer J.P., *Bioorg. Med. Chem*., **2007**, *15*, 3692.

2.1. Homoisoflavonoids: natural scaffolds with potent and selective monoamine oxidase-B inhibition properties.

Nicoletta Desideri,[†] Adriana Bolasco,[†] Rossella Fioravanti,[†] Luca Proietti Monaco,[†] Francisco Orallo,[§] Matilde Yáñez,[§] Francesco Ortuso,[‡] Stefano Alcaro.[‡]
J. Med. Chem. 54 (2011) 2155-2164.

[†]*Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza - Università di Roma, P.le Aldo Moro, 5, 00185 Rome, Italy*
[§]*Departamento de Farmacología and Instituto de Farmacia Industrial, Facultad de Farmacia, Universidad de Santiago de Compostela, Campus Universitario Sur, E-15782 Santiago de Compostela (La Coruña), Spain*
[‡]*Dipartimento di Scienze Farmacobiologiche, Università di Catanzaro "Magna Græcia", Complesso Ninì Barbieri, 88021 Catanzaro, Italy*.

The aim of the present study was the identification of novel potent MAO inhibitors that could serve as potential lead molecules for drug discovery.

Homoisoflavonoids constitute a small class of natural products prevalently isolated from the bulbs, rhizomes or roots of several genera of *Hyacinthaceae* and *Caesalpinioideae*. These compounds are structurally related to the more widespread flavonoids and, according to their structural features, can be classified into three types: 3-benzylidenechroman-4-ones, 3-benzyl-4*H*-chromen-4-ones and 3-benzylchroman-4-ones. Several natural and synthetic homoisoflavonoids, like the related flavonoids, were found to possess various biological properties such as antifungal,[1] antiviral,[2-4] antimutagenic,[5,6] antiproliferative,[7] antioxidant,[8,9] anti-allergic and anti-histaminic,[10] anti-inflammatory[11] and protein tyrosine kinase (PTK) inhibitor activities.[12] However, no data are available on the inhibitory activity of homoisoflavonoids on MAOs.

Recently, several substituted chalcones and flavanones were reported as potent and selective inhibitors of the B-isoform of human MAO (hMAO).[13,14] The structural relationship of homoisoflavonoids to chalcones and flavanones prompted us to investigate their inhibitory activity against the A and B isoforms of hMAO. Initially,

we tested the synthetic (*E*)-3-benzylidenechroman-4-ones **1a-g**, 3-benzyl-4-chromones **2a-g** and 3-benzylchroman-4-ones **3a-e**, previously studied as anti-picornavirals.[2-4] Because all the (*E*)-3-benzylidenechroman-4-ones **1a-g**, which may be regarded as rigid analogues of chalcones, were potent and selective inhibitors of the B isoform of hMAO, we planned the synthesis of a larger panel of (*E*)-3-benzylidenechroman-4-ones to further investigate the structure-activity relationships. The MAO recognition of the most active compounds, **1h** and **1l**, was investigated by docking experiments performed using available Protein Data Bank (PDB) structures as receptor models.

2.1.1. Chemistry

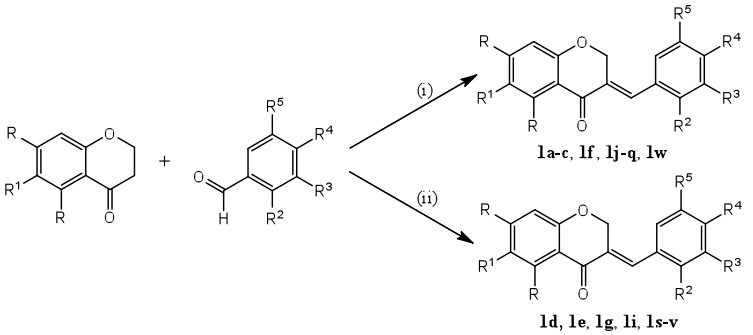
The (*E*)-3-benzylidenechroman-4-ones **1a-w** were synthesized by acid-catalyzed condensation of the appropriate chroman-4-one with substituted benzaldehydes (scheme 1).

In general, the target compounds were prepared in good yield using 85% phosphoric acid as the acid catalyst and heating the mixture at 80 °C for 6 h. Under these conditions, the reaction of chroman-4-one with N-(4-formylphenyl)acetamide gave a mixture of amide **1k** and the corresponding amine **1j**, which were easily separated by column chromatography. Starting from substituted chroman-4-ones or 2-substituted benzaldehydes, treatment with 85% phosphoric acid provided the corresponding chromanones (**1d**, **1e**, **1g**, **1i** and **1s-v**) in very low yields. To obtain these compounds more efficiently, the condensations were carried out in dry ethyl alcohol saturated with dry hydrogen chloride. A single stereoisomer was obtained with both procedures. The ^{1}H NMR spectra allow the E configuration of the double bound to be assigned on the basis of the chemical shift of the olefinic proton, ranging from 7.6 ppm to 7.9 ppm.

(*E*)-5,7-Dihydroxy-3-(4-hydroxybenzylidene) chroman-4-one (**1h**) was synthesized by saponification of the corresponding tribenzoate following published procedures.[15]

The reduction of (*E*)-3-(4-hydroxy-3-nitrobenzylidene) chroman-4-one (**1q**) with tin chloride in ethyl alcohol and concentrated hydrogen chloride provided the corresponding amino derivative (**1r**).

The (*E*)-3-benzylidenechroman-4-ones **1a-g** were converted into the corresponding 3-benzyl-4-chromones **2a-g** or 3-benzylchroman-4-ones **3a-e** according to procedures described previously.[2]



1a R = R^1 = R^2 = R^3 = R^4 = R^5 = H
1b R = R^1 = R^2 = R^3 = R^5 = H, R^4 = OH
1c R = R^1 = R^2 = R^3 = R^5 = H, R^4 = Cl
1d R = R^2 = R^3 = R^5 = H, R^1 = R^4 = Cl
1e R^1 = R^2 = R^3 = R^5 = H, R = R^4 = Cl
1f R = R^1 = R^2 = R^3 = R^5 = H, R^4 = OCH_3
1g R^1 = R^2 = R^3 = R^5 = H, R = R^4 = OCH_3
1i R = R^2 = R^3 = R^4 = R^5 = H, R^1 = Cl
1j R = R^1 = R^2 = R^3 = R^5 = H, R^4 = NH_2
1k R = R^1 = R^2 = R^3 = R^5 = H, R^4 = $NHCOCH_3$
1l R = R^1 = R^2 = R^3 = R^5 = H, R^4 = $N(CH_3)_2$

1m R = R^1 = R^2 = R^5 = H, R^3 = OCH_3, R^4 = OH
1n R = R^1 = R^2 = R^5 = H, R^3 = OH, R^4 = OCH_3
1o R = R^1 = R^2 = R^5 = H, R^3 = R^4 = OH
1p R = R^1 = R^2 = R^5 = H, R^3 = COOH, R^4 = OH
1q R = R^1 = R^2 = R^5 = H, R^3 = NO_2, R^4 = OH
1s R = R^1 = R^3 = R^5 = H, R^2 = OH, R^4 = OCH_3
1t R = R^1 = R^3 = R^5 = H, R^2 = R^4 = OCH_3
1u R = R^1 = R^4 = R^5 = H, R^2 = R^3 = OCH_3
1v R = R^1 = R^5 = H, R^2 = R^3 = R^4 = OCH_3
1w R = R^1 = R^2 = H, R^3 = R^4 = R^5 = OCH_3

Scheme1. Reagents and conditions: (i) 85% H_3PO_4, 80° C, 6h; (ii) dry EtOH sat. HCl, r.t., 48h.

2.1.2. Biochemistry

The potential effects of the tested compounds on hMAO activity were investigated by measuring their effects on the production of hydrogen peroxide (H_2O_2) from *p*-tyramine, using the Amplex Red MAO assay kit (Molecular Probes, Inc., Eugene, Oregon, USA) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B (Sigma-Aldrich Química S.A., Alcobendas, Spain). In this study hMAO activity was evaluated using the above method following the general procedure described previously by us.[16] The tested drugs (new compounds and reference inhibitors) themselves were unable to react directly with the Amplex Red reagent, which indicates that these compounds do not interfere with the measurements. On the other hand, in our experiments and under our experimental conditions, the control activity of hMAO-A and hMAO-B (using *p*-tyramine as a common substrate for both isoforms) was 165 ± 2 pmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min (n = 20).

The results of hMAO-A and hMAO-B inhibition studies are expressed as IC_{50} and reported in tables 1 and 2 together with the hMAO-B selectivity indexes (SI = IC_{50} MAO-A/IC_{50} MAO-B).

Table 1. IC_{50} and SI values for the inhibitory effects of (*E*)-3-benzylidenechroman-4-ones **1a-w** and reference inhibitors on the enzymatic activity of human recombinant MAO isoforms expressed in baculovirus infected BTI insect cells.

Comp.	R	R^1	R^2	R^3	R^4	R^5	MAO-A (IC_{50})	MAO-B (IC_{50})	SI[c]
1a	H	H	H	H	H	H	**	479.70 ± 19.37 nM	> 208[#]
1b	H	H	H	H	OH	H	**	55.37 ± 2.18 nM	> 1806[#]
1c	H	H	H	H	Cl	H	**	154.23 ± 6.93 nM	> 648[#]
1d	H	Cl	H	H	Cl	H	**	31.82 ± 1.19 nM	> 3143[#]
1e	Cl	H	H	H	Cl	H	**	13.03 ± 0.79 nM	> 7675[#]
1f	H	H	H	H	OCH_3	H	**	58.90 ± 2.09 nM	> 1698[#]
1g	OCH_3	H	H	H	OCH_3	H	**	1.57 ± 0.03 µM	> 64[#]
1h	OH	H	H	H	OH	H	11.46 ± 0.43µM[a]	8.61 ± 0.12 nM	1331
1i	H	Cl	H	H	H	H	**	331.11± 9.63 nM	> 302[#]
1j	H	H	H	H	NH_2	H	**	879.18 ± 45.31 nM	> 114[#]
1k	H	H	H	H	$NHCOCH_3$	H	2.10 ± 0.11µM[b]	1.06 ± 0.04 µM	2.0
1l	H	H	H	H	$N(CH_3)_2$	H	**	8.51 ± 0.32 nM	> 11751[#]
1m	H	H	H	OCH_3	OH	H	***	104.34 ± 2.61 nM	> 958[#]
1n	H	H	H	OH	OCH_3	H	**	476.51± 21.60 nM	> 210[#]
1o	H	H	H	OH	OH	H	4.74± 0.21µM[a]	140.52 ± 4.71 nM	34
1p	H	H	H	COOH	OH	H	**	**	
1q	H	H	H	NO_2	OH	H	**	490.84 ± 12.73 nM	> 204[#]
1r	H	H	H	NH_2	OH	H	1.44± 0.51µM[a]	483.88 ± 16.21 nM	3.0
1s	H	H	OH	H	OCH_3	H	***	1.33 ± 0.07 µM	> 75[#]
1t	H	H	OCH_3	H	OCH_3	H	**	247.51 ± 6.31 nM	> 404[#]
1u	H	H	OCH_3	OCH_3	H	H	**	1.92 ± 0.08 µM	> 52[#]
1v	H	H	OCH_3	OCH_3	OCH_3	H	**	1.31 ± 0.04 µM	> 76[#]
1w	H	H	H	OCH_3	OCH_3	OCH_3	**	20.69 ± 0.37 µM	> 4.8[#]
A							4.46± 0.32nM[a]	61.35 ± 1.13 µM	0.000073
B							67.25 ± 1.02 µM[a]	19.60 ± 0.86 nM	3431
C							6.56± 0.76 µM	7.54 ± 0.36 µM	0.87
D							361± 19.37µM	*	< 0.36

All IC_{50} values shown in this table are the mean ± S.E.M. from five experiments. Level of statistical significance: [a]$P < 0.01$ or [b]$P < 0.05$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett´s. [c] SI: hMAO-B selectivity index = IC_{50} (hMAO-A)/IC_{50} (hMAO-B).

* inactive at 1 mM (highest concentration tested). ** Inactive at 100 μM (highest concentration tested). *** 100 μM inhibits the corresponding MAO activity by approximately 40-50%. At higher concentration the compounds precipitate. # Values obtained under the assumption that the corresponding IC_{50} against MAO-B is the highest concentration tested (100 μM).
A: Clorgyline, **B:** Selegiline, **C:** Iproniazid, **D:** Moclobemide

In table 1 are presented the data obtained for the (*E*)-3-benzylidenechroman-4-ones **1a-w**. Most of these homoisoflavonoids selectively inhibited the enzymatic activity of hMAO-B in the nanomolar or micromolar range. Derivatives **1h**, **1k**, **1o** and **1r** were also able to inhibit hMAO-A in the micromolar range. Among these analogues, **1k** and **1r** were essentially non selective (SI = 2 and 3, respectively) whereas **1h** and **1o** exhibited hMAO-B selectivity to different extents (SI = 1331 and 34, respectively). Only the acid **1p** was found to be completely inactive towards both isoforms up to the highest concentration tested (100 μM). (*E*)-3-[4-(Dimethylamino)benzylidene]chroman-4-one (**1l**) was the most potent and selective hMAO-B inhibitor identified in this study exhibiting an IC_{50} value of 8.51 nM and SI > 11751. In comparison with the reference inhibitor selegiline, compound **1l** showed higher hMAO-B affinity and selectivity. The replacement of the dimethylamino group with the amino group resulted in compound **1j**, about one hundred fold less potent and selective as a hMAO-B inhibitor (IC_{50} = 879.18 nM, SI > 114) than **1l**. Interestingly, acetylation of the amino group (compound **1k**) produced a further decrease of hMAO-B inhibitory activity and a dramatic reduction of selectivity (IC_{50} = 1.06 μM, SI = 2). (*E*)-5,7-Dihydroxy-3-(4-hydroxybenzylidene)chroman-4-one (**1h**) showed an affinity for hMAO-B (IC_{50} = 8.61 nM) comparable to **1l**. Although **1h** was able to inhibit both hMAO isoforms, it still exhibited an excellent hMAO-B selectivity (SI = 1331). Although less active than **1h** and **1l**, (*E*)-5,7-dichloro-3-(4-chlorobenzylidene)chroman-4-one (**1e**) showed hMAO-B affinity and selectivity (IC_{50} = 13.03 nM, SI > 7675) better than those of the reference compound, selegiline.

The results for inhibitory effects and selectivity of the 3-benzyl-4*H*-chromen-4-ones **2a-g** and 3-benzylchroman-4-ones **3a-e** on hMAO isoforms are reported in table 2.

Several of these compounds (**2e, 2f**, **3a** and **3c-e**) showed selective hMAO-B inhibitory activities, whereas the 3-benzyl-4-chromones **2a-d** and **2g** and the 3-benzylchroman-4-one **3b** were essentially inactive towards both isoforms up to the highest concentration tested (100 μM). Disappointly, the endocyclic migration

of the double bond produces a loss of the inhibitory activity (compounds **2a-d**, **2g**) or a marked reduction in potency (compounds **2e** and **2f**) with respect to the corresponding (*E*)-3-benzylidenechroman-4-ones **1a-g**.

Table 2. IC_{50} and SI values for the inhibitory effects of 3-benzyl-4*H*-chromen-4-ones **2a-g** and 3-benzylchroman-4-ones **3a-e** and reference inhibitors on the enzymatic activity of human recombinant MAO isoforms expressed in baculovirus infected BTI insect cells.

Comp.	R	R^1	R^2	R^3	R^4	R^5	MAO-A (IC_{50})	MAO-B (IC_{50})	SI[b]
2a	H	H	H	H	H	H	**	**	
2b	H	H	H	H	OH	H	**	***	
2c	H	H	H	H	Cl	H	**	**	
2d	H	Cl	H	H	Cl	H	**	***	
2e	Cl	H	H	H	Cl	H	***	438.60 ± 27.48 nM	> 228[#]
2f	H	H	H	H	OCH_3	H	**	21.59 ± 1.06 µM	> 4.6[#]
2g	OCH_3	H	H	H	OCH_3	H	**	***	
3a	H	H	H	H	H	H	**	58.53 ± 2.24 µM	> 1.7[#]
3b	H	H	H	H	OH	H	**	***	
3c	H	H	H	H	Cl	H	**	7.37 ± 0.31 µM	> 14[#]
3d	H	Cl	H	H	Cl	H	**	3.07 ± 0.11 µM	> 33[#]
3e	Cl	H	H	H	Cl	H	**	4.59 ± 0.19 µM	> 22[#]
A							4.46 ± 0.32 nM [a]	61.35 ± 1.13 µM	0.000073
B							67.25 ± 1.02 µM [a]	19.60 ± 0.86 nM	3431
C							6.56± 0.76 µM	7.54 ± 0.36 µM	0.87
D							361± 19.37 µM	*	< 0.36

All IC_{50} values shown in this table are the mean ± S.E.M. from five experiments. Level of statistical significance: [a] $P < 0.01$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett´s. [b] SI: hMAO-B selectivity index = IC_{50} (hMAO-A)/IC_{50} (hMAO-B). * inactive at 1 mM (highest concentration tested). ** Inactive at 100 µM (highest concentration tested).*** 100 µM inhibits the corresponding MAO activity by approximately 40-50%. At higher concentration the compounds precipitate. [#] Values obtained under the assumption that the corresponding IC_{50} against MAO-B is the highest concentration tested (100 µM).
A: Clorgyline, **B:** Selegiline, **C:** Iproniazid, **D:** Moclobemide

The reduction of the double bond results in compounds **3a-e**, generally more potent and selective than the corresponding 3-benzyl-4*H*-chromen-4-ones **2a-g**. The only exception was 5,7-dichloro-3-(4-chlorobenzyl)-4*H*-chromen-4-one (**2e**) that exhibited submicromolar potency towards hMAO-B, whereas the corresponding 3-benzylchroman-4-one (**3e**) was about tenfold less active and less selective. However, all the 3-benzylchroman-4-ones **3a-e** were less

potent hMAO-B inhibitors than the corresponding (*E*)-3-benzylidenechroman-4-ones **1a-e**.

Table 3 shows the results of the reversibility and irreversibility tests for the most effective compounds **1h** and **1l**. hMAO-A and hMAO-B inhibition was irreversible in presence of **1h** as shown by the lack of enzyme activity restoration after repeated washing. Similar results were obtained for compound **1l** and selegiline against hMAO-B. On the contrary, significant recovery of hMAO-A activity was observed after repeated washing of moclobemide, indicating that this drug is a reversible inhibitor of hMAO-A.

Table 3. Reversibility and irreversibility of hMAO-B inhibition of derivatives **1h** and **1l**[a]

	% hMAO-A inhibition	
Compound	Before washing	After repeated washing
1h (50 μM)	68.26 ± 4.34	61.76 ± 4.98
Moclobemide (500 μM)	85.98 ± 4.03	11.45 ± 0.58[b]
	% hMAO-B inhibition	
Compound	Before washing	After repeated washing
1h (10 nM)	48.66 ± 6.39	36.22 ± 4.26
1l (10 nM)	56.34 ± 5.37	58.67 ± 6.60
Selegiline (20 nM)	53.28 ± 2.59	56.34 ± 2.01

[a]Each value is the mean ± S.E.M. from five experiments (n = 5). [b]Level of statistical significance: $P < 0.01$ versus the corresponding % MAO-A or MAO-B inhibition before washing, as determined by ANOVA/Dunnett´s.

2.1.3. Docking studies

Docking studies were carried out to evaluate the binding modes of this class of homoisoflavonoids with respect to both isoforms of human MAO. Crystallographic structures were selected from the PDB to build our theoretical receptors models (Experimental section). Taking into account the experimental hMAO inhibition data, compounds **1h** and **1l** were chosen for our molecular modeling investigation. Although the irreversible inhibitors usually form a covalent bond with a residue on the enzyme, they may also act by other mechanisms.[17] The so-called tight-binding inhibitors, owing to the very low dissociation constant, may show kinetics similar to covalent irreversible inhibitors. In some cases, the inhibitors may rapidly bind to the enzyme in a low-affinity enzyme-inhibitor (EI) complex and then undergoes a slower rearrangement to a very tightly bound EI* complex. For instance, *R-(-)*-deprenyl, an irreversible

MAO inhibitor, forms a non-covalent complex with MAO as an initial and reversible step. Afterwards, the interaction of *R-(-)*-deprenyl with MAO leads to a reduction of the enzyme-bound FAD, and concomitant oxidation of the inhibitor. This oxidized inhibitor is able to form a covalent bond at the N-5-position of FAD.[18] The initial non-covalent binding to MAO has been also described for other MAO inhibitors (e.g.: clorgyline derivatives).[19] Moreover, we have already docked non-covalent ligands[13,14, 20] showing an irreversible profile similar to **1h** and **1l**.

Both homoisoflavonoids were submitted to a conformational search revealing, not surprisingly, only two conformers within 10 kcal/mol from the global minimum energy. Using the AutoDock-Vina method,[21] the most populated structures of **1h** and **1l** were submitted to flexible docking simulation with respect to both hMAO-A and hMAO–B receptor models (Experimental section). The theoretical complexes were evaluated taking into account two interaction energy descriptors. Best interaction energy (IE) is the AutoDock-Vina lowest ligand-target interaction energy. ΔG_{bind} is the Boltzmann averaged binding free energy computed with the entire complex ensemble generated by the docking program. In both cases, good qualitative agreements with experimental inhibition data have been obtained (table 4).

Table 4. Comparison between experimental IC_{50} data and theoretical binding affinities of **1h** and **1l** with respect to hMAO-A and –B.

	hMAO-A		**hMAO-B**	
	1h	**1l**	**1h**	**1l**
IC_{50} [a]	11460.00	>100000.00	8.61	8.51
ΔG_{bind} [b]	-6.30	-1.88	-9.10	-9.35
Best IE[c]	-7.00	-3.00	-9.50	-10.40

[a] experimental inhibition data (nM); [b] free energy of binding (kcal/mol); [c] best interaction energy (kcal/mol)

The most stable binding modes of both homoisoflavonoids in the hMAO-A and -B active sites were graphically inspected (figure 2).

Docking simulations generated several low energy binding modes of **1h** and **1l** within both hMAO isoforms. These poses revealed the ability of these ligands to alternatively recognize the FAD with both benzyl and chromone rings (see Supporting Information). Interestingly, the hMAO-A best poses of the **1h** and **1l** chromone rings were located toward the enzymatic cofactor (figure

2a). Most of the ligand-enzyme interactions appeared to be identical. The main difference between **1h** and **1l** hMAO-A recognition was due to the presence of a phenol OH in the former compound that established a hydrogen bond with FAD N5 atom. Moreover **1h**, attracted by the FAD, resulted in exclusive contact with Tyr69; conversely **1l** was involved in attractive hydrophobic contacts with Asn181 and Tyr444. The effects of these different interactions on binding energy are highlighted in table 4.

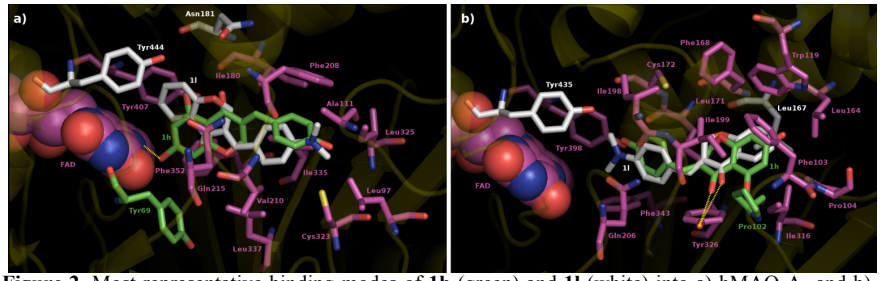


Figure 2. Most representative binding modes of **1h** (green) and **1l** (white) into a) hMAO-A and b) hMAO-B catalytic sites. Common **1h** and **1l** interacting residues are reported in magenta carbon atoms sticks. Exclusive **1h** or **1l** interacting residues are displayed according to the corresponding ligands. The FAD cofactor is depicted using the spacefill representation. The rest of the enzyme is shown in yellow, transparent cartoon. Yellow dotted lines indicate hydrogen bonds.

The best poses of these compounds in the hMAO-B binding site were different from the A isoform. These configurations displayed the chromone ring far from the FAD cofactor. In both **1h** and **1l** hMAO-B complexes the interaction pattern was similar: one hydrogen bond between the carbonyl oxygen of the ligands and Tyr326-OH and several almost identical hydrophobic contacts. The slightly better interaction of **1l** can be addressed to its more hydrophobic nature than **1h**. Actually the chromone ring was surrounded by a large lipophilic cage (Phe103, Pro104, Trp119, Leu164, Leu167, Phe168, Leu171, Ile199 and Ile316) where **1h**, due to its hydrophilic substituents, was disfavoured. Finally, the hMAO-B selectivity of both compounds can be referred to: a) the larger set of residues interacting with hMAO-B; b) the different hydrogen bond contribution, stronger in hMAO-B (Tyr326-OH--O=**1h**/**1l**) than in hMAO-A (FAD-N5--HO-**1h**).

2.1.4. Conclusion

In the current study we have synthesized and evaluated a series of (*E*)-3-benzylidenechroman-4-ones **1a-w**, 3-benzyl-4*H*-chromen-4-ones **2a-g** and 3-benzylchroman-4-ones **3a-e** as inhibitors of h-MAO

isoforms A and B. In general, the active compounds showed potent and selective activity towards the B isoform. In particular, several (*E*)-3-benzylidenechroman-4-ones exhibited potencies in the nanomolar range. Two derivatives, **1h** and **1l**, were the most potent and selective hMAO-B inhibitors with higher potency than the reference inhibitor selegiline and, in the latter case, even higher selectivity. Molecular docking studies suggest that stronger enzyme-inhibitor hydrogen bonds and hydrophobic contacts in the hMAO-B active site can explain the selectivity of both inhibitors for this isoform.

2.1.5. Experimental section

2.1.5.1. Chemistry

Chemicals were purchased from Sigma-Aldrich and used without further purification. Melting points were determined on a Stenford Research Systems OptiMelt (MPA-100) apparatus and are uncorrected. 1H NMR and 13C NMR spectra were recorded in DMSO-d_6, unless indicated otherwise, on a Bruker AM-400 spectrometer, using TMS as internal standard. IR spectra were recorded in KBr disks on an FT-IR PerkinElmer Spectrum 1000. All compounds were routinely checked by thin-layer chromatography (TLC), 1H NMR and 13C NMR. TLC was performed on silica gel or aluminium oxide fluorescent coated plates (Merck, Kieselgel or Aluminium oxide 60 F254). Components were visualised by UV light. Compound purity was determined by elemental analysis and was confirmed to be ≥ 95% for all compounds. (*E*)-3-Benzylidenechroman-4-ones (**1a-g**), 3-benzyl-4-chromones **(2a-g**) and 3-benzylchroman-4-ones (**3a-e**) were prepared as previously described by us.[2] (*E*)-5,7-Dihydroxy-3-(4-hydroxybenzylidene)chroman-4-one (**1h**) was obtained by saponification of the corresponding tribenzoate as previously reported.[25]

2.1.5.1.1. General Procedure for the Synthesis of (*E*)-3-Benzylidenechroman-4-ones 1l-q and 1w.

A mixture of the appropriate chroman-4-one (0.01 mol) and substituted benzaldehyde (0.01 mol) in 85% phosphoric acid (20 mL) was heated at 80 °C for 6h. After cooling, the mixture was diluted with ice-water (and made alkaline for the preparation of compound **1l**). The solid was filtered off, washed with water and crystallized.

2.1.5.1.1.1. (*E*)-3-(4-(Dimethylamino)benzylidene) chroman-4-one (1l). Yield: 77%, mp = 151-153 °C (lit 150-153 °C)[7] from EtOH. The compound exhibited spectroscopic data identical to those previously reported.[7]

2.1.5.1.1.2. (*E*)-3-(4-Hydroxy-3-methoxybenzylidene) chroman-4-one (1m). Yield: 71%, mp = 130-131 °C (lit 126-129 °C)[22] from ethyl alcohol. IR (KBr): 3200, 1655 cm^{-1}. ^1H NMR: δ (ppm) 9.75 (bs, 1H, OH), 7.88 (dd, 1H, H5, J_{5-6} = 8.0 Hz, J_{5-7} = 1.6 Hz), 7.71 (t, 1H, =CH, J_{all}= 1.8 Hz), 7.58 (ddd, 1H, H7, J_{6-7} = 7.2 Hz, J_{7-8} = 8.4 Hz, J_{5-7} = 1.6 Hz), 7.12 (ddd, 1H, H6, J_{6-7} = 7.2 Hz, J_{5-6} = 8.0 Hz, J_{6-8} = 1.0 Hz), 7.07 (d, 1H, H2', $J_{2'-6'}$ = 1.7 Hz), 7.05 (dd, 1H, H8, J_{7-8} = 8.4 Hz, J_{6-8} = 1.0 Hz), 6.93 (dd, 1H, H6', $J_{2'-6'}$ = 1.7 Hz), 6.90 (d, 1H, H5', $J_{5'-6'}$ = 8.4 Hz), 5.47 (d, 2H, H2, J_{all}= 1.7 Hz), 3.84 (s, 3H, OCH_3). ^{13}C NMR: δ (ppm) 180.9, 160.4, 148.8, 147.6, 137.2, 135.8, 127.7, 127.1, 125.2, 124.4, 121.8, 121.6, 117.7, 115.6, 114.8, 67.5, 55.6.

2.1.5.1.1.3. (*E*)-3-(3-Hydroxy-4-methoxybenzylidene)chroman-4-one (1n). Yield: 78%, mp = 192-193 °C from EtOH. IR (KBr): 3150, 1650 cm^{-1}. ^1H NMR: δ (ppm) 9.34 (bs, 1H, OH), 7.88 (dd, 1H, H5, J_{5-6} = 7.9 Hz, J_{5-7} = 1.8 Hz), 7.64 (t, 1H, =CH, J_{all} = 1.8 Hz), 7.58 (ddd, 1H, H7, J_{6-7} = 7.2 Hz, J_{7-8} = 8.4 Hz, J_{5-7} = 1.8 Hz), 7.13 (ddd, 1H, H6, J_{6-7} = 7.2 Hz, J_{5-6} = 7.9 Hz, J_{6-8} = 1.0 Hz), 7.07-7.03 (m, 2H, H8, H5'), 6.94-6.91 (m, 2H, H2', H6'), 5.43 (d, 2H, H2, J_{all} = 1.8 Hz), 3.85 (s, 3H, OCH_3). ^{13}C NMR: δ (ppm) 180.9, 160.4, 149.4, 146.5, 136.8, 135.9, 128.3, 127.1, 126.5, 122.9, 121.8, 121.5, 117.7, 117.2, 112.0, 67.4, 55.6.

2.1.5.1.1.4. (*E*)-3-(3,4-Dihydroxybenzylidene)chroman -4-one (1o). Yield: 54% , mp = 227-230 °C (lit 224-225 °C)[22] from EtOH. IR (KBr): 3400, 1640 cm^{-1}. ^1H NMR: δ (ppm) 9.50 (bs, 2H, 2OH), 7.87 (dd, 1H, H5, J_{5-6} = 7.8 Hz, J_{5-7} = 1.7 Hz), 7.61 (t, 1H, =CH, J_{all}= 1.8 Hz), 7.58 (ddd, 1H, H7, J_{6-7} = 7.4 Hz, J_{7-8} = 8.4 Hz, J_{5-7} = 1.7 Hz), 7.12 (ddd, 1H, H6, J_{6-7} = 7.4 Hz, J_{5-6} = 7.8 Hz, J_{6-8} = 1.0 Hz), 7.05 (dd, 1H, H8, J_{7-8} = 8.4 Hz, J_{6-8} = 1.0 Hz), 6.89-6.80 (m, 3H, H2', H5', H6'), 5.43 (d, 2H, H2, J_{all} = 1.8 Hz). ^{13}C NMR: δ (ppm) 180.9, 160.3, 147.8, 145.3, 137.2, 135.8, 127.3, 127.1, 125.2, 123.4, 121.7, 121.6, 117.7, 115.8, 67.5.

2.1.5.1.1.5. (*E*)-2-Hydroxy-5-[(4-oxochroman-3-ylidene)methyl]

benzoic acid (1p). Yield: 74%, mp = 234-238 °C from EtOH. IR (KBr): 3500-2300, 1650, 1600 cm^{-1}. ^1H NMR: δ (ppm) 11.87 (bs, 1H, COOH), 7.87-7.80 (m, 2H, H5, H2'), 7.69 (bs, 1H, =CH), 7.63-7.54 (m, 2H, H6', H7), 7.12-7.01 (m, 3H, H5', H6, H8), 5.40 (d, 2H, H2, J_{all} = 1.4 Hz). ^{13}C NMR: δ (ppm) 180.9, 171.2, 162.0, 160.5, 137.4, 136.0, 135.6, 132.7, 129.4, 127.1, 125.0, 121.8, 121.4, 117.8, 117.7, 113.5, 67.2.

2.1.5.1.1.6. (*E*)-3-(4-Hydroxy-3-nitrobenzylidene)chroman-4-one (1q). Yield: 77%, mp = 203-205 °C from EtOAc. IR (KBr): 3240, 1655, 1530, 1300 cm^{-1}. ^1H NMR: δ (ppm) 11.65 (bs, 1H, OH), 7.94 (d, 1H, H2', $J_{2'-6'}$ = 2.2 Hz), 7.88 (dd, 1H, H5, J_{5-6} = 7.9 Hz, J_{5-7} = 1.8 Hz), 7.70 (bs, 1H, =CH), 7.64 (dd, 1H, H6', $J_{2'-6'}$ = 2.2 Hz, $J_{5'-6'}$ = 8.7 Hz), 7.60 (ddd, 1H, H7, J_{6-7} = 7.3 Hz, J_{7-8} = 8.4 Hz, J_{5-7} = 1.8 Hz), 7.23 (d, 1H, H5', $J_{5'-6'}$ = 8.7 Hz), 7.13 (ddd, 1H, H6, J_{6-7} = 7.3 Hz, J_{5-6} = 7.9 Hz, J_{6-8} = 0.9 Hz), 7.05 (dd, 1H, H8, J_{7-8} = 8.4 Hz, J_{6-8} = 0.9 Hz), 5.44 (d, 2H, H2, J_{all} = 1.8 Hz).^{13}C NMR: δ (ppm) 180.5, 160.5, 152.8, 137.2, 136.5, 135.1, 134.4, 130.2, 127.2, 127.1, 124.9, 121.9, 121.3, 119.3, 117.8, 67.2.

2.1.5.1.1.7. (*E*)-3-(3,4,5-Trimethoxybenzylidene)chroman-4-one (1w). Yield: 63%, mp = 105-106 °C (lit 108-109 °C)[23] from EtOAc/petroleum ether. IR (KBr): 1660 cm^{-1}. ^1H NMR: δ (ppm) 7.90 (dd, 1H, H5, J_{5-6} = 7.8 Hz, J_{5-7} = 1.8 Hz), 7.73 (bs, 1H, =CH), 7.60 (ddd, 1H, H7, J_{7-8} = 8.4 Hz, J_{6-7} = 7.2 Hz, J_{5-7} = 1.8 Hz), 7.14 (ddd,1H, H6, J_{6-7} = 7.2 Hz, J_{5-6} = 7.8 Hz, J_{6-8} = 1.0 Hz), 7.06 (dd, 1H, H8, J_{7-8} = 8.4 Hz, J_{6-8} = 1.0 Hz), 6.77 (s, 2H, H2', H6'), 5.50 (d, 2H, H2 J_{all}= 1.8 Hz), 3.84 (s, 6H, $2OCH_3$), 3.74 (s, 3H, OCH_3). ^{13}C NMR: δ (ppm) 181.0, 160.6, 152.8, 138.9, 136.9, 136.2, 130.0, 129.2, 127.2, 121.9, 121.5, 117.8, 108.0, 67.4, 60.1, 56.0.

2.1.5.1.2. Synthesis of (*E*)-3-(4-Aminobenzylidene) chroman-4-one (1j) and (*E*)-N-(4-[(4-Oxochroman-3-ylidene)methyl]phenyl) acetamide (1k).

A mixture of chroman-4-one (0.01 mol) and 4-aminobenzaldehyde (0.01 mol) in 85% H_3PO_4 (20 mL) was heated at 80 °C for 6h. After cooling, the mixture was diluted with ice-water and alkalinized with 2N NaOH. The solid was filtered off and washed with water. The obtained mixture of (*E*)-3-(4-aminobenzylidene) chroman-4-one (**1j**) and (*E*)-N-(4-[(4-oxochroman-3-

ylidene)methyl]phenyl)acetamide (**1k**) was separated by column chromatography on silica gel eluting with AcOEt/ petroleum ether (1:1).

2.1.5.1.2.1. (*E*)-3-(4-Aminobenzylidene)chroman-4-one (1j). Yield: 30%, mp = 164-66 °C from EtOAc/petroleum ether. IR (KBr): 3430, 3340, 1650 cm^{-1}. ^1H NMR: δ (ppm) 7.85 (dd, 1H, H5, J_{5-6} = 7.8 Hz, J_{5-7} = 1.8 Hz), 7.62 (bs, 1H, = CH), 7.55 (ddd, 1H, H7, J_{6-7} = 7.2 Hz, J_{7-8} = 8.4 Hz, J_{5-7} = 1.8 Hz), 7.21 (d, 2H, H2', H6', $J_{2'-3'}$ = 8.6 Hz), 7.10 (ddd, 1H, H6, J_{6-7} = 7.2 Hz, J_{5-6} = 8.2 Hz, J_{6-8} = 1.0 Hz), 7.03 (dd, 1H, H8, J_{7-8} = 8.4 Hz, J_{6-8} = 1.0 Hz), 6.66 (d, 2H, H3', H5', $J_{2'-3'}$ = 8.6 Hz), 5.95 (bs, 2H, NH_2), 5.43 (d, 2H, H2, J_{all}= 1.8 Hz). ^{13}C NMR: δ (ppm) 180.6, 160.2, 151.2, 137.8, 135.4, 133.0, 127.0, 124.6, 121.8, 121.6, 121.0, 117.6, 113.5, 67.8.

2.1.5.1.2.2. (*E*)-N-(4-[(4-Oxochroman-3-ylidene) methyl]phenyl) acetamide (1k). Yield: 25%, mp = 175-176 °C from EtOAc/petroleum ether. IR (KBr): 3300, 1664, 1660 cm^{-1}. ^1H NMR: δ (ppm) 10.23 (s, 1H, NH), 7.88 (dd, 1H, H5, J_{5-6} = 7.8 Hz, J_{5-7} = 1.6 Hz), 7.74-7.70 (m, 3H, H2', H6', =CH), 7.59 (ddd, 1H, H7, J_{6-7} = 7.1 Hz, J_{7-8} = 8.4 Hz, J_{5-7} = 1.6 Hz), 7.44 (d, 2H, H3', H5', $J_{2'-3'}$ = 8.6 Hz), 7.13 (ddd, 1H, H6, J_{6-7} = 7.1 Hz, J_{5-6} = 7.8 Hz, J_{6-8} = 0.7 Hz), 7.06 (dd, 1H, H8, J_{7-8} = 8.4 Hz, J_{6-8} = 0.7 Hz), 5.45 (d, 2H, H2, J_{all}= 1.6 Hz), 2.09 (s, 3H, CH_3). ^{13}C NMR: δ (ppm) 181.0, 168.7, 160.5, 140.8, 136.3, 136.0, 131.5, 129.1, 128.3, 127.2, 121.9, 121.5, 118.7, 117.8, 67.5, 24.1.

2.1.5.1.3. General Procedure for the Synthesis of (*E*)-3-Benzylidenechroman-4-ones 1i and 1s-v.

A mixture of the appropriate chroman-4-one (0.01 mol) with substituted benzaldehyde (0.01 mol) in dry EtOH saturated with HCl (40 mL) was stirred at room temperature for 48h. After this time, the mixture was diluted with ice-water, the solid filtered off and washed with water. The product was purified by column chromatography on silica gel or by crystallization.

2.1.5.1.3.1. (*E*)-3-Benzylidene-6-chlorochroman-4-one (1i). Yield: 86%, mp = 158-159 °C (lit 148-150 °C)[24] from EtOAc. IR (KBr): 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ (ppm) 7.98 (d, 1H, H5, J_{5-7} = 2.6 Hz), 7.90 (bs, 1H, =CH), 7.49-7.37 (m, 4H, H7, H3'-H5'), 7.33-7.30 (m,

2H, H2', H6'), 6.93 (d, 1H, H8, $J_{7\text{-}8}$ = 8.8 Hz), 5.35 (d, 2H, H2, J_{all} = 1.8 Hz). ^{13}C NMR (DMSO d_6): δ (ppm) 180.2, 159.2, 137.3, 135.7, 133.5, 130.3, 129.8, 128.8, 126.0, 125.9, 124.8, 122.4, 120.3, 67.5.

2.1.5.1.3.2. (*E*)-3-(2-Hydroxy-4-methoxybenzylidene) chroman-4-one (1s). Purified by column chromatography eluting with AcOEt/petroleum ether (1:5). Yield: 56%, mp = 158-160 °C from EtOH. IR (KBr): 3124, 1655 cm^{-1}. ^{1}H NMR: δ (ppm) 10.34 (bs, 1H, OH), 7.89-7.86 (m, 2H, =CH, H5), 7.57 (ddd, 1H, H7, $J_{6\text{-}7}$ = 7.2 Hz, $J_{7\text{-}8}$ = 8.4 Hz, $J_{5\text{-}7}$ = 1.8 Hz), 7.12 (ddd, 1H, H6, $J_{6\text{-}7}$ = 7.2 Hz, $J_{5\text{-}6}$ = 7.9 Hz, $J_{6\text{-}8}$ = 1.0 Hz), 7.08 (d, 1H, H6', $J_{5'\text{-}6'}$ = 8.2 Hz), 7.04 (dd, 1H, H8, $J_{7\text{-}8}$ = 8.4 Hz, $J_{6\text{-}8}$ = 1.0 Hz), 6.53-6.49 (m, 2H, H3', H5'), 5.33 (d, 2H, H2, J_{all} = 1.8 Hz), 3.77 (s, 3H, OCH_3). ^{13}C NMR: δ (ppm) 181.2, 162.2, 160.5, 158.6, 135.8, 132.8, 131.7, 127.3, 127.1, 121.8, 121.7, 117.8, 114.0, 105.4, 101.1, 67.9, 55.2.

2.1.5.1.3.3. (*E*)-3-(2,4-Dimethoxybenzylidene) chroman-4-one (1t). Yield: 64%, mp = 123-125 °C (lit 133-135 °C)[7] from MeOH. The compound exhibited spectroscopic data identical to those previously reported.[7]

2.1.5.1.3.4. (*E*)-3-(2,3-Dimethoxybenzylidene) chroman-4-one (1u). Yield: 53%, mp = 108-109 °C EtOAc/petroleum ether. IR (KBr): 1673 cm^{-1}. ^{1}H NMR: δ d(ppm) 7.90 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.8 Hz, $J_{5\text{-}7}$ = 1.7 Hz), 7.82 (bs, 1H, =CH), 7.61 (ddd, 1H, H7, $J_{7\text{-}8}$ = 8.9 Hz, $J_{6\text{-}7}$ = 7.2 Hz, $J_{5\text{-}7}$ = 1.7 Hz), 7.22-7.12 (m, 3H, H6, H5', H6'), 7.05 (d, 1H, H8, $J_{7\text{-}8}$ = 8.3 Hz), 6.83 (dd, 1H, H4', $J_{4'\text{-}5'}$ = 6.9 Hz, $J_{4'\text{-}6'}$ = 2.2 Hz), 5.29 (d, 2H, H2 J_{all}= 1.8 Hz), 3.86 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3). ^{13}C NMR: δ (ppm) 181.2, 160.8, 152.5, 147.6, 136.3, 132.2, 131.3, 127.6, 127.3, 124.1, 122.0, 121.8, 121.5, 118.0, 114.8, 67.6, 60.6, 55.8.

2.1.5.1.3.5. (*E*)-3-(2,3,4-Trimethoxybenzylidene) chroman-4-one (1v). Yield: 43% , mp = 116-118 °C (lit viscous oil)[23] from acetone. IR (KBr): 1668 cm^{-1}. ^{1}H NMR: δ (ppm) 7.89 (dd, 1H, H5, $J_{5\text{-}6}$ = 7.8 Hz, $J_{5\text{-}7}$ = 1.8 Hz), 7.79 (bs, 1H, =CH), 7.60 (ddd, 1H, H7, $J_{7\text{-}8}$ = 8.9 Hz, $J_{6\text{-}7}$ = 7.2 Hz, $J_{5\text{-}7}$ = 1.8 Hz), 7.13 (ddd, 1H, H6, $J_{6\text{-}7}$ = 7.2 Hz, $J_{5\text{-}6}$ = 7.8 Hz, $J_{6\text{-}8}$ = 1.0 Hz), 7.05 (dd, 1H, H8, $J_{7\text{-}8}$ = 8.9 Hz, $J_{6\text{-}8}$ = 1.0 Hz), 6.98 (s, 1H, H6', $J_{2'\text{-}6'}$ = 8.7 Hz), 6.92 (s, 1H, H5', $J_{2'\text{-}6'}$ = 8.7 Hz), 5.33 (d, 2H, H2, J_{all}= 1.8 Hz), 3.88 (s, 3H, OCH3), 3.83 (s, 3H, OCH3), 3.80 (s, 3H, OCH3). ^{13}C NMR: δ (ppm) 181.1, 160.6, 155.3,

152.7, 141.7, 136.0, 132.0, 129.5, 127.2, 125.6, 121.9, 121.6, 120.3, 117.8, 107.7, 67.7, 61.3, 60.5, 56.0.

2.1.5.1.4. Synthesis of (*E*)-3-(3-Amino-4-hydroxybenzylidene) chroman-4-one (1r).

Tin (II) chloride (0.03 mol) in EtOH (15 mL) was added to a suspension of (*E*)-3-(4-hydroxy-3-nitrobenzylidene) chroman-4-one (**1q**) (0.005 mol) in EtOH (35 mL) and concentrated HCl (75 mL). The mixture was refluxed for 2 h under stirring. After cooling, the mixture was neutralized with 2N NaOH and the solid was filtered off. The product was purified by column chromatography on silica gel (AcOEt/ petroleum ether 1:1). Yield: 46%, mp = 192-195 °C from EtOAc/petroleum ether. IR (KBr): 3460, 3380, 1655 cm^{-1}. ^1H NMR: δ (ppm) 9.80 (bs, 1H, OH), 7.86 (d, 1H, H5, $J_{5\text{-}6}$ = 7.6 Hz), 7.59-7.55 (m, 2H, = CH, H7), 7.12 (t, 1H, H6, $J_{5\text{-}6} = J_{6\text{-}7} =$ 7.6 Hz), 7.04 (d, 1H, H8, $J_{7\text{-}8}$ = 8.3 Hz), 6.78-6.75 (m, 2H, H2', H6'), 6.61 (d, 1H, H5', $J_{5'\text{-}6'}$ = 7.9 Hz), 5.43 (d, 2H, H2, J_{all}= 1.5 Hz), 4.76 (bs, 2H, NH_2). ^{13}C NMR: δ (ppm) 181.0, 160.4, 146.5, 137.9, 137.0, 135.7, 127.1, 126.8, 125.3, 121.8, 121.7, 120.9, 117.7, 115.9, 114.3, 67.7.

2.1.6. Pharmacological Studies. Determination of hMAO Isoform Activity.

The effects of the test compounds on hMAO isoform enzymatic activity were evaluated by a fluorimetric method following the experimental protocol previously described.[25]

2.1.6.1. Reversibility and Irreversibility Experiments.

To evaluate whether some of the tested compounds (**1h** and **11**) are reversible or irreversible hMAO-B inhibitors, an effective centrifugation-ultrafiltration method (so-called repeated washing) was used.[13]

2.1.6.2. Molecular Modeling.

Compounds **1h** and **1l** were built by means of the Maestro GUI.[26] Conformational properties of both molecules have been investigated by means of 1000 steps of Monte Carlo (MC) search as implemented in Macromodel ver. 7.2.[27] Each conformer was energy

minimized using the AMBER* [28] force field in united atoms notation. Water solvent effects have been taken into account by means of the implicit model GB/SA.[29] The global minimum energy structures of **1h** and **1l** were considered for the next docking simulations. Crystallographic structures, deposited into the Protein Data Bank (PDB)[30] with codes 2Z5X[31] and 2V60,[32] were considered, after graphical manipulation (fixing missing atoms and/or bond order, adding non-aliphatic H atoms), as receptor model of hMAO-A and hMAO-B, respectively. The co-crystallized ligands, harmine and 7-(3-chlorobenzyloxy)-4-carboxaldehyde-coumarin respectively for 2Z5X and 2V60, were removed, FAD double bonds were fixed and hydrogen atoms were added onto both proteins and cofactors.
According to the Autodock-Vina[21] docking methodology, a regular box of 15,625 $Å^3$, centered on the FAD N5, was considered as the active site in both hMAO-A and B receptor models. The global minimum energy conformers of **1h** and **1l** were submitted to flexible docking simulations. The estimation of the ΔG_{bind} was carried out according to the following equation:

$$\Delta G_{bind} = \sum_{c=1}^{n} \frac{IE_c \cdot P_c}{100}$$

ΔG_{bind} = Boltzmann averaged binding free energy, c = configuration of the ligand-target complex, n = maximum number of configurations, IE_c = AutoDock-Vina interaction energy of the configuration c, P_c = Boltzman population at 300 °K for configuration c.

Acknowledgment. We thank MURST (Italy) for financial support.

References

1. Al Nakib T., Bezjak V., Meegan, M.J., Chandy, R.. *Eur. J. Med. Chem.* **1990**, *25*, 455.
2. Desideri N., Olivieri S., Stein M.L., Sgro R., Orsi N., Conti C.,. *Antiviral Chem. Chemother.* **1997**, *8*, (1997) 545.
3. Quaglia M.G., Desideri N., Bossu E., Sgro R., Conti C.,. *Chirality;* **1999**, *11*(5/6), 495.
4. Tait S., Salvati A.L., Desideri N., Fiore L., *Antiviral Res.* **2006**, *72*(3), 252.
5. Wall M.E., Wani M.C., Manikumar G., Taylor H., McGivney R.,. *J. Nat. Prod.* **1989**, *52*, 774.
6. Miadokova E., Masterova I., Vlckova V., Duhova V., Toth J., *J. Ethnopharmacol.* **2002**, *81*, 381.

7. Perjési P., Das U., De Clercq E., Balzarini J., Kawase M., Sakagami H., Stables J.P., Lorand T., Rozmer Z., Dimmock J.R., *Eur. J. Med. Chem.* **2008**, *43*, 839.
8. Siddaiah V., Rao C.V., Venkateswarlu S., Krishnaraju A.V., Subbaraju G.V., *Bioorg. Med. Chem.* **2006**, *14*, 2545.
9. Siddaiah V., Maheswara M., Rao C.V, Venkateswarlu S., Subbaraju G.V., *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1288.
10. Kirkiacharian S., Tongo H.G., Bastide J., Bastide P., Grenie, M.M., *Eur. J. Med. Chem.* **1989**, *24*, 541.
11. Hung T.M., Thu C.V., Dat N.T., Ryoo S.W., Lee J.H., Kim J.C., Na M., Jung H.J., Bae K., Min B.S., *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2412.
12. Lin L.G., Xie H., Li H.L., Tong L.J., Tang C.P., Ke C.Q., Liu Q.F., Lin L.P., Geng M.Y., Jiang H., Zhao W.M., Ding J., Ye Y., *J. Med. Chem.* **2008**, *51*, 4419.
13. Chimenti F., Fioravanti R., Bolasco A., Chimenti P., Secci D., Rossi F., Yañez M., Orallo F., Ortuso F., Alcaro, S., *J. Med. Chem.* **2009**, *52*, 2818.
14. Chimenti F., Fioravanti R., Bolasco A., Chimenti P., Secci D., Rossi F., Yañez M., Orallo F., Ortuso F., Alcaro, S., Cirilli R., Ferretti R., Sanna M.L., *Bioorg. Med. Chem.* **2010**, *18*, 1273-1279.
15. Krishnamurty H.G., Parkash B., Seshadri T.R.,. *Indian J. Chem.* **1974**, *12*(6), 554.
16. Yáñez M., Fraiz N., Cano E., Orallo F., *Biochem. Biophys. Res. Commun.* **2006**, *344*(2), 688.
17. Silverman R.B., In *Wiley Encyclopedia of Chemical Biology*. John Wiley & Sons, Inc., **2009**, *1*, 663.
18. Gerlach M., Riederer P., Youdim M.B., *Eur. J. Pharmacol.* **1992**, *226*, 97.
19. O'Brien E.M., Tipton K.F., Meroni M., Dostert *J. Neural. Transm. Suppl*. **1994**, *41*, 295.
20. Chimenti F., Maccioni E., Secci D., Bolasco A., Chimenti P., Granese A., Carradori S., Alcaro S., Ortuso O., Yañez M., Orallo F., Cirilli R., Ferretti R., La Torre F., *J. Med. Chem.* **2008,** *51,* 4874.
21. Trott O., Olson J.A., *J. Comput. Chem.* **2010**, *31*, 455.
22. Pfeiffer P., Breit E., Hoyer H., *J. prakt. Chem*. **1931**, *129*, 31.
23. Sathunuru R., Koh B., Zhang H., Biehl E., *Heterocycles;* **2005**, *65*(10), 2493.
24. Bennett P., Donnelly J.A., Meaney D.C., O'Boyle P., *J. Chem. Soc., Perkin Trans. 1* **1972**, *12*, 1554.
25. Chimenti F., Secci D., Bolasco A., Chimenti P., Bizzarri B., Granese A., Carradori S., Yañez M., Orallo F., Ortuso F., Alcaro S., *J. Med. Chem.* **2009**, *52*(7), 1935.
26. Maestro ver. 4.1 Schroedinger Inc.: Portland, OR, **1998-2001**.
27. Mohamadi F., Richards N.G.J., Guida W.C., Liskamp R., Lipton M., Caufield C., Chang G., Hendrickson T., Still W.C., *J. Comput. Chem*. **1990**, *11*, 440.
28. (a) McDonald D.Q., Still, W.C. *Tetrahedron Lett.* **1992**, *33*, 774. (b) Weiner S.J., Kollman P.A., Case D.A., Singh U.C., Ghio C., Alagona G., Profeta S., Weiner P., *J. Am. Chem. Soc.* **1984**, *106*, 765.
29. Hasel W., Hendrickson T.F., Still W.C., *Tetrahedron Comput. Methodol*. **1988**, *1*, 103.
30. Berman H.M., Westbrook J., Feng Z., Gilliland G., Bhat T.N., Weissig H., Shindyalov I.N., Bourne P.E., *Nucleic Acids Res*. **2000**, *28*, 235.

31. Son S.Y., Ma J., Kondou Y., Yoshimura M., Yamashita E., Tsukihara T., *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 5739.
32. Binda C., Wang J., Pisani L., Caccia C., Carotti A., Salvati P., Edmondson D.E., Mattevi A., *J. Med. Chem.* **2007**, *50*, 5848.

Supporting Information

SI1. Elemental analyses of derivatives **1a-w**.

Comp	C		H		Cl		N	
	Calc %	Found %	Calc %	Found %	Calc %	Found %	Calc %	Found %
1a	81.34	81.49	5.12	5.13				
1b	76.18	76.28	4.79	4.81				
1c	70.99	71.07	4.10	4.12	13.10	13.05		
1d	62.97	62.86	3.30	3.31	23.24	23.28		
1e	56.59	56.41	2.67	2.65	31.32	31.36		
1f	76.68	76.53	5.30	5.33				
1g	69.93	69.77	5.56	5.54				
1h	67.60	67.42	4.25	4.26				
1i	70.99	70.81	4.10	4.11	13.10	13.07		
1j	76.48	76.24	5.21	5.22			5.57	5.55
1k	73.71	73.56	5.15	5.14			4.78	4.79
1l	77.40	77.23	6.13	6.11			5.01	5.03
1m	72.33	72.58	5.00	5.01				
1n	72.33	72.56	5.00	5.00				
1o	71.64	71.53	4.51	4.52				
1p	68.92	68.70	4.08	4.10				
1q	64.65	64.80	3.73	3.74			4.71	4.69
1r	71.90	71.81	4.90	4.89			5.24	5.26
1s	72.33	72.27	5.00	5.01				
1t	72.96	73.24	5.44	5.45				
1u	72.96	72.83	5.44	5.47				
1v	69.93	69.82	5.56	5.57				
1w	69.93	70.04	5.56	5.55				

SI2. Elemental analyses of derivatives **2a-g** and **3a-e.**

Comp	C		H		Cl	
	Calc %	Found %	Calc %	Found %	Calc %	Found %
2a	81.34	81.63	5.12	5.11		
2b	76.18	76.07	4.79	4.78		
2c	70.99	70.78	4.10	4.08	13.10	13.14
2d	62.97	63.09	3.30	3.29	23.24	23.13
2e	56.59	56.48	2.67	2.68	31.32	31.37
2f	76.68	76.61	5.30	5.29		
2g	69.93	69.82	5.56	5.57		
3a	80.65	80.43	5.92	5.93		
3b	75.57	75.68	5.55	5.54		
3c	70.46	70.65	4.80	4.79	13.00	13.02
3d	62.56	62.75	3.94	3.93	23.08	23.11
3e	56.25	56.34	3.25	3.26	31.13	31.06

Molecular modeling details of derivatives **1h** and **1l.**

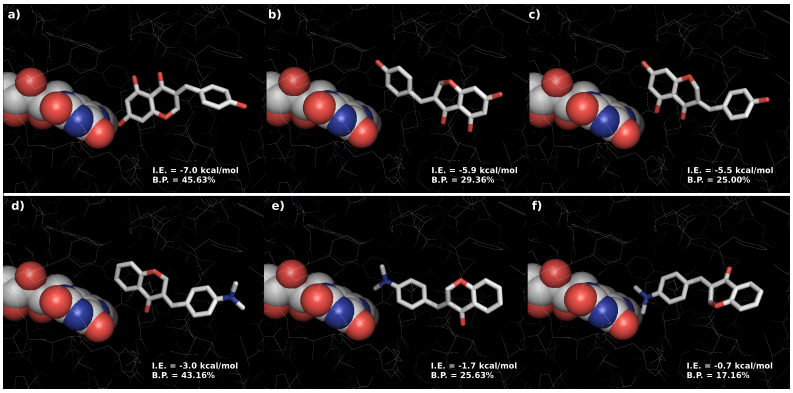


Figure SI1: most stable complexes of compounds **1h** (a-c) and **1l** (d-f) into the hMAO-A active site and related interaction energies (I.E.) and Boltzman population (B.P.). The inhibitors are depicted in polytube, FAD cofactor in spacefill and enzyme residues in wireframe. Atoms are coloured following the CPK notation. I.E.

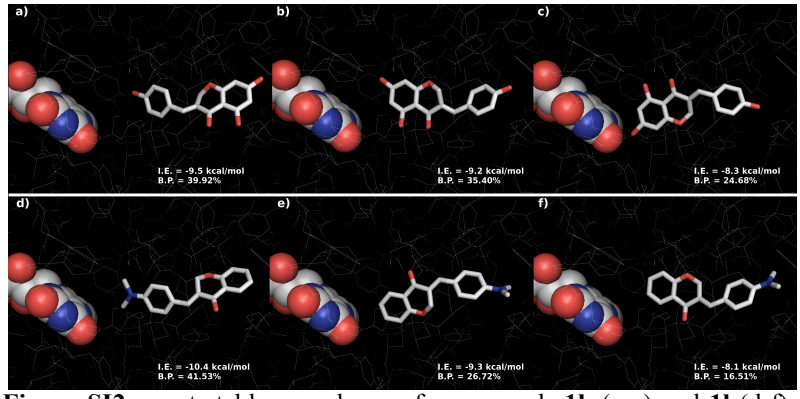


Figure SI2: most stable complexes of compounds **1h** (a-c) and **1l** (d-f) into the hMAO-B active site and related interaction energies (I.E.) and Boltzman population (B.P.). The inhibitors are depicted in polytube, FAD cofactor in spacefill and enzyme residues in wireframe. Atoms are coloured following the CPK notation.

2.2. 1,5-Diphenylpenta-2,4-dien-1-ones as potent and selective monoamine oxidase-B inhibitors.

Nicoletta Desideri[a], Rossella Fioravanti[a], Luca Proietti Monaco[a], Mariangela Biava[a], Matilde Yáñez[b], Francesco Ortuso[c], Stefano Alcaro[c].

Eur. J. Med. Chem. 59 (2013), 91-100.

[a] *Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza - Università di Roma, P.le Aldo Moro, 5, 00185 Rome, Italy*
[b] *Departamento de Farmacología and Instituto de Farmacia Industrial, Facultad de Farmacia, Universidad de Santiago de Compostela, Campus Universitario Sur, E-15782 Santiago de Compostela (La Coruña), Spain*
[c] *Dipartimento di Scienze della Salute, Università "Magna Græcia" di Catanzaro, Campus Universitario "S. Venuta", Viale Europa, 88100 Catanzaro, Italy.*

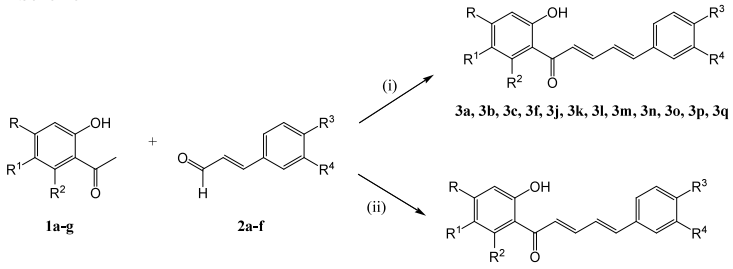
Recently, we reported new series of MAO inhibitors related to natural compounds as flavones, thioflavones and flavanones[1], homoisoflavones[2], and chalcones[3], the biogenetic precursors of flavonoids. The potent and selective hMAO-B inhibitory activity of several synthetic chalcones[3] and of 1,4-diphenyl-2-butene[4], prompted us to designed and tested against hMAO-A and hMAO-B isoforms a series of structurally related (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**3a-r**) and (2*Z*,4*E*)-3-hydroxy-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one (**6a-j**).

2.2.1. Results and discussion

2.2.1.1. Chemistry

(2*E*,4*E*)-1-(2-Hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**3a-r**) were synthesized by base-catalysed reaction of the appropriate 2-hydroxyacetophenone (**1a-g**) and substituted cinnamaldehyde (**2a-f**). The condensation was performed in hydro-alcoholic sodium hydroxide or in methanol using barium hydroxide octahydrate as base (scheme 1). The deprotection of hydroxyl groups to obtain the compounds **3h, 3n, 3o, 3p** and **3q** was achieved during the work up of the reaction mixture.

Scheme 1[a]



1a R = R^1 = R^2 = H
1b R = R^2 = H, R^1 = Cl
1c R = R^2= H, R^1 = F
1d R = R^1 = H, R^2 = OCH_3
1e R = OCH_3, R^1 = R^2 = H
1f R = (tetrahydro-2*H*-pyran-2-yl)oxy, R^1 = R^2 = H
1g R = R^1 = H, R^2 = (tetrahydro-2*H*-pyran-2-yl)oxy

2a R^3 = R^4 = H
2b R^3 = Cl, R^4 = H
2c R^3 = OCH_3, R^4 = H
2d R^3 = NO_2, R^4 = H
2e R^3 = R^4 = OCH_3
2f R^3 = $OCOCH_3$, R^4 = OCH_3

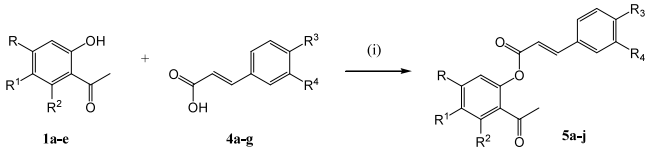
3a R = R^1 = R^2 = R^3 = R^4 = H
3b R = R^2 = R^3 = R^4 = H, R^1 = Cl
3c R = R^2 = R^4 = H, R^1 = Cl, R^3 = OCH_3
3d R = R^2 = R^4 = H, R^1 = Cl, R^3 = NO_2
3e R = R^2 = R^4 = H, R^1 = R^3 = Cl
3f R = R^1 = R^2 = R^4 = H, R^3 = Cl
3g R = OCH_3, R^1 = R^2 = R^4 = H, R^3 = Cl
3h R = OH, R^1 = R^2 = R^4 = H, R^3 = Cl
3i R = R^2 = R^3 = R^4 = H, R^1 = F
3j R = R^2 = R^4 = H, R^1 = F, R^3 = OCH_3
3k R = R^1 = R^2 = R^4 = H, R^3 = OCH_3
3l R = R^1 = R^2 = H, R^3 = R^4 = OCH_3
3m R = R^1 = H, R^2 = R^3 = R^4 = OCH_3
3n R = R^1 = R^2 = H, R^3 = OH, R^4 = OCH_3
3o R = R^1 = H, R^3 = OH, R^2 = R^4 = OCH_3
3p R = R^1 = H, R^2 = OH, R^3 = R^4 = OCH_3
3q R = R^1 = H, R^2 = R^3 = OH, R^4 = OCH_3
3r R = R^1 = R^2 = R^4 = H, R^3 = NO_2

[a]Reagents and conditions: (i) 60% NaOH, MeOH, r.t. 48 h; for **3n** and **3o** 60 °C 5 h; (ii) $Ba(OH)_2$ $8H_2O$, MeOH, 50 °C, 5 h.

(2*Z*,4*E*)-3-Hydroxy-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**6a-j**) were prepared according to the two step procedure shown in scheme 2. The appropriate 2-hydroxyacetophenone (**1a-e**) was first converted into the corresponding 2-acetylphenyl (*E*)-cinnamate (**5a-j**) by treatment with substituted cinnamic acid (**4a-g**) in the presence of phosphoryl chloride, in dry pyridine. In the second step, the Baker–Venkataraman rearrangement of the formed ester (**5a-j**) into desired **6a-j** was achieved in the presence of potassium carbonate, in dry acetone.

The hydroxy-analogues **6k** and **6l** were achieved by treatment with boron tribromide of a solution of the corresponding methoxy derivatives **6e** and **6f** in dry dichloromethane (scheme 3).

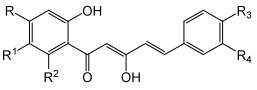
Scheme 2[a]



1a-e **4a-g** **5a-j**

1a R = R^1 = R^2 = H
1b R = R^2 = H, R^1 = Cl
1c R = R^2= H, R^1 = F
1d R = R^1 = H, R^2 = OCH_3
1e R = OCH_3, R^1 = R^2 = H

4a R^3 = R^4 = H
4b R^3 = Cl, R^4 = H
4c R^3 = OCH_3, R^4 = H
4d R^3 = CH_3, R^4 = H
4e R^3 = NO_2, R^4 = H
4f R^3 = R^4 = OCH_3
4g R^3 = $OCOCH_3$, R^4 = OCH_3

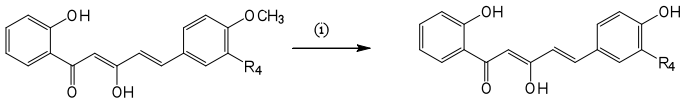


6a-k

5a, 6a R = R^1 = R^2 = R^3 = R^4 = H
5b, 6b R = R^2 = R^4 = H, R^1 = R^3 = Cl
5c, 6c R = R^1 = R^2 = R^4 = H, R^3 = Cl
5d, 6d R = R^2 = R^3 = R^4 = H, R^1 = F
5e, 6e R = R^1 = R^2 = R^4 = H, R^3 = OCH_3
5f, 6f R = R^1 = R^2 = H, R^3 = R^4 = OCH_3
5g, 6g R = R^1 = H, R^2 = R^3 = R^4 = OCH_3
5h R = R^1 = H, R^3 = $OCOCH_3$, R^2 = R^4 = OCH_3
6h R = R^1 = H, R^3 = OH, R^2 = R^4 = OCH_3
5i, 6i R = R^1 = R^2 = R^4 = H, R^3 = NO_2
5j, 6j R = R^1 = R^2 = R^4 = H, R^3 = CH_3

[a]Reagents and conditions: (i) $POCl_3$, Py, r.t., 4 h; (ii) K_2CO_3, dry acetone, reflux, 24 h.

Scheme 3[a]



6e, **6f** **6k**, **6l**

6e R^4 = H
6f R^4 = OCH_3

6k R^4 = H
6l R^4 = OH

[a]Reagents and conditions: (i) Boron Tribromide, CH_2Cl_2 dry, 1h on ice bath and then 20h r.t;

2.2.1.2. Biochemistry

The inhibitory potencies of the compounds on hMAO activity were investigated by measuring their effects on the production of hydrogen peroxide from *p*-tyramine using the Amplex Red MAO assay kit and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B. The hMAO activity was

evaluated by measuring the fluorescence generated by resorufin using the general procedure described previously by us[5].

The hMAO-A and hMAO-B inhibition data and the selectivity indexes (SI = IC_{50} hMAO-A /IC_{50} hMAO-B) of the (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**3a-r**) and (2*Z*,4*E*)-3-hydroxy-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**6a-l**) are reported in table 1 and 2, respectively, together with the results obtained for the reference inhibitors (clorgyline, selegiline, isatin, iproniazid and moclobemide).

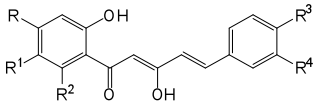
Generally, the new tested compounds selectively inhibited the enzymatic activity of hMAO-B in the nanomolar or low micromolar range. The most interesting compound of the entire series of inhibitors was (2*E*,4*E*)-5-(4-chlorophenyl)-1-(2-hydroxy-4-methoxyphenyl)penta-2,4-dien-1-one **3g**, showing potent hMAO-B activity and high selectivity (IC_{50} = 4.51 nM, SI > 22173). Within the series of (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**3a-r)**, in addition to the affinity for hMAO-B, some derivatives (**3f**, **3h**, **3n**, **3o**, **3p** and **3q**) are also able to inhibit hMAO-A in the micromolar range. These compounds demonstrate a different extent of hMAO-B selectivity, showing SIs ranging from 1.5 to 1354. With the exception of **3f**, these analogues bear at least another hydroxyl group in the aromatic rings in addition to the hydroxyl group in position 2. The same behavior was observed for related chalcones and homoisoflavones previously studied by us[2,3]. It is noteworthy that the replacement of the methoxyl group in the potent hMAO-B inhibitor **3g** with the hydroxyl group in the same position resulted in compound **3h**, able to inhibit both hMAO isoforms. Although about 2-fold less potent than **3g** as hMAO-B inhibitor, **3h** remain potent in the nanomolar range and endowed with an excellent selectivity (IC_{50} = 11.35 nM, SI = 1354). In this series of penta-2,4-dien-1-ones (**3a-r**), only the trimethoxy analogue **3m** was essentially inactive against both isoforms up to the highest concentration tested (100 μM). The replacement of one or two methoxyl groups in **3m** with hydroxyl groups results in compounds (**3o**, **3p** and **3q**) with affinity toward both hMAO isoforms. On the contrary, the removal of one or two methoxyl groups (compounds **3l** and **3k**, respectively) restored only the MAO-B affinity in the micromolar range. The replacement of the 3-methoxyl group in **3l** with the hydroxyl group to obtain compound **3n**, confirmed the ability of poli-hydroxy substituted analogues to inhibit both isoforms.

Table 1. hMAO Inhibitory activities and selectivity indexes of (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**3a-r**), (*E*)-1-(5-fluoro-2-hydroxyphenyl)-3-phenylprop-2-en-1-one (**7**) and reference inhibitors.

Comp.	R	R^1	R^2	R^3	R^4	MAO-A (IC_{50})[a]	MAO-B (IC_{50})[a]	SI[d]
3a	H	H	H	H	H	[e]	105.51 ± 5.68 nM	> 948[h]
3b	H	Cl	H	H	H	[e]	54.77 ± 2.63 nM	> 1826[h]
3c	H	Cl	H	OCH_3	H	[e]	153.14 ± 6.21 nM	> 653[h]
3d	H	Cl	H	NO_2	H	[e]	1.08 ± 0.08 µM	> 93[h]
3e	H	Cl	H	Cl	H	[e]	1.46 ± 0.13 µM	>68[h]
3f	H	H	H	Cl	H	37.73 ± 1.57 µM[b]	860.46 ± 43.18 nM	44
3g	OCH_3	H	H	Cl	H	[e]	4.51 ± 0.28 nM	> 22173[h]
3h	OH	H	H	Cl	H	15.37 ± 1.18 µM[b]	11.35 ± 0.73 nM	1354
3i	H	F	H	H	H	[e]	75.84 ± 7.66 nM	> 1319[h]
3j	H	F	H	OCH_3	H	[e]	725.92 ± 31.56 nM	> 138[h]
3k	H	H	H	OCH_3	H	[f]	1.81 ± 0.15 µM	> 55[h]
3l	H	H	H	OCH_3	OCH_3	[f]	14.61 ± 2.18 µM	> 6.8[h]
3m	H	H	OCH_3	OCH_3	OCH_3	[f]	[e]	
3n	H	H	H	OH	OCH_3	1.56 ± 0.19 µM[b]	501.66 ± 49.19 nM	3.1
3o	H	H	OCH_3	OH	OCH_3	1.03 ± 0.25 µM[b]	486.35 ± 43.36 nM	2.1
3p	H	H	OH	OCH_3	OCH_3	25.16 ± 1.96 µM[b]	1.45 ± 0.16 µM	17
3q	H	H	OH	OH	OCH_3	1.43 ± 0.22 µM[c]	969.44 ± 87.45 nM	1.5
3r	H	H	H	NO_2	H	[e]	160.32 ± 12.48 nM	> 624[h]
7	H	F	H	H	H	[f]	667.89 ± 59.32 nM	> 150[h]
A						4.46 ± 0.32 nM[b]	61.35 ± 1.13 µM	0.000073
B						67.25±1.02 µM[b]	19.60 ± 0.86 nM	3431
C						[f]	21.07 ± 1.47 µM	> 4.7[h]
D						6.56 ± 0.76 µM	7.54 ± 0.36 µM	0.87
E						361.38±19.3 µM	[g]	< 0.36[i]

[a]All IC_{50} values shown in this table are the mean ± S.E.M. from five experiments. Level of statistical significance: [b] $P <$ 0.01 or [c] $P < 0.05$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett´s. [d] SI: hMAO-B selectivity index = IC_{50}(hMAO-A)/IC_{50}(hMAO-B). [e] Inactive at 100 µM (highest concentration tested). [f] 100 µM inhibits the corresponding MAO activity by approximately 40-50%. At higher concentration the compounds precipitate. [g] Inactive at 1 mM (highest concentration tested). [h] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is the highest concentration tested (100 µM). [i]Values obtained under the assumption that the corresponding IC_{50} against MAO-B is the highest concentration tested (1 mM).
A: Clorgyline, **B:** Selegiline, **C:** Isatin, **D:** Iproniazid, **E:** Moclobemide.

Table 2. hMAO Inhibitory activities and selectivity indexes of (2*Z*,4*E*)-3-hydroxy-1-(2-hydroxyphenyl)-5-phenylpenta-2,4 dien-1-ones (**6a-l**) and reference inhibitors.



6a-l

Compd	R	R^1	R^2	R^3	R^4	MAO-A (IC_{50})[a]	MAO-B (IC_{50})[a]	SI[d]
6a	H	H	H	H	H	[e]	1.16 ± 0.03 µM	> 86[h]
6b	H	Cl	H	Cl	H	[e]	[e]	
6c	H	H	H	Cl	H	[e]	[f]	
6d	H	F	H	H	H	[e]	50.44 ± 4.61 nM	> 1983[h]
6e	H	H	H	OCH_3	H	[e]	[f]	
6f	H	H	H	OCH_3	OCH_3	[e]	[e]	
6g	H	H	OCH_3	OCH_3	OCH_3	[f]	[e]	
6h	H	H	OCH_3	OH	OCH_3	3.76 ± 0.42 µM[b]	1.68 ± 0.21 µM	2.2
6i	H	H	H	NO_2	H	[e]	2.67 ± 0.28 µM	> 37[h]
6j	H	H	H	CH_3	H	[e]	3.77 ± 0.19 µM	> 27[h]
6k	H	H	H	OH	H	1.52 ± 0.04 µM[b]	26.46 ± 1.97 nM	57
6l	H	H	H	OH	OH	431.07 ± 52.45 nM[c]	654.32 ± 61.05nM[b]	0.66
A						4.46 ± 0.32 nM[b]	61.35 ± 1.13 µM	0.000073
B						67.25 ± 1.02 µM[b]	19.60 ± 0.86 nM	3431
C						[f]	21.07 ± 1.47 µM	> 4.7[h]
D						6.56 ± 0.76 µM	7.54 ± 0.36 µM	0.87
E						361.38 ± 19.3 µM	[g]	< 0.36[i]

[a] All IC_{50} values shown in this table are the mean ± S.E.M. from five experiments. Level of statistical significance: [b] $P < 0.01$ or [c] $P < 0.05$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett´s. [d] SI: hMAO-B selectivity index = IC_{50}(hMAO-A)/IC_{50}(hMAO-B). [e] Inactive at 100 µM (highest concentration tested). [f] 100 µM inhibits the corresponding MAO activity by approximately 40-50%. At higher concentration the compounds precipitate. [g]Inactive at 1 mM (highest concentration tested). [h] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is the highest concentration tested (100 µM). [i]Values obtained under the assumption that the corresponding IC_{50} against MAO-B is the highest concentration tested (1 mM).
A: Clorgyline, **B:** Selegiline, **C:** Isatin, **D:** Iproniazid, **E:** Moclobemide.

The introduction of a hydroxyl group in position 3 of the chain results in the (2*Z*,4*E*)-3-hydroxy-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**6a-l**). This structural modification produced a loss of the inhibitory activity or a marked reduction in potency with respect to unsubstituted (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-

ones (**3a-r**) (table 2). The only exception was represented by (2*Z*,4*E*)-1-(5-fluoro-2-hydroxyphenyl)-3-hydroxy-5-phenylpenta-2,4-dien-1-one (**6d)** that showed a potent hMAO-B inhibitory activity slightly higher than the corresponding (2*E*,4*E*)-1-(5-fluoro-2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one (**3i)** (IC_{50} = 50.44 nM and 75.84 nM, respectively) and better selectivity (SI > 1983 and > 1319, respectively). Also in this series of inhibitors, the compounds substituted with more than one hydroxyl group in the aromatic rings (**6h**, **6k** and **6l**) are able to inhibit both hMAO isoforms. In particular, **6l** was the only hMAO-A inhibitor of both series active in the submicromolar range. Moreover, its potency toward B isoform was slightly higher than the potency toward A isoform, resulting in an essentially nonselective compound.

To evaluate whether the most potent hMAO-B inhibitors-(**3b, 3g, 3h, 6d, 6k**) are reversible or irreversible hMAO-B inhibitors, the so-called repeated washing method was used[3]. The results obtained using selegiline (irreversible inhibitor) and isatin (reversible inhibitor) as reference compounds are reported in table 3.

Table 3. Reversibility and irreversibility of hMAO-B inhibition of derivatives **3b**, **3g**, **3h**, **6d** and **6k**.

	% MAO-B inhibition	
Comp	**Before washing**	**After repeated washing**[a]
3b (100 nM)	58.10 ± 0.10	60.30 ± 2.53
3g (10 nM)	61.34 ± 2.38	37.51 ± 1.13
3h (10 nM)	51. 52 ± 3.18	42.02 ± 0.70
6d (100 nM)	62.35 ± 0.45	68.80 ± 3.33
6k (100 nM)	66.70 ± 1.52	68.55 ± 4.39
isatin (30 μM)	64.31 ± 2.08	31.22 ± 0.91
selegiline (20 nM)	53.08 ± 1.64	54.63 ± 1.72

Each value is the mean ± S.E.M. from five experiments (n = 5).
[a]Level of statistical significance: $P < 0.01$ versus the corresponding MAO-B inhibition before washing, as determined by ANOVA/Dunnett´s.

The reversibility tests revealed the lack of enzyme activity restoration after repeated washing for compounds **3b, 6d** and **6k**. Similar results were obtained for selegiline a well-known irreversible MAO-B inhibitor. On the contrary, significant recovery of MAO-B activity was observed after repeated washing of isatin (reversible inhibitor) and compound **3g.** The enzymatic inhibition lowered in a minor extent after repeated washing in the presence of **3h** (table 3).

These results suggest that the novel potent hMAO-B inhibitors **3g** and **3h** are reversible inhibitors.

2.2.1.3. Molecular modeling studies

With the aim to rationalize the different hMAO inhibition of the analogues **3g** and **3h**, molecular modeling studies were carried out. Following our previous experience[6] we have adopted both molecular mechanics and quantum chemistry methods to elucidate the mechanism of inhibition of previously reported compounds. In particular, the recognition of hMAO-A and –B and the role of the *p*-methoxy substituent, available into the **3g** in replacement of the **3h** hydroxy group, were investigated.

In order to obtain reasonable configuration of both inhibitors into the hMAO-A and –B binding clefts, docking experiment were performed. The top ranked theoretical complex revealed remarkable similarities, actually, in all cases the ligands showed the phenolic ring and the *p*-chlorophenyl moieties respectively positioned towards the FAD cofactor and the entrance gorge surrounded by hydrophobic residues. With the aim to improve the analysis of **3g** and **3h** hMAO recognition, new complexes between our inhibitors and both target isoform models were manually built by means of a 180° rotation of the corresponding top ranked poses. Resulting structures, after energy optimisation and re-scoring procedure, revealed interaction energy remarkably weaker than the orginal top ranked poses (see table 1 in Supplementary content). Such a scenario and the docking score (see Figure S1 in Supplementary content) were not in agreement to the experimental IC_{50} data, as a consequence, each top ranked docking complex was submitted to 12 ns of molecular dynamics (MD). The resulting four trajectories were analyzed in terms of inhibitor target interaction energy and geometry (Experimental section) reporting a good qualitative agreement to the experimental inhibition data. Actually, hMAO-B·**3g** resulted as the most energy favored complex with an advantage of about 10 kcal/mol with respect to the hMAO-A **3g**, conversely predicted as the worst one. The hMAO-B binding energies of **3h** indicated a disadvantage of 3.4 kcal/mol with respect to **3g** on the contrary, into the hMAO-A, 1.2 kcal/mol demonstrated its better recognition than **3g** (see figure S2 in Supplementary content). The hMAO-B selectivity of the selected compounds was also remarked by computing, during the molecular dynamics runs, the target backbone root mean square deviation (RMSd) with respect to

the corresponding starting structures (see figure S3 in Supplementary content). As reported by the average RMSd values, the target isoform B accommodate both inhibitors with lower perturbation (**3g** = 1.86 Å; **3h** = 2.44 Å) than hMAO-A (**3g** = 3.79 Å; **3h** = 4.12 Å). Such data were not a surprise because it is known[7] that hMAO-B active site (~700 $Å^3$) is larger than hMAO-A one (~400 $Å^3$), and contributed to rationalize the isoform selectivity of our compounds. With the aim to identify the most relevant residues interacting to **3g** and **3h**, all MD trajectory frames were investigated. The contribution of each residue to the inhibitor binding was expressed as percentage of frames reporting its interaction to the ligand (figure 1).

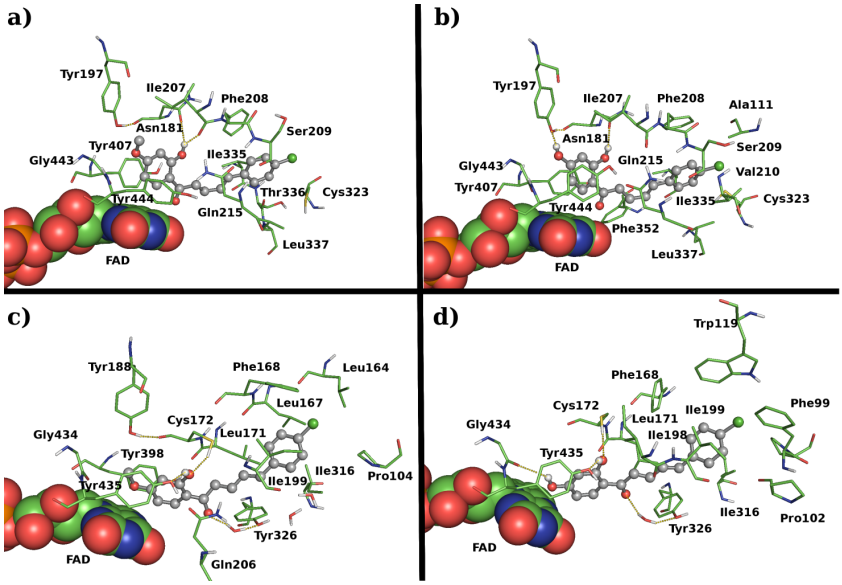


Figure 1. Representative molecular dynamics binding modes of **3g** (left side) and **3h** (right side) into the hMAO-A (top side) and hMAO-B (bottom side) active sites. Residues interacting to the inhibitors with a frequency higher than 60% are reported in green carbons sticks or spacefill, ligands are depicted in gray carbons balls and sticks. Yellow dotted lines indicate hydrogen bonds.

Due to wide movements into the hMAO-A cleft, both compounds showed a larger number of interacting residues in this isozyme (see figures S4 and S5 in Supplementary content). The percentage of frames reporting **3g** or **3h** interacting to the FAD remarked that our ligands were located into the hMAO-B binding cleft (**3g** = 93.78%; **3h** = 92.12%) deeper than into the hMAO-A (**3g** =

82.16%; **3h** = 82.99%). Such an information suggested to investigate the stacking interaction among the phenolic ring of our compounds and the hMAO-A Tyr407 and 444 and the corresponding hMAO-B Tyr398 and 435. The compound **3g** into the hMAO-B showed stacking with both residues for more than 90% of the simulation time, while in all other cases a preference for one of the two tyrosines was observed. The stacking stability could be attributed to the **3g** *p*-methoxy phenyl group. In order to deep investigate the role of such a moiety, quantum mechanics calculations of both ligands molecular orbital were performed (Experimental section).

As expected, even if molecular orbital distribution of our compounds was similar, **3g** revealed HOMO/LUMO areas surrounding the *p*-methoxy phenyl ring larger than **3h** (see Figure S5 in Supplementary content) suggesting stronger interaction to the tyrosine residues. Moreover, such a substituent highlighted a different role depending on the target. In the hMAO-A *p*-methoxy phenyl ring cannot interact to Tyr197 and allowed the hydrogen bonding between **3g** *o*-phenolic group and both Asn181 sidechain and Ile207 backbone. Unlike **3h**, the interaction network has forced the removal of the **3g** aromatic ring from the FAD and consequently from the Tyr407-444 stacking. Into the hMAO-B, the substitution of the Asn181 with the Cys172 was mainly responsible of our compounds isoform selectivity. In fact, the flexibility of the Cys sidechain was lower than Asn and the hydrogen bond to Cys172 was unable to withdraw from the FAD both our inhibitors that, therefore, established stacking contacts to Tyr398 and 435. The hMAO-B active site, larger than hMAO-A, allowed the entrance of solvent water molecules that contributed to the complexes stabilization performing hydrogen bond bridge between the keto-group of our compounds and the Tyr326 side chain.

2.2.1.4. Conclusion

A new class of highly potent and selective hMAO-B inhibitors was identified. The most potent derivatives **3g** and **3h** showed inhibitory activity in the low nanomolar range coupled with high selectivity. Moreover, the results of reversibility tests suggest that these two compounds are reversible inhibitors of MAO-B. Based on their potent and selective MAO-B inhibitory properties, these compounds could be useful for the development of promising drug candidate for the therapy of neurodegenerative diseases.

2.2.1.5. Experimental section

2.2.1.5.1. Chemistry

Chemicals were purchased from Sigma-Aldrich 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 (Fluka, DC-Alufolien Kieselgel or aluminum oxide F254). Compound purity was determined by elemental analysis and was confirmed to be >95% for all the tested compounds. Analytical results are within ±0.40% of the theoretical values (Table 2 in Supplementary content). The synthetic intermediates 2-hydroxy-4-[(tetrahydro-2H-pyran-2-yl)oxy]acetophenone (**1f**)[3], 2-hydroxy-6-[(tetrahydro-2H-pyran-2-yl)oxy]acetophenone (**1g**)[8], (*E*)-3,4-dimethoxycinnamaldehyde (**2e**)[9], and (*E*)-3-(4-acetoxy-3-methoxy)cinnamic acid (**2f**)[10] were synthesized following the procedure previously described. (2*E*,4*E*)-1-(2-Hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**3a-f**, **3i**, **3k**, **3r**)[11,12], 2'-cinnamoyloxyacetophenones (**5a-e**, **5i**, **5j**) [12,13], (2*Z*,4*E*)-3-hydroxy-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (**6a-e**, **6i**, **6j**, **6k**)[12,13] , and (*E*)-1-(5-fluoro-2-hydroxyphenyl)-3-phenylprop-2-en-1-one (**7**) [12] were prepared as previously reported by us.

2.2.1.5.2. General procedure for the synthesis of (2E,4E)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-ones (3j, 3l-q).

An aqueous solution of 60% sodium hydroxide (50 mL) was added dropwise to a stirred suspension of the appropriate 2′-hydroxyacetophenone (**1a**, **1c**, **1d, 1g**) (10 mmol) and substituted cinnamaldehyde (**2c**, **2e**, **2f**) (11 mmol) in methanol (50 mL), cooled in ice bath. The mixture was stirred at room temperature for 48 h (**3j, 3l, 3m**, **3p**, **3q**) or heated at 60 °C for 5 h (**3n, 3o**). After that period, the suspension was diluted with ice and water and acidified with 2N hydrochloric acid. For the eventual deprotection of the hydroxyl group (synthesis of compounds **3n-q**), the mixture was stirred at room

temperature for 1 h. The resulting suspension was filtered (**3j**) or extracted with AcOEt **(3l-q)**. In the latter case, the combined organic phases were washed with saturated $NaHCO_3$ solution and brine, then dried over Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by crystallization from AcOEt (**3j**) or chromatographed on a silica gel column eluting with AcOEt/petroleum ether 1:1 (**3l** and **3m**), AcOEt/petroleum ether 1:3 (**3p**), AcOEt/petroleum ether 1:4 (**3q**) or CH_2Cl_2 (**3n** and **3o**).

2.2.1.5.2.1. (2*E*,4*E*)-1-(5-Fluoro-2-hydroxyphenyl)-5-(4-methoxyphenyl)penta-2,4-dien-1-one (3j). Yield: 78%; mp 191-193 °C from AcOEt. IR (KBr) 3450, 1620 cm^{-1}. ^1H NMR ($CDCl_3$): δ (ppm) 12.70 (s, 1H, OH), 7.75 (dd, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 14.5 Hz, $J_{\beta\text{-}\gamma}$ = 10.3 Hz), 7.55-7.47 (m, 3H, H6, H2', H6'), 7.23 (dt, 1H, H4, $J_{4\text{-}F}$ = 8.8 Hz, $J_{3\text{-}4}$ = 8.8 Hz, $J_{4\text{-}6}$ = 2.8 Hz), 7.12-6.86 (m, 6H, Hα, Hγ, Hδ, H3, H3', H5'), 3.87 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$): δ (ppm) 192.8 (d, $J_{C\text{-}F}$ = 2.3 Hz), 161.1, 159.7, 154.9 (d, $J_{C\text{-}F}$ = 236.1 Hz), 147.0, 143.6, 129.2, 128.7, 124.4, 123.5 (d, $J_{C\text{-}F}$ = 23.7 Hz), 121.7, 119.7 (d, $J_{C\text{-}F}$ = 7.3 Hz), 119.6 (d, $J_{C\text{-}F}$ = 6.1 Hz), 114.5, 114.4 (d, $J_{C\text{-}F}$ = 21.3 Hz), 55.4.

2.2.1.5.2.2. (2*E*,4*E*)-5-(3,4-Dimethoxyphenyl)-1-(2-hydroxyphenyl) penta-2,4-dien-1-one (3l). Yield: 48%; mp 193-196 °C from acetone. IR (KBr) 1632 cm^{-1}. ^1H MNR ($CDCl_3$): δ (ppm) 12.96 (s, 1H, 2OH), 7.84 (dd, 1H, H6, $J_{5\text{-}6}$ = 8.1 Hz, $J_{4\text{-}6}$ = 1.6 Hz), 7.72 (dd, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 14.6 Hz, $J_{\beta\text{-}\gamma}$ = 10.6 Hz), 7.47 (ddd, 1H, H4, $J_{4\text{-}5}$ = 7.2 Hz, $J_{3\text{-}4}$ = 8.5 Hz, $J_{4\text{-}6}$ = 1.6 Hz), 7.19 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 14.6 Hz), 7.08 (dd, 1H, H6', $J_{5'\text{-}6'}$ = 8.3 Hz, $J_{2'\text{-}6'}$ = 1.9 Hz), 7.05-6.86 (m, 6H, Hγ, Hδ, H3, H5, H2', H5'), 3.95 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$): δ (ppm) 193.7, 163.6, 150.7, 149.4, 145.9, 143.0, 136.1, 129.4, 129.2, 124.9, 122.5, 121.7, 120.2, 118.7, 118.6, 111.3, 109.5, 56.0, 55.9.

2.2.1.5.2.3 (2*E*,4*E*)-5-(3,4-Dimethoxyphenyl)-1-(2-hydroxy-6-methoxyphenyl)penta-2,4-dien-1-one (3m). Yield: 48%; mp 136-139 °C from AcOEt/petroleum ether. IR (KBr) 1621 cm^{-1}. ^1H MNR ($CDCl_3$): δ (ppm) 13.28 (s, 1H, OH), 7.66 (dd, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 14.8 Hz, $J_{\beta\text{-}\gamma}$ = 10.4 Hz), 7.40 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 14.8 Hz), 7.34 (t, 1H, H4, $J_{3\text{-}4}$ = $J_{4\text{-}5}$ = 8.3 Hz), 7.08 (dd, 1H, H6', $J_{5'\text{-}6'}$ = 8.3 Hz, $J_{2'\text{-}6'}$ = 1.9 Hz), 7.05 (d, 1H, H2', $J_{2'\text{-}6'}$ = 1.9 Hz), 6.97 (d, 1H, Hδ, $J_{\gamma\text{-}\delta}$ = 15.3 Hz), 6.89 (dd, 1H, Hγ, $J_{\beta\text{-}\gamma}$ = 10.4 Hz, $J_{\gamma\text{-}\delta}$ = 15.3 Hz), 6.86 (d, 1H, H5', $J_{5'\text{-}6'}$ = 8.3

Hz), 6.60 (d, 1H, H5, J_{4-5} = 8.3 Hz), 6.41 (d, 1H, H3, J_{3-4} = 8.3 Hz), 3.95 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$): δ (ppm) 194.2, 164.9, 160.9, 150.4, 149.3, 144.2, 141.8, 135.6, 129.9, 129.5, 125.6, 121.5, 112.0, 111.3, 111.0, 109.5, 101.5, 55.99, 55.96, 55.85.

2.2.1.5.2.4. (2*E*,4*E*)-5-(4-Hydroxy-3-methoxyphenyl)-1-(2-hydroxyphenyl)penta-2,4-dien-1-one (3n). Yield: 80%; mp 124-126 °C from AcOEt/petroleum ether. IR (KBr) 3455, 1630 cm^{-1}. ^1H NMR ($CDCl_3$): δ (ppm) 12.96 (s, 1H, 2OH), 7.84 (dd, 1H, H6, J_{4-6} = 1.5 Hz, J_{5-6} = 8.1 Hz), 7.71 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 14.6 Hz, $J_{\beta-\gamma}$ = 10.7 Hz), 7.47 (ddd, 1H, H4, J_{4-5} = 7.3 Hz, J_{3-4} = 8.5 Hz, J_{4-6} = 1.5 Hz), 7.18 (d, 1H, Hα, $J_{\alpha-\beta}$ = 14.6 Hz), 7.07 (dd, 1H, H6', $J_{5'-6'}$ = 8.2 Hz, $J_{2'-6'}$ = 1.8 Hz), 7.03-6.87 (m, 6H, Hγ, Hδ, H3, H5, H2', H5'), 5.84 (s, 1H, 4'OH), 3.95 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$): δ (ppm) 193.7, 163.6, 147.5, 146.9, 146.0, 143.2, 136.1, 129.4, 128.7,124.6, 122.2, 122.1, 120.1, 118.7, 113.6, 114.9, 109.1, 56.0.

2.2.1.5.2.5. (2*E*,4*E*)-5-(4-Hydroxy-3-methoxyphenyl)-1-(2-hydroxy-6-methoxyphenyl)penta-2,4-dien-1-one (3o). Yield: 58%; mp 113-115 °C from AcOEt/ petroleum ether. IR (KBr) 3443, 1620 cm^{-1}. ^1H MNR ($CDCl_3$): δ (ppm) 13.29 (s, 1H, 2OH), 7.65 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 14.8 Hz, $J_{\beta-\gamma}$ = 10.3 Hz), 7.39 (d, 1H, Hα, $J_{\alpha-\beta}$ = 14.8 Hz), 7.33 (t, 1H, H4, J_{4-5} = J_{3-4} = 8.3 Hz), 7.06 (dd, 1H, H6', $J_{5'-6'}$ = 8.2 Hz, $J_{2'-6'}$ = 1.9 Hz), 7.00 (d, 1H, H2', $J_{2'-6'}$ = 1.9 Hz), 6.95-6.83 (m, 3H, Hγ, Hδ, H5'), 6.60 (dd, 1H, H5, J_{4-5} = 8.3 Hz, J_{3-5} = 0.8 Hz), 6.40 (dd, 1H, H3, J_{3-4} = 8.3 Hz, J_{3-5} = 0.8 Hz), 5.83 (s, 1H, 4'OH), 3.95 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$): δ (ppm) 194.2, 164.9, 160.9, 147.1, 146.8, 144.3, 142.0, 135.6, 129.7, 129.0, 125.3, 121.8, 114.8, 112.0, 110.9, 109.1, 101.5, 56.0, 55.8.

2.2.1.5.2.6. (2*E*,4*E*)-1-(2,6-Dihydroxyphenyl)-5-(3,4-dimethoxyphenyl)penta-2,4-dien-1-one (3p). Yield: 38%; mp 185-189 °C from EtOH. IR (KBr) 3405, 1625 cm^{-1}. ^1H MNR (CD_3COCD_3): δ (ppm) 11.58 (s, 2H, OH), 7.75-7.64 (m, 2H, Hα, Hβ), 7.31 (d, 1H, H2', $J_{2'-6'}$ = 2.0 Hz), 7.26 (t, 1H, H4, J_{3-4} = 8.2 Hz), 7.17-7.10 (m, 3H, Hγ, Hδ, H6'), 6.97 (d, 1H, H5', $J_{5'-6'}$ = 8.3 Hz), 6.43 (d, 2H, H3, H5, J_{3-4} = 8.2 Hz), 3.87 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3). ^{13}C NMR (DMSO D_6): δ (ppm) 194.0, 160.6, 150.2, 149.0, 144.7, 142.2, 134.8, 129.5, 129.0, 125.4, 121.8, 111.7, 111.6, 109.8, 107.1, 55.56, 55.57.

2.2.1.5.2.7. (2*E*,4*E*)-1-(2,6-Dihydroxyphenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-2,4-dien-1-one (3q). Yield: 45%; mp 176-179 °C from AcOEt/petroleum ether. IR (KBr) 3374, 1628 cm^{-1}. ^1H MNR (CD_3COCD_3): δ (ppm) 11.5 (s, 2H, OH), 8.05 (s, 1H, OH), 7.73-7.64 (m, 2H, Hα, Hβ), 7.31 (d, 1H, H2', $J_{2'\text{-}6'}$ = 1.9 Hz), 7.26 (t, 1H, H4, $J_{3\text{-}4}$ = 8.2 Hz), 7.12-7.09 (m, 3H, Hγ, Hδ, H6'), 6.85 (d, 1H, H5', $J_{5'\text{-}6'}$ = 8.2 Hz), 6.43 (d, 2H, H3, H5, $J_{3\text{-}4}$ = 8.2 Hz), 3.91 (s, 3H, OCH_3). ^{13}C NMR (DMSO D_6): δ (ppm) 194.0, 160.6, 148.5, 148.0, 145.0, 142.8, 134.8, 128.9, 127.8, 124.5, 122.1, 115.6, 111.6, 110.5, 107.1, 55.7.

2.2.1.5.3. Synthesis of (2*E*,4*E*)-5-(4-chlorophenyl)-1-(2-hydroxy-4-methoxyphenyl)penta-2,4-dien-1-one (3g). Barium hydroxide octahydrate (15 mmol) was added to a mixture of 2-hydroxy-4-methoxyacetophenone (**1e**) (10 mmol) and 4-chlorocinnamaldehyde (**2b**) (10 mmol) in EtOH (200 mL). The mixture was stirred at 50 °C for 5 h and at room temperature overnight. After removal of ethanol in vacuum, water was added to the residue and the pH was adjusted to 2 with 2N HCl. The mixture was extracted with AcOEt. The combined organic phases were washed with saturated $NaHCO_3$ solution and brine, than dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by silica gel column chromatography eluting with AcOEt/petroleum ether 1:4. Yield: 40%; mp 182-183 °C from AcOEt. IR (KBr) 3454, 1621 cm^{-1}. ^1H MNR (DMSO D_6): δ (ppm) 13.46 (s, 1H, OH), 7.92 (d, 1H, H6, $J_{5\text{-}6}$ = 9.4 Hz), 7.65 (dd, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 14.8 Hz, $J_{\beta\text{-}\gamma}$ = 8.4 Hz), 7.43 (d, 2H, H2', H6', $J_{2'\text{-}3'}$ = 8.4 Hz), 7.34 (d, 2H, H3', H5', $J_{2'\text{-}3'}$ = 8.4 Hz), 7.14 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 14.8 Hz), 7.03-6.94 (m, 2H, Hγ, Hδ), 6.48-6.45 (m, 2H, H3, H5), 3.85 (s, 3H, OCH_3). ^{13}C MNR (DMSO D_6): δ (ppm) 191.7, 166.7, 166.2, 144.0, 140.5, 135.1, 134.6, 131.1, 129.1, 128.5, 127.4, 124.3, 114.1, 107.7, 101.1, 55.6.

2.2.1.2.3.1. Synthesis of (2*E*,4*E*)-5-(4-chlorophenyl)-1-(2,4-dihydroxyphenyl)penta-2,4-dien-1-one (3h). Barium hydroxide octahydrate (15 mmol) was added to a mixture of 2-hydroxy-4-[(tetrahydro-2H-pyran-2-yl) oxy]acetophenone (**1g**)[3] (10 mmol) and 4-chlorocinnamaldehyde (**2b**) (10 mmol) in EtOH (200 mL). The mixture was stirred at 50 °C for 5 h and at room temperature overnight. After removal of ethanol in vacuum, water was added to the residue, the pH was adjusted to 2 with 2N HCl, and the mixture was stirred at room temperature for 1 h. After this period, the solid

was removed by filtration, washed with water and purified by silica gel column chromatography eluting with AcOEt/ petroleum ether 1:4. Yield: 61%; mp 217-220 °C from AcOEt/ petroleum ether. IR (KBr) 3440, 3241, 1636 cm^{-1}. ^1H MNR (DMSO D_6): δ (ppm) 13.36 (s, 1H, OH), 10.80 (bs, 1H, OH), 7.92 (d, 1H, H6, $J_{5\text{-}6}$ = 8.9 Hz), 7.62 (d, 2H, H2', H6', $J_{2'\text{-}3'}$ = 8.4 Hz), 7.58 (dd, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 14.8 Hz, $J_{\beta\text{-}\gamma}$ = 9.6 Hz), 7.50-7.46 (m, 3H, Hα, H3', H5'), 7.27 (dd, 1H, Hγ, $J_{\gamma\text{-}\delta}$ = 15.5 Hz, $J_{\beta\text{-}\gamma}$ = 9.6 Hz), 7.22 (d, 1H, Hδ, $J_{\gamma\text{-}\delta}$ = 15.5 Hz), 6.42 (dd, 1H, H5, $J_{5\text{-}6}$ = 8.9 Hz, $J_{3\text{-}5}$ = 2.3 Hz), 6.30 (d, 1H, H3, $J_{3\text{-}5}$ = 2.3 Hz). ^{13}C MNR (DMSO D_6): δ (ppm) 191.1, 165.6, 165.1, 143.5, 140.1, 134.9, 133.6, 132.3, 128.9, 128.8, 127.9, 125.1, 112.9, 108.3, 102.6.

2.2.5.2. General procedure for the synthesis of 2-acetylphenyl (E)-cinnamates (5f-h).

Phosphorus oxychloride (30 mmol) were added to a solution of the appropriate 2-hydroxyacetophenone (**1a, 1d**) (10 mmol) and substituted cinnamic acid (**4f, 4g**) (12 mmol) in dry pyridine (20 mL) cooled in ice bath. The solution was stirred at room temperature for 4 h, than it was poured into ice and water and acidified with 2N HCl. The precipitate was removed by filtration, washed with water and purified by silica gel column chromatography eluting with AcOEt/light petroleum (1:3).

2.2.5.2.1. 2-Acetylphenyl(*E*)-3,4-dimethoxycinnamate (5f). Yield: 73%; mp 102-104 °C from EtOH. The compound exhibited spectroscopic data identical to those previously reported[14].

2.2.5.2.2. 2-Acetyl-3-methoxyphenyl(*E*)-3,4-dimethoxycinnamate (5g). Yield: 90%; mp 96-98 °C from EtOH. The compound exhibited spectroscopic data identical to those previously reported[14].

2.2.5.2.3. 2-Acetyl-3-methoxyphenyl(*E*)-4-acetoxy-3-methoxycinnamate (5h). Yield: 48%; mp 143-146 °C from AcOEt/ petroleum ether. IR (KBr) 1767, 1715, 1633 cm^{-1}. ^1H MNR ($CDCl_3$): δ (ppm) 7.78 (d, 1H, Hβ, $J_{\alpha\text{-}\beta}$ = 15.9 Hz), 7.38 (t, 1H, H5, $J_{4\text{-}5}$ = $J_{5\text{-}6}$ = 8.3 Hz), 7.18-7.14 (m, 2H, H6', H2'), 7.07 (d, 1H, H5', $J_{5'\text{-}6'}$ = 8.4 Hz), 6.85 (d, 1H, H4, $J_{4\text{-}5}$ = 8.3 Hz), 6.81 (d, 1H, H6, $J_{3\text{-}4}$ = 8.3 Hz), 6.52 (d, 1H, Hα, $J_{\alpha\text{-}\beta}$ = 15.9 Hz), 3.89 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 2.51 (s, 3H, $COCH_3$), 2.32 (s, 3H, $OCOCH_3$).

2.2.5.2.4. General procedure for the synthesis of (2*Z*,4*E*)-3-hydroxy-1-(2-hydroxyphenyl)-5 phenylpenta-2,4-dien-1-ones (6f, 6g)

Anhydrous K_2CO_3 (50 mmol) was added to a solution of the appropriate 2-acetylphenyl (*E*)-cinnamate (**5f**, **5g**) (10 mmol) in dry acetone (100 mL), and the mixture was refluxed for 24 h with stirring. After cooling, the mixture was poured into ice and the pH was adjusted to 4 with 2N HCl. The solid was removed by filtration, dissolved in chloroform and washed with brine. The organic phase was dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by crystallization.

2.2.5.2.5. (2*Z*,4*E*)-5-(3,4-Dimethoxyphenyl)-3-hydroxy-1-(2-hydroxyphenyl)penta-2,4-dien-1-one (6f) Yield: 40%; mp 126-128 °C from EtOH. The compound exhibited spectroscopic data identical to those previously reported.[14]

2.2.5.2.6. (2*Z*,4*E*)-5-(3,4-Dimethoxyphenyl)-3-hydroxy-1-(2-hydroxy-6-methoxyphenyl)penta-2,4-dien-1-one (6g). Yield: 40%; mp 131-132 °C from AcOEt. The compound exhibited spectroscopic data identical to those previously reported.[14]

2.2.5.2.7. (2*Z*,4*E*)-3-Hydroxy-5-(4-hydroxy-3-methoxyphenyl)-1-(2-hydroxy-6-methoxyphenyl) penta -2,4-dien-1-one (6h). Anhydrous K_2CO_3 (50 mmol) was added to a solution of 2-acetyl-3-methoxyphenyl (*E*)-3-(4-acetoxy-3-methoxyphenyl) cinnamate (**5h**) (10 mmol) in dry acetone (100 mL), and the mixture was refluxed for 24 h with stirring. After cooling, the mixture was poured into ice (50 g) and water (50 mL), and stirred at room temperature for 1 h. After that period, the pH was adjusted to 4 with 2N HCl, the solid was removed by filtration and purified by silica gel column chromatography eluting with CH_2Cl_2. Yield: 25%; mp 162-164 °C from AcOEt/ petroleum ether. IR (KBr) 3406 1626 cm^{-1}. ^{1}H MNR ($CDCl_3$): δ (ppm) ($CDCl_3$): 14.85 (s, 1H, OH), 12.77 (s, 1H, OH), 7.58 (d, 1H, Hδ, $J_{\gamma\text{-}\delta}$ = 15.8 Hz), 7.32 (t, 1H, H4, $J_{3\text{-}4} = J_{4\text{-}5}$ = 8.3 Hz), 7.15 (dd, 1H, H6', $J_{5'\text{-}6'}$ = 8.2 Hz, $J_{2'\text{-}6'}$ = 1.9 Hz), 7.06 (d, 1H, H2', $J_{2'\text{-}6'}$ = 1.9 Hz), 6.95 (d, 1H, H5', $J_{5'\text{-}6'}$ = 8.2 Hz), 6.77 (s, 1H, Hα), 6.60 (d, 1H, H3, $J_{3\text{-}4}$ = 8.3 Hz), 6.45 (d, 1H, Hγ, $J_{\gamma\text{-}\delta}$ = 15.8 Hz), 6.43 (d, 1H, H5, $J_{4\text{-}5}$ = 8.3 Hz), 5.99 (s, 1H, OH), 3.97 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$): δ

(ppm) 194.7, 175.2, 164.0, 160.3, 147.8, 146.8, 139.6, 134.9, 127.9, 122.7, 120.4, 114.9, 111.1, 110.7, 109.7, 103.2, 101.7, 56.0, 55.9.

2.2.5.3. (2Z,4E)-5-(3,4-Dihydroxyphenyl)-3-hydroxy-1-(2-hydroxyphenyl)penta-2,4-dien-1-one (6m).

A solution 1M of boron tribromide in dichloromethane (100 mL) was added dropwise to a stirred solution of **6f** (10 mmol) in dry dichloromethane (200 mL) cooled in ice bath. The reaction mixture was stirred in ice bath for 1h and then at room temperature for 20 h. After this period, the mixture was poured into ice and water and vigorously stirred. The obtained precipitate was filtered, washed with water, and purified by silica gel column chromatography eluting with AcOEt/petroleum ether 1:1. Yield: 40%; mp 200-201 °C from AcOEt. IR (KBr) 3385, 1635 cm^{-1}. ^{1}H MNR (acetone D6): δ (ppm) 14.73 (s, 1H, OH), 12.21 (s, 1H, OH), 8.45 (bs, 2H, 2OH), 7.92 (dd, 1H, H6, $J_{5\text{-}6}$ = 8.3 Hz, $J_{4\text{-}6}$ = 1.5 Hz), 7.60 (d, 1H, Hδ, $J_{\gamma\text{-}\delta}$ = 15.8 Hz), 7.51 (ddd, 1H, H4, $J_{3\text{-}4}$ = 8.4 Hz, $J_{4\text{-}5}$ = 7.2 Hz, $J_{4\text{-}6}$ = 1.5 Hz), 7.21 (d, 1H, H2', $J_{2'\text{-}6'}$ = 2.0 Hz), 7.09 (dd, 1H, H6', $J_{5'\text{-}6'}$ = 8.2 Hz, $J_{2'\text{-}6'}$ = 2.0 Hz), 6.97-6.94 (m, 2H, H3, H5), 6.90 (d, 1H, H5', $J_{5'\text{-}6'}$ = 8.2 Hz), 6.69 (d, 1H, Hγ, $J_{\gamma\text{-}\delta}$ = 15.8 Hz), 6.68 (s, 1H, Hα). ^{13}C MNR (DMSO D_6): δ (ppm) 191.4, 177.7, 159.9, 148.5, 145.7, 140.5, 134.9, 129.2, 126.3, 121.6, 120.1, 119.2, 117.7, 115.8, 114.7, 114.4, 98.5.

2.2.5.4. Biochemistry.

2.2.5.4.1. Determination of hMAO Isoform Activity.

The effects of the tested compounds on hMAO isoform enzymatic activity were evaluated by measuring their effects on the production of hydrogen peroxide from *p*-tyramine using the Amplex Red MAO assay kit (Molecular Probes, Inc., Eugene, Oregon, USA) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B (Sigma-Aldrich Química S.A., Alcobendas, Spain). Briefly, various concentrations of the test drugs or reference inhibitors and adequate amounts of recombinant hMAO-A or hMAO-B (required to obtain the same reaction velocity) were incubated for 15 min at 37 ºC in a sodium phosphate buffer (0.05 M, pH 7.4). The reaction was started by adding (final concentrations) 200 μM Amplex

Red reagent, 1 U/mL horseradish peroxidase and 1 mM *p*-tyramine which is totally sufficient to saturate both MAO isoforms in these experimental conditions. The hMAO activity was evaluated by measuring the fluorescence generated by resorufin using the general procedure described previously by us.[5] The possible capacity of the above test drugs to modify the fluorescence due to non-enzymatic inhibition (e.g., for directly reacting with Amplex Red reagent) was determined by adding these drugs to solutions containing only the Amplex Red reagent in a sodium phosphate buffer. For fluorescent compounds, incubations with 60 μM kynuramine (Sigma-Aldrich Química S.A., Alcobendas, Spain) were performed at 37 ºC and pH 7.4 in a sodium phosphate buffer (Na_2HPO_4/KH_2PO_4 isotonized with KCl). The rate of oxidation of the non selective substrate into 4-hydroxyquinolone was monitored at 314 nm using a Pharmacia Biotech Ultrospec 4000 UV/Visible Spectrophotometer.[15] IC_{50} values were estimated by non-linear regression analysis using GraphPad Prism software (San Diego, USA), with X = log molar concentration of tested compound and Y = percentage of inhibition of control resorufin production. This regression was performed using data obtained with 4–6 different concentrations of each tested compound. IC_{50} values are the mean ± S.E.M. from five experiments.

2.2.5.4.2. Reversibility and Irreversibility Experiments.

To evaluate whether some of the tested compounds (**3b, 3g, 3h, 6d** and **6k**) are reversible or irreversible hMAO-B inhibitors, an effective centrifugation-ultrafiltration method (so-called repeated washing) was used.[3]

2.2.5.4.3. Molecular Modeling.

Theoretical 3D models of the most active and hMAO-B selective compounds, **3g** and **3h** were built by means of Maestro[16] GUI version 9.1. The inhibitor structures were optimized by means of energy minimization carried out with the OPLS-2005 force field[17] and the GB/SA[18] water implicit solvation model. All these preliminary calculation were computed by the version 9.8 of the MacroModel[19] software. Docking simulation were performed using the ligand flexible algorithm of Glide[20] at XP precision level. The target models of hMAO-A and hMAO-B were two high resolution crystal structures corresponding to the Protein Data Bank codes 2Z5X[21] and 2V5Z[22]

respectively. Co-crystallized ligands, harmine for 2Z5X and safinamide for 2V5Z, and water molecules were removed from both target models and their binding clefts were defined by means of a regular box of about 1,000 $Å^3$, centred onto the N5 FAD cofactor. Molecular dynamics (MD) simulations were carried out, up to 12 ns at 300 °K, applying the version 2.4 of the DESMOND[23] package and the OPLS-AA force field onto the most stable complex of both inhibitors in hMAO-A and -B. The explicit solvation model TIP3 was adopted for taking into account water solvent effects. The four MD trajectories, each consisting of 240 structures sampled at regular interval equal to 5 ps, were graphical inspected and analysed using VMD version 1.8.7.[24] The molecular orbital (MO) analysis of compounds **3g** and **3h** was carried out at DFT level of theory using the hybrid functional B3LYP and the 6-31G** basis set. For mimicking the enzyme environment, the implicit solvation model PBF chloroform was adopted. The structure of both inhibitors, selected for the MO study, were obtained from the last molecular dynamics sampled frame and energy minimized during the DFT calculation. Quantum mechanics calculations were performed by means of the Jaguar.[25] Molecular modelling figures were depicted by PyMol version 1.3.[26]

Acknowledgment

This work was supported by the Italian MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca). We thank dr Ivano Pindinello for technical assistance.

References

1. Chimenti F., Fioravanti R., Bolasco A., Chimenti P., Secci D., Rossi F., Yanez M., Orallo, F., Ortuso F., Alcaro S., s, *Bioorg. Med. Chem*., **2010**, *18*, 1273.
2. Desideri N., Bolasco A., Fioravanti R., Proietti Monaco L., Orallo F., Yañez M., Ortuso F., Alcaro S., *J. Med. Chem*., **2011**, *54*, 2155.
3. Chimenti F., Fioravanti R., Bolasco A., Chimenti P., Secci D., Rossi F., Yañez M., Orallo F., Ortuso F., Alcaro S., *J. Med. Chem*, **2009**, *52* 2818.
4. Binda C., Li M., Hubalek F., Restelli N., Edmondson D.E., Mattevi A., *Proc. Natl. Acad. Sci. USA*, **2003**, *100*, 9750.
5. Yáñez M., Fraiz N., Cano E., Orallo F., *Biochem. Biophys. Res. Commun*., **2006**, *344*, 688.
6. Chimenti F., Maccioni, E. Secci D, Bolasco A., Chimenti P., Granese A., Carradori S., Alcaro S., Ortuso F.,Yáñez M., Orallo F., Cirilli R., Ferretti R., La Torre F., *J. Med. Chem*., **2008**, *51*, 4874.

7. Edmondson D.E., Binda C., Wang J., Upadhyay A.K., Mattevi, A., *Biochemistry*., **2009**, *20*, 4220.
8. Deodhar M., StC. Black D., Kumar, N., *Tetrahedron*., **2007**, *63*, 5227.
9. Vedernikov Al., Gromov S.P., *Synthesis*, **2001**, 889.
10. Jr. Condo A.M., Baker D.C., Moreau R.A., Hicks K.B., *J. Agric. Food Chem*.,**2001**, *49*, 4961 .
11. Desideri N., Mastromarino,P.,. Conti C., *Antiviral Chem. Chemother*, **2003**, *14*, 195.
12. Conti C., Mastromarino P., Goldoni P., Portalone G., Desideri, N., *Antiviral Chem. Chemother*., **2005**, *16* , 267.
13. Desideri N., Conti C., Mastromarino P., Mastropaolo F., *Antiviral Chem. Chemother.*, **2000**, *11*, 321.
14. Santos C.M.M., Silva A.M.S., Cavaliero J.A.S., *Eur. J. Org. Chem*., **2009**, 2642.
15. Thull U., Kneubühler S., Testa B., Borges M.F., Pinto M.M., *Pharm. Res*., **1993**, *10*, 1187.
16. Maestro, version 9.1, Schrodinger, LLC, New York, NY, **2010.**
17. Kaminski G., Friesner R.A., Tirado-Rives J., Jorgensen W.L., Phys J., *Chem. B*, **2001**, *105*, 6474.
18. Hasel W., Hendrickson T.F., W.C., *Tetrahedron Comput. Methodol*,. **1988**, *1*, 103.
19. (a) MacroModel, version 9.8, Schrodinger, LLC, New York, NY, **2010**. (b) Mohamadi F., Richards N.G.J., Guida W.C., Liskamp R., Lipton M., Caufieldn C., Chang G., Hendrickson T., Still W.C., *J. Comput. Chem.* **1990**, *11*, 440.
20. (a) Glide, version 5.6, Schrodinger, LLC, New York, NY, **2010**. (b) Friesner R.A., Banks J.L., Murphy R.B., Halgren T.A., Klicic J.J., Mainz D.T., Repasky M.P., Knoll E.H., Shaw D.E., Shelley M., Perry J.K., Francis P., Shenkin, P.S.. *J. Med. Chem*., **2004**, *47* 1739. (c) Halgren T.A., Murphy R.B., Friesner R.A., Beard H.S., Frye L.L., Pollard W.T., Banks J.L., *J. Med. Chem*., **2004**, *47*, 1750. (d) Friesner R.A., Murphy R.B., Repasky M.P., Frye L.L., Greenwood J.R., Halgren T.A., Sanschagrin P.C., Mainz D.T., *J. Med. Chem*., **2006**, *49*, 6177.
21. Son S.Y., Ma J., Kondou Y., Yoshimura M., Yamashita E., Tsukihara T., *Proc. Natl. Acad. Sci. Usa*., **2008**, *105*, 5739.
22. Binda C., Wang J., Pisani L., Caccia C., Carotti A., Salvati P., Edmondson D.E., Mattevi A., *J. Med. Chem*., **2007**, *50*, 5848.
23. Bowers K.J., Chow E., Xu H., Dror R.O., Eastwood M.P., Gregersen B.A., Klepeis J.L., Kolossváry I., Moraes M.A., Sacerdoti F.D., Salmon J.K., Shan Y., Shaw D.E., *Proceedings of the ACM/IEEE Conference on Supercomputing (SC06), Tampa, Florida, November*, **2006**, 11–17.
24. Humphrey W., Dalke A., Schulten K., VMD -, *J. Molec. Graphics*., **1996**, *14*, 33.
25. Jaguar, version 5.6, Schrodinger, LLC, New York, NY, **2010**.
26. The PyMOL Molecular Graphics System, version 1.3, Schrödinger, LLC.

Supplementary content

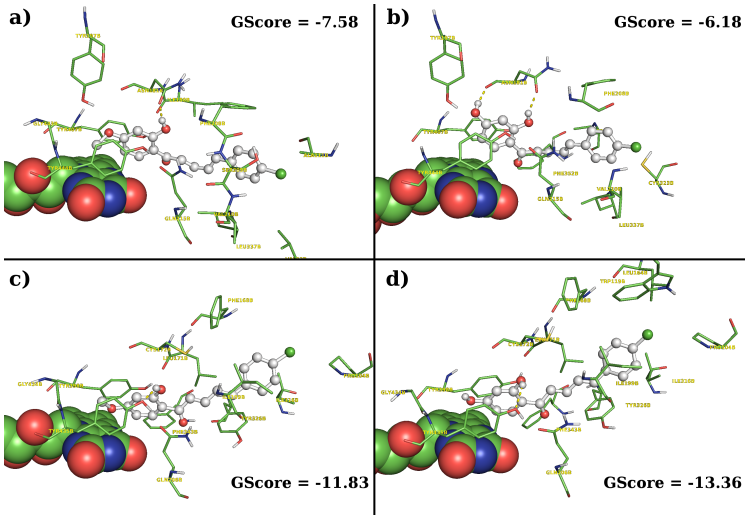


Figure S1. Top ranked docking pose of **3g** and **3h** into hMAO-A (a, b) and hMAO-B (c, d) respectively. Inhibitors are depicted in white carbons balls & sticks, interacting residues are in green carbons sticks, FAD cofactor in spacefill. Yellow dotted lines indicate intermolecular hydrogen bonds. GScore is the Glide XP score value.

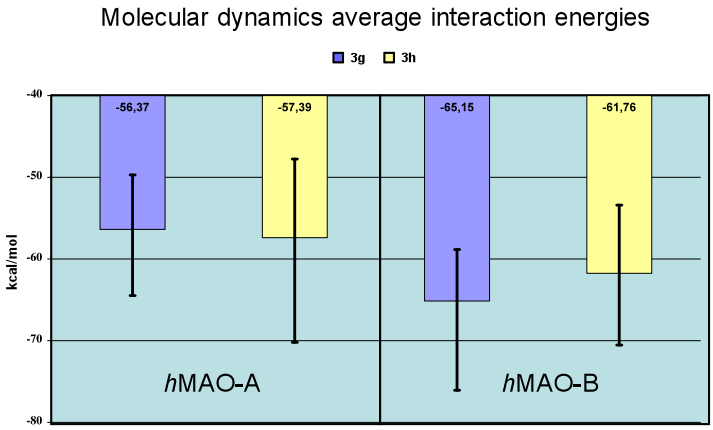


Figure S2. 3g and **3h** molecular dynamics average interaction energies and the corresponding deviations with respect to hMAO-A and -B.

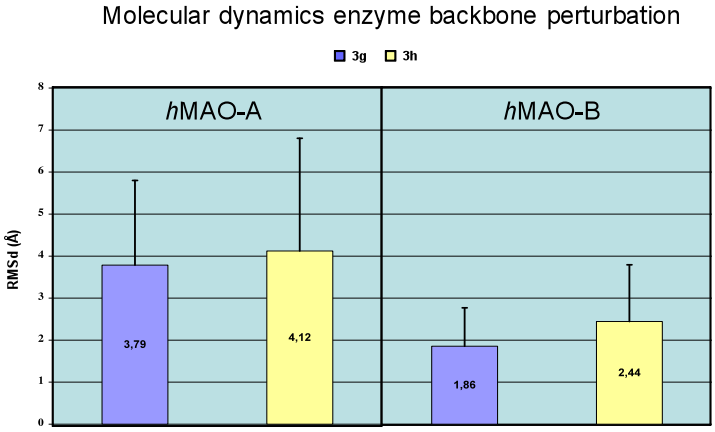


Figure S3. Molecular dynamics **3g** and **3h** induced perturbation of the target backbone conformation. The measurement was computed as average RMSd with respect to the starting structures.

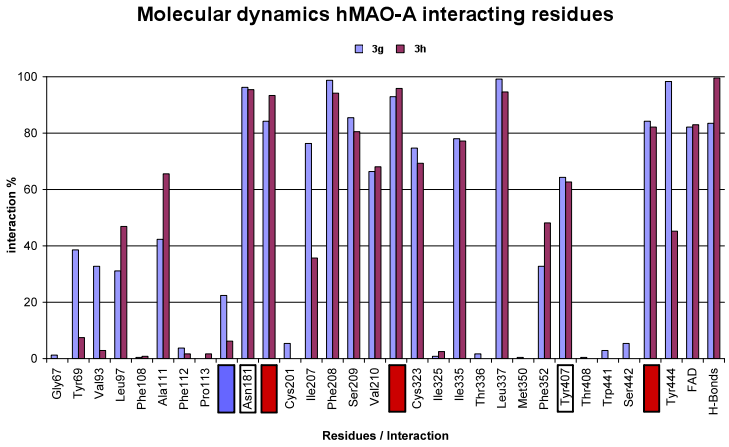


Figure S4. Contribution (%) of each hMAO-A **3g** and **3h** interacting residue. Ligand hydrogen bonding aminoacids are violet, brown and white highlighted to indicate their involvement in **3g**/**3h**/both inhibitors recognition respectively.

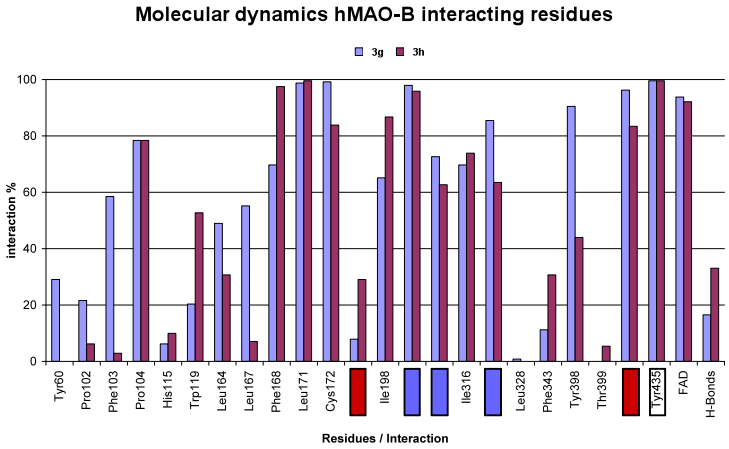


Figure S5. Contribution (%) of each hMAO-B **3g** and **3h** interacting residue. Ligand hydrogen bonding aminoacids are violet, brown and white highlighted to indicate their involvement in **3g**/**3h**/both inhibitors recognition respectively.

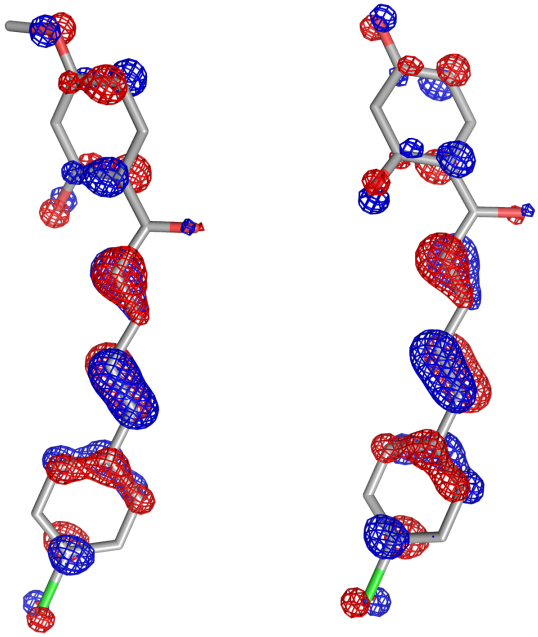


Figure S6. Mesh representation of compounds **3g** (left) and **3h** (right) highest occupied (blue) and lowest unoccupied (red) molecular orbitals.

Table 1. Inhibitor target interaction energy gap between the top ranked docking poses and their corresponding, manually built, head-tail configuration. Values are reported in kcal/mol.

	hMAO-A	**hMAO-B**
3g	8.0	3.4
3h	22.8	10.2

Table 2. Elemental analyses of derivatives **3a-r** and **6a-l.**

Comp	**C**		**H**		**F**		**Cl**		**N**	
	Calc	Found	Calc	Found	Calc	Found	Calc	Found		
3a	81.58	81.55	5.64	5.60						
3b	71.71	71.67	4.60	4.56			12.45	12.47		
3c	68.68	68.65	4.80	4.77			11.26	11.28		
3d	61.92	61.89	3.67	3.64			10.75	10.77	4.25	4.25
3e	63.93	64.01	3.79	3.76			22.21	22.25		
3f	71.71	71.67	4.60	4.56			12.45	12.47		
3g	68.68	68.65	4.80	4.77			11.26	11.28		
3h	67.89	67.86	4.36	4.32			11.79	11.81		
3i	76.11	76.08	4.88	4.85	7.08	7.09				
3j	72.47	72.45	5.07	5.03	6.37	6.37				
3k	77.13	77.10	5.75	5.71						
3l	73.53	73.51	5.85	5.81						
3m	70.57	70.55	5.92	5.88						
3n	72.96	72.93	5.44	5.40						
3o	69.93	69.90	5.56	5.52						
3p	69.93	69.90	5.56	5.52						
3q	69.22	69.19	5.16	5.13						
3r	69.15	69.12	4.44	4.40					4.74	4.74
6a	76.68	76.65	5.30	5.26						
6b	60.92	60.87	3.61	3.58			21.15	21.19		
6c	67.89	67.85	4.36	4.32			11.79	11.81		
6d	71.82	71.80	4.61	4.57	6.69	6.69				
6e	72.96	72.93	5.44	5.40						
6f	69.93	69.90	5.56	5.52						
6g	67.41	67.34	5.66	5.61						
6h	66.66	66.63	5.30	5.26						
6i	65.59	65.56	4.21	4.20					4.50	4.50
6j	77.12	77.09	5.75	5.71						
6k	72.33	72.30	5.00	4.96						
6l	68.42	68.43	4.73	4.69						