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Original article

Semisynthesis, *ex vivo* evaluation, and SAR studies of coumarin derivatives as potential antiasthmatic drugs

Amanda Sánchez-Recillas [a,1], Gabriel Navarrete-Vázquez [b,*,2], Sergio Hidalgo-Figueroa [b], María Yolanda Rios [c], Maximiliano Ibarra-Barajas [d], Samuel Estrada-Soto [a,*,2]

[a] *Laboratorio de Farmacognosia y Química de Productos Naturales, Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Col. Chamilpa, 62209 Cuernavaca, Morelos, Mexico*
[b] *Laboratorio de Química Farmacéutica, Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Col. Chamilpa, 62209 Cuernavaca, Morelos, Mexico*
[c] *Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Col. Chamilpa, 62209 Cuernavaca, Morelos, Mexico*
[d] *Unidad de Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, 54090 Tlalnepantla, Estado de México, Mexico*

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ABSTRACT

Asthma is a chronic inflammatory disorder that causes contraction in the smooth muscle of the airway and blocking of airflow. Reversal the contractile process is a strategy for the search of new drugs that could be used for the treatment of asthma. This work reports the semisynthesis, *ex vivo* relaxing evaluation and SAR studies of a series of 18 coumarins. The results pointed that the ether derivatives **1**–**3**, **7**–**9** and **13**–**15** showed the best activity (E_{max} = 100%), where compound **2** (42 μM) was the most potent, being 4-times more active than theophylline (positive control). The ether homologation (methyl, ethyl and propyl) in position 7 or positions 6 and 7 of coumarins lead to relaxing effect, meanwhile formation of esters generated less active compounds than ethers. The SAR analysis showed that it is necessary the presence of two small ether groups and the methyl group at position 4 (site 3) encourage biological activity through soft hydrophobic changes in the molecule, without drastically affecting the cLogP.

1. Introduction

Asthma is a chronic inflammatory disorder of the airways. The chronic inflammation of airway is characterized by the hyperreactivity causing limited airflow (by bronchoconstriction, mucus plug and increased inflammation) when airways are exposed to allergens such as those from house dust mites, animals with fur, cockroaches, pollens and molds, occupational irritants, tobacco smoke, respiratory infections, exercise, chemical irritants and drugs [1]. These affect 300 million people worldwide, especially to child population [2]. In the first place, drug therapy is centered on counteracting bronchoconstriction and/or inflammation processes using β2-agonist and inhaled glucocorticoids, respectively. Besides, there are some other therapeutic alternatives as leukotriene receptor antagonist, phosphodiesterases inhibitors, anticholinergics and anti-IgE, which are indicated for the successful treatment of asthma. However, they are not completely effective in chronic treatment [1]. Plants, especially those with ethnopharmacological uses, have been the primary source for early drug discovery.

Abbreviations: $(CH_3CO)_2O$, acetic anhydride; μL, microliters; μM, micromolar; $C_4H_7BrO_2$, ethyl bromoacetate; $CaCl_2$, calcium chloride; cAMP, cyclic adenosine monophosphate; $CDCl_3$, Deuterated chloroform; cGMP, cyclic guanosine monophosphate; CH_3CN, acetonitrile; CO_2, carbon dioxide; CRC, concentration–response curve; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EC_{50}, media effective concentration; EDTA, ethylenediaminetetraacetic acid; EI–MS [M^+], electron impact–mass spectrometry; E_{max}, maximum effect; Et_3N, triethylamine; g, gram; H, hour; H_2SO_4, sulfuric acid; Hz, hertz; *J*, coupling constants; K_2CO_3, potassium carbonate; KCl, potassium chloride; KH_2PO_4, potassium phosphate monobasic; KI, potassium iodide; KOH, potassium hydroxide; Me_2SO_4, dimethyl sulfate; $MgSO_4$, magnesium sulfate; cLogP, partition coefficient calculated; mL, milliliters; mm, millimeters; Mp, melting point; NaCl, sodium chloride; $NaHCO_3$, Sodium bicarbonate; NMR, nuclear magnetic resonance; O_2, oxygen; Oct/wat, octanol–water mixture; ppm, parts per million; SM1, starting material 1; SM2, starting material 2; SM3, starting material 3; SAR, structure–activity relationship; SN_2, bimolecular nucleophilic substitution; TLC, thin layer chromatography; TMS, tetramethylsilane; UV, ultra violet.

* Corresponding authors. Facultad de Farmacia, Avenida Universidad 1001, Col. Chamilpa, 62209 Cuernavaca, Morelos, Mexico.
E-mail addresses: gabriel_navarrete@uaem.mx (G. Navarrete-Vázquez), enoch@uaem.mx (S. Estrada-Soto).

[1] Taken from the. Ph. D. thesis of Amanda Sánchez-Recillas.
[2] These authors contribute equally in current work.

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Furthermore, many bioactive compounds have been isolated from these or have served as a prototype to develop new molecules with therapeutic interest [3], e.g. coumarins that are phenolic compounds with a common structure of 2-*H*-1-benzopyran-2-one with multiple biological effects such as anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant, and neuroprotective properties [4–6]. Therefore, the search for novel molecules based on coumarin scaffold with therapeutic activity represents pharmacological alternatives to treat prevalent diseases such as asthma.

2. Results and discussion

2.1. Chemistry

In this work was carried out the semisynthesis of 18 coumarins (**1**–**18**), on the basis of 6,7-dihydroxycoumarin (compounds **1**–**6**), 7-hydroxycoumarin (compounds **7**–**12**) and 4-methyl-7-hydroxycoumarin (compounds **13**–**18**) through alkylation and esterification of hydroxyl groups in position 6, or 6 and 7 of coumarin structure as previously described (**1**–**3**) (Table 1). Compounds were obtained in discrete to moderate yields, and were purified by recrystallization. The chemical structures of the synthesized compounds were determined based on their spectral data (^{1}H, ^{13}C NMR and mass spectra MS–EI [M$^+$]).

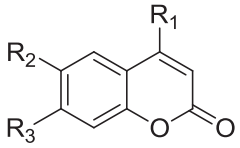
2.2. Rat tracheal ring assay

All the compounds were evaluated [0.1–500 μM] on the contraction induced by carbachol (1 μM, muscarinic cholinergic agonist) in the trachea rat rings; and theophylline (phosphodiesterases inhibitor) was used as positive control. Table 2 shows values of median effective concentration (EC_{50}), maximum effect (E_{max}) as well as calculated LogP for each compound (cLogP).

Esculetin (6,7–dihydroxycoumarin, **SM1**) is a *di*-hydroxylated coumarin, which has significant pharmacological effects such as anticoagulant, anti-inflammatory, vasoconstriction inhibitor, hepatoprotective, anti-obesity, hypolipidemic, and neuroprotective agent [7–12]. Compounds designed from **SM1** have great potential to show important pharmacological effects [4]. The most active compounds derived from **SM1** were *di*-ethers: (**1**) methoxy-, (**2**) ethoxy- and (**3**) propoxy-, which achieved 100% of relaxation and the CRC moved toward left with respect to **SM1** (E_{max} = 48%) and theophylline (E_{max} = 100%, EC_{50} = 144 μM) used as positive control (Fig. 1); this fact suggests that compounds were more potent than **SM1** and theophylline. Compounds **1** and **3** are equipotent (EC_{50} = 80 μM), and the compound **2** (EC_{50} = 42 μM) achieved the most potent tracheal relaxation of the entire series, which was 4-times more potent than theophylline. Compounds **4**, **5** and **6** (CCR not shown) didn't showed significant relaxant effect on the contraction induced by carbachol.

Umbelliferone (7-hydroxycoumarin, **SM2**) is a *mono*-hydroxylated coumarin that also showed significant pharmacological effects such as antidiabetic, hypolipidemic, anti-inflammatory, anti-allergic, anti-oxidant, and bronchodilator [13–16]. This compound was previously isolated from plant species that are used in traditional medicine as anti-asthmatic [17]. Therefore, compounds designed from umbelliferone have great potential to show important pharmacological effects, including as antiasthmatic [4]. The *mono* derivatives of **SM2** such as **7**, **8** and **9** reached 100% of relaxation, but were less potent than the *di*-ethers, however their CCR were moved to the left compared than **SM2** (Fig. 2). Compound **9** was the most potent (EC_{50} = 133 μM) in this series, it is considered to be equipotent with theophylline. The ester derivatives **10**, **11** and **12** didn't show significant relaxing effect (E_{max} = <30%) on the contraction induced by carbachol.

Table 1
Physicochemical data of the semisynthetic coumarin derivatives.



Compound number	R_1	R_2	R_3	Molecular weight (g/mol)	Melting point (°C)	Reaction time (h)	Unoptimized yield (%)	M.W. EI MS: (*m*/*z*) [M]$^+$
SM1	–H	–OH	–OH	178	271.0–273.0	–	–	178
1	–H	–OMe	–OMe	206	128.0–130.0	80	38	206
2	–H	–OEt	–OEt	234	105.5–106.5	48	54	234
3	–H	–OPr	–OPr	262	79.5–81.5	48	85	262
4	–H	–OAc	–OAc	262	127.6–130.4	48	78	178
5	–H	–OCOPh	–OCOPh	386	185.1–186.6	48	85	386
6	–H	–OCH_2COOEt	–OCH_2COOEt	350	107.0–108.8	6	91	350
SM2	–H	–H	–OH	162	230.0	–	–	162
7	–H	–H	–OMe	176	117.8–119.8	24	95	176
8	–H	–H	–OEt	190	86.7–88.7	60	46	190
9	–H	–H	–OPr	204	62.6–63.9	36	27	204
10	–H	–H	–OAc	204	133.5–135.0	0.5	90	204
11	–H	–H	–OCOPh	266	158.0–159.3	12	78	266
12	–H	–H	–OCH_2COOEt	248	112.2–114.2	6	96	248
SM3	–Me	–H	–OH	176	190.0–192.0	–	–	176
13	–Me	–H	–OMe	190	158.6–161.7	48	77	190
14	–Me	–H	–OEt	204	108.2–110.5	80	37	204
15	–Me	–H	–OPr	218	74.4–75.2	240	96	218
16	–Me	–H	–OAc	218	152.5–153.5	0.5	92	219
17	–Me	–H	–OCOPh	280	161.5–163.5	12	86	280
18	–Me	–H	–OCH_2COOEt	262	100.1–103.1	8	71	262

SM: Starting Materials, [M]$^+$ = molecular ion found.

Table 2
Ex vivo pharmacological parameters of the relaxant effect of coumarin derivates used in the SAR study.

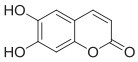
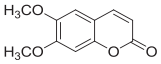
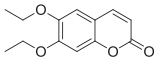
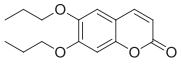
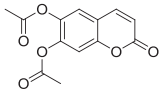
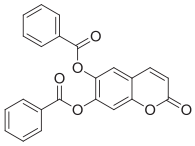
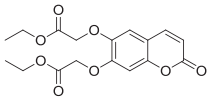
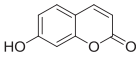
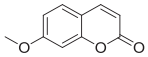
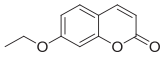
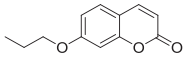
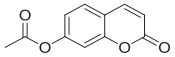
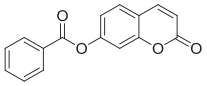
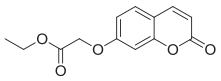
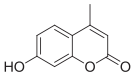
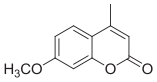
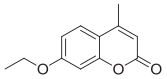
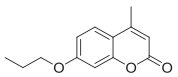
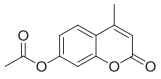
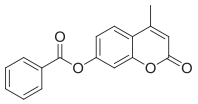
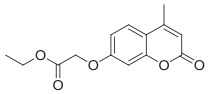
Compound number	Structure	E_{max} (%)	EC_{50} (μM)	miLogP
SM1		47.9	NS	1.021
1		100	80	1.636
2		**100**	**42**	2.388
3		100	89	3.394
4		77	231	0.627
5		12	NS	5.259
6		33	NS	2.113
SM2		67	449	1.511
7		100	244	2.046
8		100	245	2.422
9		**100**	**133**	2.925
10		16	NS	1.542
11		26	NS	3.858
12		31	NS	2.285
SM3		90	260	1.887
13		100	230	2.423
14		**100**	**80**	2.799
15		100	267	3.302
16		62	360	1.918

Table 2 (*continued*)

Compound number	Structure	E_{max} (%)	EC_{50} (μM)	miLogP
17		34	NS	4.234
18		70	200	2.661

Bold represents the most active compounds.
E_{max}: maximum effect; EC_{50}: median effective concentration; RM: Raw materials; NS. Not shown; given that $E_{max} < 40\%$.

4-Methylumbelliferone (**SM3**) is a coumarin that has demonstrated pharmacological effects as analgesic, anti-arthritis, anti-inflammatory, antipyretic, antibacterial, anti-viral, anti-cancer, among others [18]. However, it has not been demonstrated the possible relaxing effect of the tracheal smooth muscle. **SM3** showed significant relaxing effect ($E_{max} = 90\%$), and the ether derivatives **13**, **14** and **15** showed 100% of relaxation. Compound **14** ($EC_{50} = 80$ μM) was the most potent in this series. The CCR of **14** is shifted to the left compared with theophylline (Fig. 3). Once again, the ester derivatives **16**, **17** and **18** of **SM3** also did not showed significant relaxant effect.

These findings suggest that increasing in the lipophilic chain in position 6 and/or 7 of the coumarin skeleton increases the relaxing effect in rat tracheal rings, while reducing the lipophilic chain the effect reduces too. As described above, one of the strategies to treat asthma is to reduce the bronchoconstriction. The main target could be to generate relaxation of the smooth muscle cells of the airway by the increasing of cyclic nucleotide second messengers (cAMP and cGMP), decrease of intracellular calcium (calcium channel blockers), the inhibition of excitatory response (anti-cholinergic, antihistamines, anti-leukotriens) or induce relaxing direct response (β-agonists or phosphodiesterases inhibition) [19]. The coumarin ether derivatives may act by inhibition of cyclic nucleotide phosphodiesterases that generate cAMP and cGMP increasing, as established by Hoult and Payá [20] and Billington et al. [21]. Also, can increase the intracellular concentration of cAMP by activation of β_2-adrenergic receptors or prostaglandins-E_2 production [19], with subsequent decrease of the influx of calcium through the

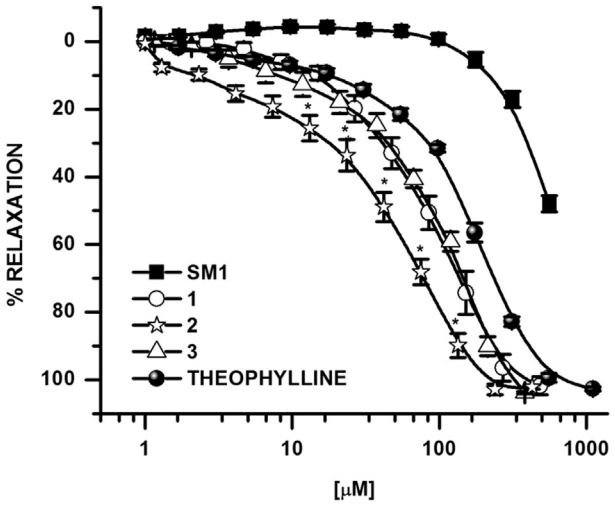


Fig. 1. Concentration–response curves of ether compounds **1**–**3** and **SM1** on trachea rings pre-contracted with carbachol [1 μM]. All results are expressed as the mean ± S.E.M of six experiments ($^*p < 0.05$).

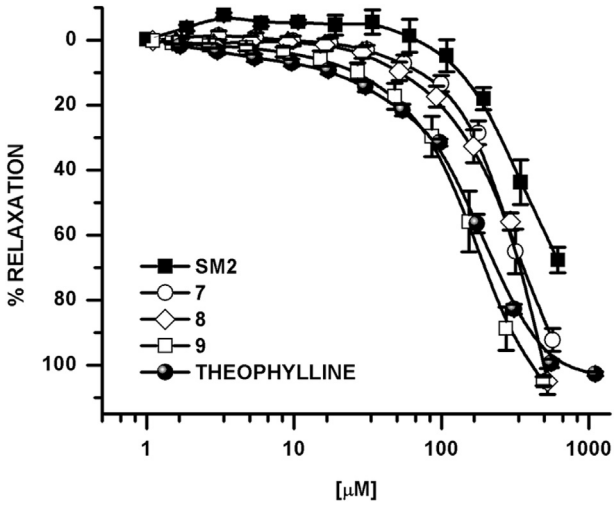


Fig. 2. Concentration–response curves of ether compounds **7**–**9** and **SM2** on trachea rings pre-contracted with carbachol [1 μM]. All results are expressed as the mean ± S.E.M of six experiments (*$p < 0.05$).

blockade of calcium channels of membrane [22], as well as blocking of calcium channels from intracellular, generating the decrease in intracellular calcium concentrations [23,24]. Meurs et al. [25] suggest that the importance of muscarinic receptors not only involves the blockage of the airway, but also have an important regulatory role in the function of β_2-adrenergic receptors agonists and inflammation; thus, the coumarin derivatives can act through a muscarinic antagonism [26]. It is worth mentioning that previous studies have shown that the compound **1** has significant vasorelaxant [27] and anti-allergic [28] effects. Finally, the underlying mechanism of relaxant action and the possible anti-inflammatory activity of the most active compounds **1**, **2**, **3**, **9** and **14** are current studying in order to develop new potential drugs for the treatment of asthma [24,29].

2.3. Structure–activity relationship (SAR) of coumarin derivatives

For SAR analysis it was used descriptors of LogP values (Table 2) calculated with different programs and after that were averaged

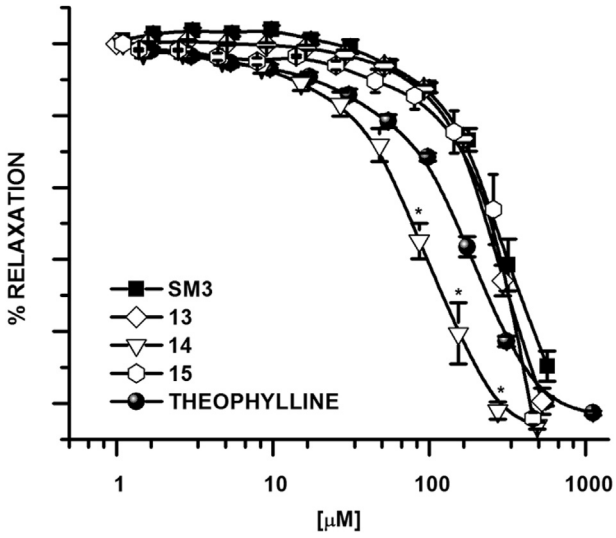


Fig. 3. Concentration–response curves of ether compounds **13**–**15** and **SM3** on trachea rings pre-contracted with carbachol [1 μM]. All results are expressed as the mean ± S.E.M of six experiments (*$p < 0.05$).

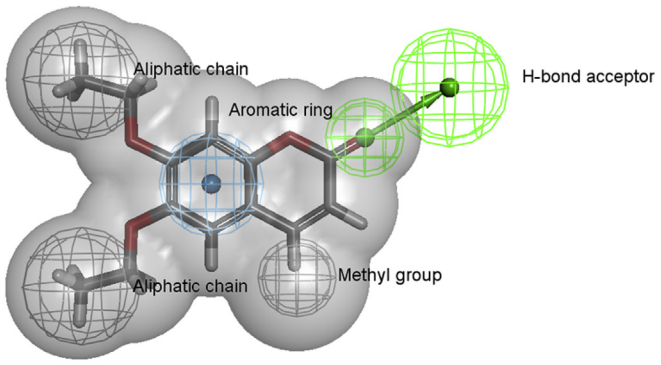


Fig. 4. Pharmacophoric pattern based on experimental results.

and grouped in the ALOGPS 2.1 software available online [30,31]. In addition, the experimental EC_{50} and E_{max} values from relaxant effect were considered. **SM1** and **SM2** bear hydroxyl groups (OH) in position 6 and 7 or 7, respectively, and it seems that these polar substituents did not contributes with the relaxing effect in the trachea, while the **SM3**, with a hydroxyl group in position C-7 and a small lipophilic group ($-CH_3$) in position C-4, increased the relaxatory effect. Homologated ether derivatives (with a maximum of three carbons) in the position C-6 and C-7 of the coumarins, drastically increase relaxant effect generating the most potent compounds as **1**, **2** and **3**. The ether formation only in position C-7 with and without a methyl group in C-4 also increases the relaxant effect; however, all of them are less potent than **1**, **2** and **3**, which indicates that is necessary to add liphophilic groups such as diether derivatives to improve the relaxant effect. Ester derivatives (acetyl and benzoyl) reduced relaxant effect. SAR analysis revealed the minimal stereo-electronic requirements for good relaxation activity: 1) Positions C-6 and C-7 substituted with homologous aliphatic ether chains (1–3 carbon atoms, preferable two) reflect possible steric and lipophilic molecular recognition from the cavities in the active site. Strong improvements of ligand affinity for many therapeutic targets are frequently accompanied by modest lipophilicity increases [32]. 2) Sometimes, a methyl group with its lipophilic surface well included into a protein hydrophobic pocket is expected to provide 1.5 kcal/mol binding energy and so give up to a 10-fold improvement in binding energy [33]. In this case, position C-4 of the coumarin replaced with a methyl group (compound **14**), improved the activity 3-fold versus compound **8**, but it was not as active as the most potent compound (**2**).

Based on the most active molecules treated in this study (compounds **2** and **14**), we examine the effect of different substituents in the molecular scaffold of coumarin derivatives. The construction of pharmacophoric model (Fig. 4) and a proposed new molecule designed (Fig. 5) was based on experimental results and is

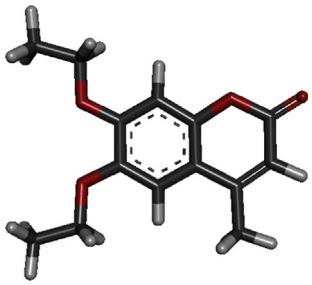


Fig. 5. New coumarin derivative molecule designed based on the data generated in this work.

describing the main chemical features shared by the most active coumarin derivatives. A chemical inspection of molecular structure suggest an incorporation of methyl group at position 4, as well as ethoxy substitutions at positions 6 and 7. The three dimensional display was built with the program DSV3.5. [34].

3. Conclusions

The skeleton of 2*H*-1-benzopyran-2-one (coumarin) represents a remarkable scaffold with interesting airway relaxant properties to develop new therapeutic alternatives that are useful in the treatment of asthma. Compounds **2**, **9** and **14**, from each series, respectively, showed the best activity, being compound **2** the most potent of all of three series. The SAR analysis shown that the presence of two small ether groups (6 and/or 7 position) and the methyl group at position 4, increases biological activity through soft changes in the hydrophobic characteristic of the molecule, since it doesn't drastically affects the LogP. Last should be between 2.0 and 2.7 and/or less than 3, because after that value the hydrophobic molecule would lose effectiveness and potency.

4. Experimental

4.1. Chemistry

All chemicals were ACS grade and all starting materials and reagents were obtained commercially from Sigma–Aldrich and were used as received. Melting points were determined on an EZ-Melt MPA120 automated melting point apparatus from Stanford Research Systems and are uncorrected. TLC monitored reactions on 1.5 × 3.0 cm pre-coated silica gel 60 F_{254} plates (E. Merck KGaA, Darmstadt, Germany) and visualized with 254-nm UV light. The reactions that lasted for more than 24 h were kept in refrigerator for overnight.

NMR studies were carried out with a Varian Inova 400 instrument. Chemical shifts (δ_H, δ_C) and coupling constants values (*J*) are given in ppm and Hz, respectively. The following abbreviations for signal multiplicities are represented by s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Standard reference was used: TMS ($\delta_H = 0$, $\delta_C = 0$) in $CDCl_3$. Mass spectra were recorded on a Jeol JMS-700 equipment (JEOL USA Inc., Peabody, MA, USA).

4.1.1. General method of semisynthesis of compounds *1*–*3*, *7*–*9* and *13*–*15*

Compounds were prepared using bimolecular nucleophilic substitution (S_N2) reaction, starting from 6,7-dihydroxycoumarin (**SM1**), 7-hydroxycoumarin (**SM2**) or 7-hydroxy-4-methylcoumarin (**SM3**) as starting materials (**SM**), polar aprotic solvents [acetone, acetonitrile (CH_3CN), dimethylformamide (DMF)] and potassium carbonate (K_2CO_3) or potassium hydroxide (KOH) as a base. All reactions were carried out with moderate heating (<40 °C). The crude products were washed with cold water, filtered, dried and purified according to the procedures reported [35].

4.1.2. Preparation of *1*, *7* and *13* (Scheme 1a)

0.4 g of **SM1** (2.2 mmol), **SM2** (2.4 mmol) and **SM3** (2.2 mmol), respectively, were dissolved with CH_3CN (2.0 mL). After that, solid KOH (1.5 or 3 equivalents) was added, and stirred during thirty minutes. 2.1 equivalents of dimethyl sulfate (Me_2SO_4) were added to the mixture and heating for 48 h at 40 °C. Finally, when reaction was completed (monitored by TLC analysis), the solid was filtered off and dried to afford the corresponding compounds **1** (6,7-dimethoxy-2*H*-chromen-2-one), **7** (7-methoxy-2*H*-chromen-2-one) and **13** (7-methoxy-4-methyl-2*H*-chromen-2-one), respectively.

*4.1.2.1. 6,7-Dimethoxy-2H-chromen-2-one (**1**).* Pale yellow powder, 60% yield, mp: 128.0–130.0 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 3.93 (3H, s, CH_3), 3.95 (3H, s, CH_3), 6.28 (1H, d, $J = 9.6$ Hz), 6.83 (1H, s), 6.87 (1H, s) 7.64 (1H, d, $J = 9.6$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ_C 56.2 CH_3, 100.1 (CH), 108.1 (CH), 111.5 (C), 113.5 (CH), 143.4 (CH), 146.4 (C), 150.1 (C), 152.9 (C), 161.4 (C).

*4.1.2.2. 7-Methoxy-2H-chromen-2-one (**7**).* White crystals, 95% yield, mp: 117.8–119.8 °C, recrystallized from 100% methanol. ^{1}H NMR ($CDCl_3$) δ: 3.88 (3H, s), 6.25 (1H, d, $J = 9.6$ Hz), 6.86 (1H, d, $J = 1.6$ Hz), 7.63 (2H, d, $J = 9.6$ Hz), 7.37 (1H, dd, $J = 9.6$ Hz, 1.6 Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 55.9 (CH_3), 101.0 (CH), 112.7 (CH), 113.2 (CH), 113.2 (C), 128.9 (CH), 143.5 (CH), 160.0 (C), 161.2 (C), 163.0 (C).

*4.1.2.3. 7-Methoxy-4-methyl-2H-chromen-2-one (**13**).* White powder, 77% yield, mp: 158.6–161.7 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 2.40 (3H, s), 3.88 (3H, s), 6.14 (1H, s), 6.82 (1H, d, $J = 2.6$ Hz), 6.86 (1H, dd, $J = 8.8$, 2.6 Hz), 7.50 (1H, d, $J = 8.8$ Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 18.9 (CH_3), 55.9 (CH_3), 101.1 (CH), 112.0 (CH), 112.5 (CH), 113.8 (C), 125.7 (CH), 152.7 (C), 155.4 (C), 161.4 (C), 162.8 (C).

4.1.3. Preparation of *2*, *8* and *14* (Scheme 1b)

0.4 g of **SM1** (2.2 mmol), **SM2** (2.4 mmol) and **SM3** (2.2 mmol), respectively, were dissolved with 2 mL of DMF (compound **2**) or acetone (compound **8** and **14**). After that, solid K_2CO_3 (1.9 or 3.8 equivalents) were add, and stirred during thirty minutes. 2.2 equivalents of ethyl iodide and potassium iodide (as catalyst) were added to the mixture and heating for 2, 2.5 and 5 days at 40 °C, respectively. Finally, when reaction was completed (monitored by TLC analysis), the solid was filtered off and dried to afford the corresponding compounds **2** (6,7-diethoxy-2*H*-chromen-2-one), **8** (7-ethoxy-2*H*-chromen-2-one) and **14** (7-ethoxy-4-methyl-2*H*-chromen-2-one).

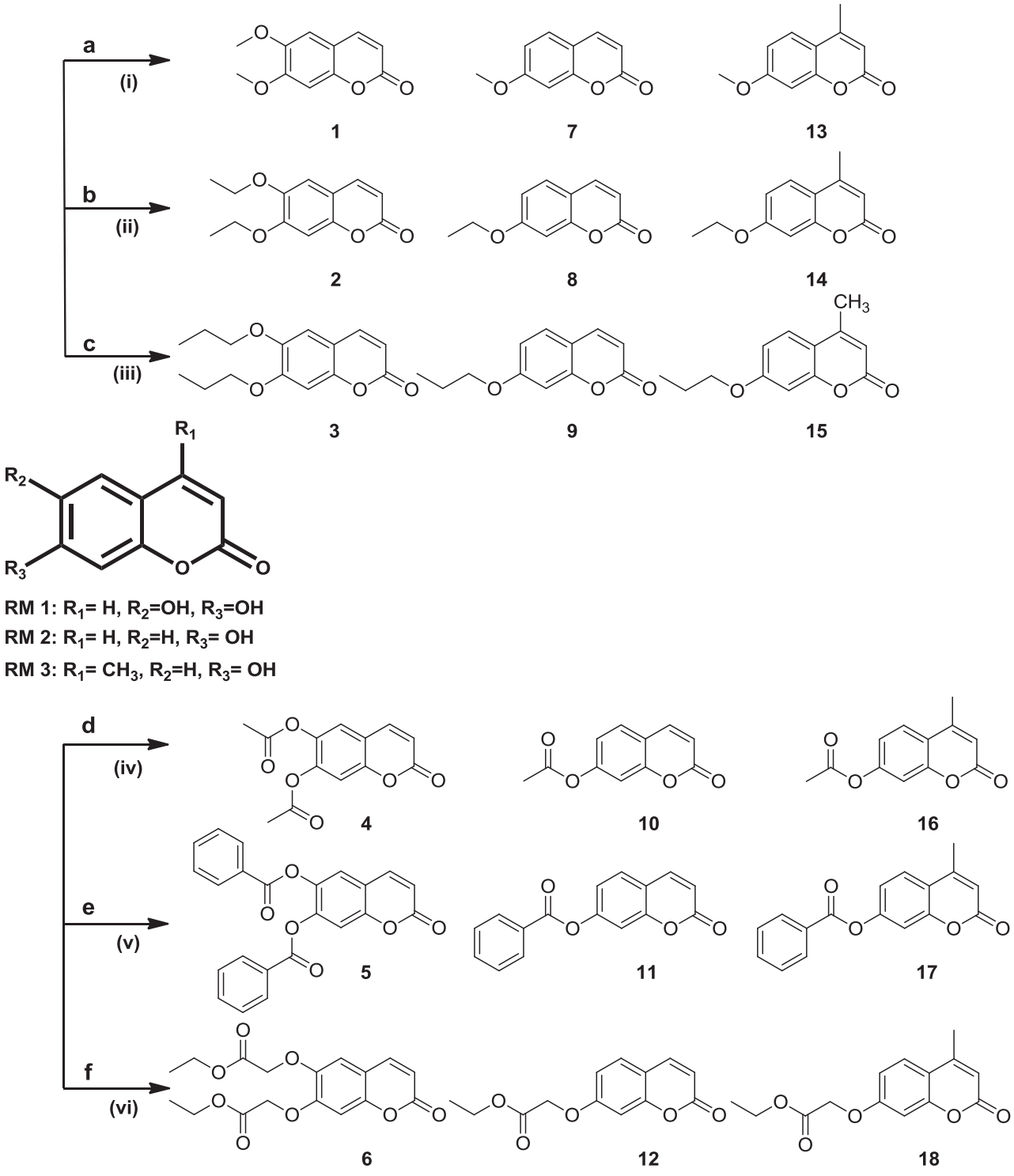
*4.1.3.1. 6,7-Diethoxy-2H-chromen-2-one (**2**).* Yellow powder, 54% yield, mp: 105.5–106.5 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 1.48 (3H, t, $J = 7.2$ Hz), 1.51 (3H, t, $J = 7.2$ Hz), 4.10 (2H, q, $J = 7.2$ Hz), 4.15 (2H, q, $J = 7.2$ Hz), 6.26 (1H, d, $J = 9.6$ Hz), 6.82 (1H, s), 6.87 (1H, s), 7.60 (1H, d, $J = 9.6$ Hz); ^{13}C NMR (400 MHz, $CDCl_3$): δ_C 14.6 (CH_2), 14.9 (CH_2), 65.0 (CH_2), 65.4 (CH_2), 101.2 (CH), 110.3 (CH), 111.5 (C), 113.4 (CH), 143.6 (CH), 145.9 (C), 150.2 (C), 152.9 (C), 161.7 (C).

*4.1.3.2. 7-Ethoxy-2H-chromen-2-one (**8**).* White powder, 46% yield, mp: 86.7–88.7 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 1.46 (3H, t, $J = 7.0$ Hz), 4.10 (2H, q, $J = 7.0$ Hz), 6.24 (1H, d, $J = 9.4$ Hz), 6.81 (1H, s), 7.34 (1H, d, $J = 8.8$ Hz), 7.63 (1H, d, $J = 9.4$ Hz), 7.82 (1H, dd, $J = 8.8$, 2.4 Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 14.8 (CH_3), 64.4 (CH_2), 101.5 (CH), 112.5 (C), 113.1 (CH), 113.1 (CH), 128.8 (CH), 143.6 (CH), 156.0 (C), 161.4 (C), 162.3 (C).

*4.1.3.3. 7-Ethoxy-4-methyl-2H-chromen-2-one (**14**).* White powder, 37% yield, mp: 108.2–110.5 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 1.42 (3H, t, $J = 7.2$ Hz), 2.36 (3H, d, $J = 1.2$ Hz), 4.06 (2H, q, $J = 7.2$ Hz), 6.09 (1H, s), 6.76 (1H, d, $J = 2.8$ Hz), 6.82 (1H, dd, $J = 8.8$, 2.8 Hz), 7.45 (1H, d, $J = 8.8$ Hz); ^{13}C NMR (400 MHz, $CDCl_3$): δ_C 14.5 (CH_3), 18.7 (CH_3), 64.1 (CH_2), 101.3 (CH), 111.8 (CH), 112.6 (CH), 113.5 (C), 125.5 (CH), 152.7 (C), 155.2 (C), 161.5 (C), 162.0 (C).

4.1.4. Preparation of *3*, *9* and *15* (Scheme 1c)

0.4 g of **SM1** (2.2 mmol), **SM2** (2.4 mmol) and **SM3** (2.2 mmol), respectively, were dissolved with 2 mL of acetone (for compound **9** and **15**) and DMF (for compound **3**). After that, solid K_2CO_3 (4 molar



Scheme 1. Synthesis of homologous coumarin derivates: methyl (**a**), ethyl (**b**), propyl (**c**), acetyl (**d**), benzoyl and (**e**) Ethoxy-2-oxoethyl (**f**). Reagents and conditions: (**i**) CH_3CN, KOH, Me_2SO_4/reflux, (**ii**) DMF or acetone, K_2CO_3, ethyl iodide, KI/reflux, (**iii**) DMF or acetone, K_2CO_3, *n*-propyl bromide, Et_3N or KI/reflux, (**iv**) acetic anhydride, H_2SO_4, Et_3N, (**v**) benzoyl chloride, Et_3N (**vi**), acetone, K_2CO_3, ethyl bromoacetate.

equivalent) was added and stirred during thirty minutes. Then 3 equivalents of triethylamine (Et_3N) and 3 equivalents of *n*-propyl bromide were added. All of three reactions were heating for 2, 2.5 and 5 days at 40 °C, respectively. After reactions were completed (analyzed by TLC), the solid was filtered off and dried to afford the corresponding compounds **3** (6,7-dipropoxy-2*H*-chromen-2-one), **9** (7-propoxy-2*H*-chromen-2-one) and **15** (4-methyl-7-propoxy-2*H*-chromen-2-one).

*4.1.4.1. 6,7-Dipropoxy-2H-chromen-2-one (**3**).* Beige powder, 85% yield, mp: 79.2–81.8 °C, recrystallized from 100% methanol. ^1H NMR ($CDCl_3$) δ: 1.06 (3H, t, $J = 7.6$), 1.08 (3H, t, $J = 7.6$), 1.85 (2H, q,

J = 7.2 Hz), 1.90 (2H, q, J = 7.2 Hz), 3.98 (2H, t, J = 6.4 Hz), 4.01 (2H, t, J = 6.4 Hz), 6.25 (1H, d, J = 9.6 Hz), 6.81 (1H, s), 6.87 (1H, s), 7.60 (1H, d, J = 9.6 Hz); ^{13}C NMR (400 MHz, $CDCl_3$): δ_C 10.6 (CH_3), 10.6 (CH_3), 22.4 (CH_2), 22.5 (CH_2), 70.9 (CH_2), 71.6 (CH_2), 101.1 (CH), 110.8 (CH), 111.5 (C), 113.3 (CH), 143.6 (CH), 146.3(C), 150.3 (C), 153.4 (C), 161.7 (C).

*4.1.4.2. 7-Propoxy-2H-chromen-2-one (**9**).* White powder, 27% yield, mp: 62.6–63.9 °C, recrystallized from 1:1 methanol: ethanol mixture. ^{1}H NMR ($CDCl_3$) δ: 1.05 (3H, t, J = 7.6 Hz), 1.83 (2H, q, J = 7.2 Hz), 3.97 (2H, t, J = 6.4 Hz), 6.23 (1H, d, J = 9.6 Hz), 6.80 (1H, d, J = 2.4 Hz), 6.84 (1H, d, 2.4 Hz), 7.36 (1H, d, J = 8.4 Hz), 7.63 (1H, d, J = 9.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 10.6 (CH_3), 22.5 (CH_2), 70.3 (CH_2), 101.5 (CH), 112.5 (CH), 113.1 (C), 113.1 (CH), 128.9 (CH), 143.7 (CH), 156.9 (C), 161.5 (C), 162.6 (C).

*4.1.4.3. 4-Methyl-7-propoxy-2H-chromen-2-one (**15**).* White crystals, 96% yield, mp: 74.4–75.2 °C, recrystallized from 100% methanol. ^{1}H NMR ($CDCl_3$) *δ:* 1.03 (3H, t, J = 7.6 Hz), 1.81 (2H, m), 1.83 (2H, q, J = 7.2 Hz), 2.36 (3H, d, J = 1.2 Hz), 3.94 (2H, t, J = 6.4 Hz), 6.23 (1H, s), 6.82 (1H, dd, J = 2.4, 2.4 Hz), 6.75 (1H, d, J = 2.4 Hz), 7.44 (1H, d, J = 8.8 Hz); ^{13}C NMR (400 MHz, $CDCl_3$): δ_C 10.41 (CH_3), 18.7 (CH_3), 22.3 (CH_2), 70.0 (CH_2), 101.3 (CH), 111.8 (CH), 112.6 (CH), 113.4 (C), 125.4 (CH), 152.5 (C), 155.3 (C), 161.3 (C), 162.2 (C).

*4.1.5. General methods of synthesis of compounds **4**–**5**, **10**–**11** and **16**–**17***

Esterification of **SM1** (2.2 mmol), **SM2** (2.4 mmol) and **SM3** (2.2 mmol) were carried out with acetic anhydride [$(CH_3CO)_2O$] and sulfuric acid as catalyst, or benzoyl chloride and Et_3N. All reactions were carried out in agitation at room temperature. The crude reactions were washed with cold water, filtered, dried and purified according to the procedures reported [35].

*4.1.6. Preparation of **4**, **10** and **16** (Scheme 1d)*

0.4 g of **SM1** (2.2 mmol), **SM2** (2.4 mmol) and **SM3** (2.2 mmol), respectively, were stirred with 4 molar equivalents of acetic anhydride and two drops of H_2SO_4 (as catalyst) under ice bath. After that, 2.5 molar equivalents of Et_3N were added to the mixture and agitated for 2 days, 10 min and 30 min, respectively, at room temperature. After reactions were completed (analyzed by TLC), the solid was filtered off and dried to afford the corresponding compounds **4** (2-oxo-2*H*-chromene-6,7-diyl diacetate), **10** (2-oxo-2*H*-chromen-7-yl acetate) and **16** (4-methyl-2-oxo-2*H*-chromen-7-yl acetate), respectively.

*4.1.6.1. 2-Oxo-2H-chromene-6,7-diyl diacetate (**4**).* Gray powder, 78% yield, mp: 127.6–130.4 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) *δ:* 2.32 (3H, s), 2.34 (3H, s), 6.43 (1H, d, J = 9.6 Hz), 7.26 (1H, s), 7.35 (1H, s), 7.64 (1H, d, J = 9.6 Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 20.8 (CH_3), 112.5 (CH), 117.0 (C), 117.1 (CH), 121.8 (CH), 138.9 (C), 142.5 (CH), 144.9 (C), 151.8 (C), 160.0 (C), 167.7 (C).

*4.1.6.2. 2-Oxo-2H-chromen-7-yl acetate (**10**).* White powder, 90% yield, mp: 133.5–135.0 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 2.34 (3H, d, J = 2.4 Hz), 6.40 (1H, d, J = 10.2 Hz), 7.05 (1H, dd, J = 8.4, 2.2 Hz), 7.12 (1H, d, J = 2.2 Hz), 7.49 (1H, d, J = 8.4 Hz), 7.70 (1H, d, J = 10.2 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ_C 20.4 (CH_3), 110.6 (CH), 116.2 (CH), 116.8 (C), 118.5 (CH), 128.7 (CH), 143.0 (CH), 153.3 (C), 154.8 (C), 160.4 (C), 168.8 (C).

*4.1.6.3. 4-Methyl-2-oxo-2H-chromen-7-yl acetate (**16**).* White powder, 92% yield, mp: 152.5–153.5 °C, recrystallized from ethanol. ^{1}H NMR ($CDCl_3$) δ: 2.34 (3H, s), 2.43 (3H, d, J = 1.2 Hz), 6.27 (1H, d, J = 1.2 Hz), 7.06 (1H, d, J = 1.8 Hz), 7.11 (1H, dd, J = 8.4, 1.8 Hz), 7.61 (1H, d, J = 8.4 Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 18.9 (CH_3), 21.4 (CH_3), 110.6 (CH), 114.7 (CH), 118.0 (C), 118.2 (CH), 125.5 (CH), 152.0 (C), 153.2 (C), 154.3 (C), 160.5 (C), 168.8 (C).

*4.1.7. Preparation of **5**, **11** and **17** (Scheme 1e)*

0.4 g of **SM1** (2.2 mmol), **SM2** (2.4 mmol) and **SM3** (2.2 mmol), respectively, were stirred in 2 mL of CH_2Cl_2. After that, 2.5 molar equivalents of Et_3N, and 5 molar equivalents of benzoyl chloride were added to the mixture. Reaction was agitated for 2 days and 12 h for compounds **5**, **11** and **17**, respectively. After reactions were completed (analyzed by TLC), the solid was filtered off and dried to afford the corresponding compounds **5** (2-oxo-2*H*-chromen-7-yl benzoate), **11** (2-oxo-2*H*-chromene-7-yl benzoate) **17**. (4-methyl-2-oxo-2*H*-chromene-7-yl benzoate) **17**.

*4.1.7.1. 2-Oxo-2H-chromene-6,7-diyl dibenzoate (**5**).* Translucent powder, 82% yield, mp: 185.1–186.6 °C, recrystallized from 100% acetonitrile. ^{1}H NMR ($CDCl_3$) δ: 6.48 (1H, d, J = 9.6 Hz), 7.26 (1H, s), 7.39 (4H, dd, J = 8.0, 8.0 Hz), 7.44 (1H, s), 7.56 (2H, m), 7.72 (1H, d, J = 9.6 Hz), 8.05 (4H, d, J = 7.6 Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 113.2 (CH), 117.7 (C), 117.7 (CH), 121.6 (CH), 128.2 (C), 129.4 (CH), 131.1 (CH), 134.8 (CH), 134.9 (CH), 139.4 (C), 143.2 (CH), 146.7 (C), 152.7 (C), 160.8 (C), 164.9 (C).

*4.1.7.2. 2-Oxo-2H-chromen-7-yl benzoate (**11**).* White powder, 78% yield, mp: 158.0–159.3 °C, recrystallized from 100% methanol. ^{1}H NMR ($CDCl_3$) δ: 6.42 (1H, d, J = 9.6 Hz), 7.19 (1H, dd, J = 8.4, 2.2 Hz), 7.53 (1H, d, J = 2.2 Hz), 7.48–7.60 (3H, m), 7.66 (1H, d, J = 8.4 Hz), 7.72 (1H, d, J = 9.6 Hz), 8.21 (2H, d, J = 7.6 Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 110.8 (CH), 116.3 (CH), 116.9 (C), 118.7 (CH), 128.8 (CH), 128.9 (CH), 130.4 (CH), 134.2 (C), 134.2 (CH), 143.0 (CH), 153.6 (C), 154.9 (C), 160.4 (C), 164.6 (C).

*4.1.7.3. 4-Methyl-2-oxo-2H-chromen-7-yl benzoate (**17**).* White powder, 86% yield, mp: 161.5–163.5 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 2.46 (3H, d, J = 1.2 Hz), 6.30 (1H, d, J = 1.2 Hz), 7.21 (1H, dd, J = 8.4, 1.8 Hz), 7.25 (1H, d, J = 1.8 Hz), 7.48–7.50 (3H, m), 7.67 (1H, d, J = 8.4 Hz), 8.22 (2H, dd, J = 8.4, 1.4 Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 19.0 (CH_3), 110.8 (CH), 114.7 (CH), 119.0 (C), 118.4 (CH), 125.6 (CH), 128.9 (CH), 130.4 (CH), 134.2 (CH), 152.0 (C), 153.5 (C), 154.4 (C), 160.6 (C), 164.6 (C), 190.0 (C).

*4.1.8. General methods of synthesis of compounds **6**, **12** and **18***

Reactions were carried out by etherification of **SM1** (2.2 mmol), **SM2** (2.4 mmol) and **SM3** (2.2 mmol) with ethyl bromoacetate ($C_4H_7BrO_2$), K_2CO_3, and acetone as solvent. The mixture was stirred at room temperature. The crude products were washed with cold water, filtered, dried and purified according to the procedures reported [35].

*4.1.9. Preparation of **6**, **12** and **18** (Scheme 1f)*

0.4 g of **SM1** (2.2 mmol), **SM2** (2.4 mmol) and **SM3** (2.2 mmol), respectively, were dissolved in acetone (2.0 mL) and 1.75 or 3.5 molar equivalents of K_2CO_3 was add, and stirred during thirty min. After that, it was added drop wise 1.75 molar equivalents of ethyl bromoacetate. The mixture was reacted at room temperature during 6 h for compounds **6** and **12**, and 8 h for **18**. After reactions were completed (analyzed by TLC), the solid was filtered off and dried to afford the corresponding compounds **6** ethyl {[6-(2-ethoxy-2-oxoethoxy)-2-oxo-2*H*-chromen-7-yl]oxy}acetate, **12** [ethyl 2-(2-oxo-2*H*-chromen-7-yloxy)acetate] and **18** [ethyl 2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy)acetate].

*4.1.9.1. Ethyl {[6-(2-ethoxy-2-oxoethoxy)-2-oxo-2H-chromen-7-yl]oxy}acetate (**6**).* White powder, 91% yield, mp: 107.0–108.8 °C,

recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 1.30 (3H, t, J = 7.0 Hz), 1.32 (3H, t, J = 7.0 Hz), 4.27 (2H, q, J = 7.0, 3.4 Hz), 4.28 (2H, q, J = 7.0, 3.4 Hz), 4.75 (4H, d, J = 7.6 Hz), 6.30 (1H, d, J = 9.6 Hz), 6.76 (1H, s), 7.01 (1H, s), 7.59 (1H, d, J = 9.6 Hz); ^{13}C NMR (200 MHz, $CDCl_3$): δ_C 14.4 (CH_3), 61.6 (CH_2), 61.9 (CH_2), 66.2 (CH_2), 67.6 (CH_2), 102.4 (CH), 112.7 (C), 114.2 (CH), 114.6 (CH), 143.1 (CH), 144.9 (C), 150.5 (C), 151.9 (C), 161.0 (C), 167.8 (C), 168.8 (C).

*4.1.9.2. Ethyl 2-(2-oxo-2H-chromen-7-yloxy)acetate (**12**).* Yellow powder, 96% yield, mp: 112.2–114.4 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 1.32 (3H, t, J = 7.0 Hz), 4.29 (2H, q, J = 7.0 Hz), 4.68 (2H, s), 6.28 (1H, d, J = 9.6 Hz), 6.78 (1H, d, J = 2.2 Hz), 6.90 (1H, dd, J = 8.4, 2.2 Hz), 7.40 (1H, d, J = 8.4 Hz), 7.64 (1H, d, J = 9.6 Hz); ^{13}C NMR (200 MHz, Acetone-d_6): δ_C 14.4 (CH_3), 61.9 (CH_2), 65.6 (CH_2), 101.9 (CH), 113.0 (CH), 113.5 (C), 113.9 (CH), 129.1 (CH), 143.3 (CH), 155.8 (C), 161.0 (C), 168.0 (C).

*4.1.9.3. Ethyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate (**18**).* White crystals, 71% yield, mp: 100.1–103.2 °C, recrystallized from 100% ethanol. ^{1}H NMR ($CDCl_3$) δ: 1.29 (3H, t, J = 7.2 Hz), 2.37 (3H, d, J = 1.2 Hz), 4.25 (2H, q, J = 7.2 Hz), 4.65 (2H, s), 6.13 (1H, q, 1.2 Hz), 6.75 (1H, d, J = 2.8 Hz), 6.88 (1H, dd, J = 8.8, 2.8 Hz), 7.49 (1H, d, J = 8.8 Hz); ^{13}C NMR (400 MHz, $CDCl_3$): δ_C 14.1 (CH_3), 18.6 (CH_3), 61.7 (CH_2), 65.3 (CH_2), 101.7 (CH), 112.5 (CH), 112.5 (C), 114.4 (CH), 125.7 (CH), 152.3 (C), 155.0 (C), 160.6 (C), 161.0 (C), 167.9 (C).

4.2. Pharmacological evaluation

4.2.1. Chemicals and drugs

Carbamylcholine (carbachol), teophylline, dimethyl sulfoxide (DMSO) and ethyl ether were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

4.2.2. Animals

Healthy male Wistar rats (250–300 g) were used and maintained under standard laboratory conditions with free access to food and water. All animal procedures were conducted in accordance with our Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZOO-1999, México), and approved by the Institutional Animal Care and Use Committee based on US National Institute of Health publication (No. 85-23, revised 1985). All experiments were carried out using six animals per group. Animals used were euthanized by exposure to ethylic ether and cervical dislocation.

4.2.3. Rat trachea ring assay

A previous protocol was used [36]. The trachea was dissected and cleaned out of connective tissue and mucus, and immediately was cut into 4–5 mm length rings (each containing 2–3 cartilaginous rings). Then, tissue segments were mounted in stainless steel hooks, under optimal tension of 2 g, in 10 mL organ baths containing warmed (37 °C) and oxygenated (O_2/CO_2, 95:5) Krebs solution (composition, mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; EDTA, 0.026 and glucose, 11.1, pH 7.4). Changes in tension were recorded by Grass-FT03 forced transducers (Astromed, West Warwick, RI, USA) connected to a MP100 analyzer (BIOPAC Instruments, Santa Barbara, CA, USA) as previously described. After equilibration, rings were contracted by carbachol [1 μM] and washed every 40 min for 2 h. After contraction with carbachol, the test samples (organics extracts, vehicle and positive control) were added to the bath in a volume of 100 μL; then cumulative concentration–response curves were obtained for each ring (1–500 μM). The relaxant effect of extracts and positive control (theophylline, an inhibitor of phosphodiesterase; 1.67–550 μM) were determined by comparing the muscular tone of the contraction before and after addition of the test materials. Muscular tone was calculated from the tracings, using Acknowledge software (Biopac®).

4.3. Theoretical evaluation of lipophilicity

For a better understanding of the overall properties of the described compounds, the lipophilicity values were estimated trough theoretical determination with miLogP (expressed as the oct/wat) using the Molinspiration calculation program [37,38], also were calculated with different programs, and after that were averaged and grouped in the ALOGPS 2.1 software available online [30,31].

4.4. Data analysis

Data are expressed as mean of six experiments ± standard error of mean (SEM). Concentration responses curves (CRC) were plotted and experimental data of the CRC were adjusted by the nonlinear, curve-fitting program Microcal TM origin 8.0 (Microcal Software Inc., USA). Significance was evaluated using the Student's t test. Values of $^*p < 0.05$ imply significance of the pharmacological effects in the experiments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.029.

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