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Graphical Abstract

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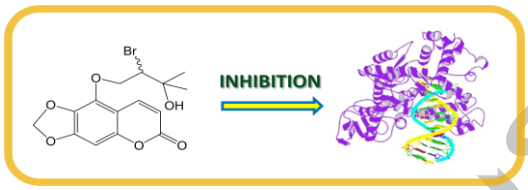

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Inhibition of reverse transcriptase and *Taq* DNA polymerase by compounds possessing the coumarin framework.

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ABSTRACT

Coumarin derivatives were prepared using natural products isolated from plants belonging in the *Pterocaulon* genus (Asteraceae) and commercial drugs. Some molecules have displayed interesting activity against myeloid murine leukemia virus-reverse transcriptase (MMLV-RT) (compound **20** and **28** produced inhibition with IC_{50} values of **38.62 μM** and **50.98 μM**, respectively) and *Taq* DNA polymerase (analogues **13** and **14** produced inhibition with IC_{50} values of **48.08 μM** and **57.88 μM**, respectively). Such inhibitors may have importance as antiretroviral chemotherapeutic agents and also in the development of anticancer drugs.

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Human Immunodeficiency Virus (HIV) is the viral agent of acquired immunodeficiency syndrome (AIDS), and at present, there is no effective vaccine against HIV.[1] Reverse Transcriptase (RT) is an essential enzyme for retroviral replication, such as HIV as well as for other RNA infectious viruses like Human T lymphocyte virus.[2] Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), a class of antiretroviral chemotherapeutic agents, act by binding to an allosteric pocket showing, generally, low toxicity.[3] Another potential molecular target is DNA polymerases, which represent important cellular targets in the development of anticancer and antiviral agents.[4]

Natural coumarins have attraction due to bioactive properties such as tumor promotion inhibitory effects,[5] anti-bacterial activity,[6] antituberculosis,[7] anti-influenza,[8] anti-alzheimer,[9] anti-inflammatory,[10] and as anti-coagulants.[11] Coumarins and derivates exhibit potent inhibitory effects on HIV-1 replication in H9 lymphocytes and compounds isolated from *Calophyllum inophyllum* showed inhibitory activity against human RT.[12-14] Furthermore, natural isocoumarins isolated from cultures of fungi were able to inhibit human DNA polymerase λ.[15] The object of the present work was to examine the ability of compounds possessing the coumarin framework to inhibit RT from myeloid murine leukemia virus (MMLV) and *Taq* DNA polymerase.

For the present study we selected two plant species from genus *Pterocaulon* (Asteraceae); *P. virgatum* (L.) D.C and *P. alopecuroides* (L.) D.C, known as coumarin´s producers.[16, 17] We describe the isolation and characterization of four coumarins: sabandinine **1**; 5-(3',3'-dimethylallyloxy)-6,7-methylendioxycoumarin **2**; 7-(3'-methyl-2',3'-dihydroxybutoxy)-6,8-dimethoxycoumarin **3** and 7-(3'-methyl-2',3'-dihydroxybutoxy)-6-methoxycoumarin **4** (Fig.1).

We evaluate the potential antiviral therapeutic properties from natural and semi-synthetic analogues derivatives. The family of compounds was screened by retro-transcription polymerase chain reaction (RT-PCR) and classical polymerase chain reaction (PCR) inhibition.[18]

In order to increase the number of compounds for biological tests we performed chemical transformation over products **2** and **4**. Thus, 7-(3'-methyl-2',3'-dihydroxybutoxy)-6-methoxycoumarin (**4**) was treated with H_2SO_4 in presence of acetone, leading to the formation of compound **5**, with a typical acetonide moiety with a yield of 93% (Scheme 1). This group is also present in natural coumarins **1** and **2**, resembles in part their structure.

Besides, acetylating of hydroxyl groups in compound **4** was carried out in a *regio*-selective manner. Thus, secondary hydroxyl group was preferentially acetylated (**7**) to tertiary hydroxyl (**6**) when the reaction was carried out in the absence of DMAP. Using DMAP as a catalyst the *per-O*-acetylated derivative (**6**) was obtained as the sole product, with 94 % yield (Scheme 1).

In another group of reactions the γ,γ-dimethylallyl moiety present in compound **2** was subjected to epoxidation whit *m*-

CPBA to obtain the corresponding oxirane **8** and generate a new stereogenic center. Also, by a simple reduction using hydrogen and 5% palladium a hydrogenated derivative **9** was obtained with *regio*-selection at carbons 2´-3´ (63% yield). We also envisaged allylic oxidations, initially using $KMnO_4$ and ammonium salt to achieve the exchange of phases, giving a mixture of compounds **10** (59% yield) and **11** (33% yield). Now, employing SeO_2 to get a carbonyl function was possible to prepare a Michael acceptor **12** at the side chain. It is well known the high degree of reactivity of α,β-unsaturated carbonyls against the nucleophiles nitrogen, sulfur and oxygen, present in enzymes, or even the same DNA molecule.[19, 20] In this reaction it was also observed the appearance of a more polar product **13**, corresponding to a primary alcohol at the isoprenoid chain. It was also envisaged to introduce halogen atom (bromination) on the double bond, mainly at the γ,γ-dimethylallyl function. Simple addition of bromine performed with NBS in carbon tetrachloride or acetic acid furnished a complex mixture of compounds. Surprisingly, halohydrin **14** was obtained in 72% rather than the expected dibromoderivative. This result can be explained in case the dihalogenated product initially obtained could suffer SN_1 reaction on C-3' in the water washing process (Scheme 2). The different reactivity of carbons 2' and 3' can be explained considering the C-3' is a tertiary carbon, capable of forming a more stable carbocation that C-2'.

In another set of reactions we used commercial 4-hydroxycoumarin **15**, 4-hydroxy-3-nitrocoumarin **16**, coumaranone **17**, 3-acetylcoumarin **18**, 3-hydroxycoumarin **19** and coumarin **20** to increase the number of derivatives for inhibition assays (Fig. 2). We were able to methylate the enol group of **15** using Me_2SO_4 as alkylation agent producing compound **21** (67% yield). In same substrate we can generate a benzoyl analogue (**22**) in a 57% yield, employing BzOCl. In order to incorporate a heteroatom on compound **15**, it was subjected to reaction with methanesulfonyl chloride (**23**) (92% yield) and with *p*-chlorobenzoyl chloride (**24**) (78% yield) and both of them showed as white crystals (Scheme 3). However, compound **16** was only capable to reacting with this last protecting group, generating the derivative **25** (46% yield) (Scheme 4). This low reactivity of **16** may be explained by the high electro-drawing effect of the nitro group, which would decrease the nucleophilic character of the enol function. Finally, we proposed a prenylation reaction on **15** to achieve a structurally related derivative to the naturally 5-(3',3'-dimethylallyloxy)-6,7-methylendioxycoumarin **2**. In this reaction, we obtained two products: the expected analogue **26** (59% yield) and a less polar dicarbonylic compound **27** (24% yield). Hybridization change and the incorporation of two isoprenoid chains, both on third position can be explained mainly by the substantial electron density present in this carbon and keto-enol equilibrium of **15**. In the same way, we used allyl bromide in turbo cooler equipment, giving a mixture of compounds (**28**-**30**) with different degrees of substitution (Scheme 5). All synthesized compounds and natural coumarins isolated were well characterized by ^{1}H and ^{13}C NMR.[21]

Different natural coumarins as well as synthetic analogs were found to display potent anti-HIV activity and can serve as potent NNRTIs in the development of anti-AIDS leads.[22] It has been amply documented that DNA polymerases and reverse transcriptases serve as molecular targets for antiviral chemotherapy.[23] Assessment of the activity of *Taq* DNA polymerase was performed by PCR technique. In an initial screening we used a concentration equal to 500 μM; the results revealed that analogues **13** and **14** produced inhibition with IC_{50} values of **48.08 ± 9.26 μM** and **57.88 ± 1.26 μM** respectively. Against expectations, the derivative bearing a Michael acceptor **12** was not active. However, the alcohol **13** obtained during the same reaction, was active. Compound **10** has a carbonyl group at side chain, and as **12** also shown inactive. On the other hand, the incorporation of bromide seems to be significant for protein recognition and inhibition,[24-26] because compound **11** also have a tertiary alcohol and similar stereo-electronic factors as **14**, but it was inactive. Compound **14** has a particular structural feature in their side chain. The bromine atom can act as a leaving group in a nucleophilic attack by nucleophilic residues of proteins. The presence of oxygen containing functional groups at both C-β can lead to neighboring group participation in the formation of the intermediate carbocation. This situation would result in a higher rate to the nucleophilic substitution (S_N1) in relation to the intramolecular substitution reaction (S_{Ni}) giving rise to the epoxide. This center might be the determining factor in the inactivation of the enzyme. Moreover, in compound **12**, where it would be possible to expect a conjugate Michael addition, the oxygen atom of the ether function can be protected from nucleophilic attack by the proximity of their unshared electron pairs to the electrophilic carbon. This situation has previously been reported for α,β-unsaturated cyclopentenone moieties in sesquiterpene lactones.[27] Finally, the similar values of activity obtained with products **13** and **14** could be partially attributed to their related polarity.

Furthermore, we used all compounds obtained (except **13** and **14** because the RT-PCR experiment involve the *Taq* DNA polymerase activity) to evaluate reverse transcription process using a concentration of 500 μM for initial screening**.** Herein could be observed that compound **20** and **28** produced inhibition with IC_{50} values of **38.62 ± 3.25 μM** and **50.98 ± 1.79 μM** respectively. These results indicate that in reverse transcription, simple molecules like coumarin and **28** are able to inhibit this event. Taking into account the structure, is hard to think that coumarins act recognizing the enzymatic active site. Probably they can place allosterically, for example at some hydrophobic pocket.[28] Furthermore, it is interesting to note that none of the naturally occurring compound was active against *Taq* DNA polymerase and RT-MMLV. However, these compounds are important synthons and skeletons for obtaining active compounds. Also, it is possible to observe that the compounds inhibit the DNA polymerase present methylendioxy ring; different from that observed with RT inhibitor compounds which do not possess the mentioned moety. These results convert to the methylendioxy ring in necessary for activity against *Taq* DNA polymerases but it is not the only required condition because compounds as **1**-**2** and **10**-**12** are not active.

The aim of the present study was to evaluate the inhibition by coumarin, because of these compounds have showed important inhibitory activity against HIV-infected lymphocytes.[29] There is a high relationship between RNA dependent enzymes as RT-MMLV and DNA dependent polymerases like *Taq* polymerase. These structures resemble a right hand domain and conserve amino acid sequence inside catalytic region.[30] We also found that some coumarins can inhibit the *Taq* DNA polymerase activity and in this way could be cataloged as new polymerase inhibitors (see Table 1). Taking into account the results from this paper, it is possible to say that the coumarin analogs merit special attention as potential antivirals.

Table 1. Activity data of compounds against enzymes.

	IC_{50} [a] (μM)	
Compound	***Taq* DNA pol**	**RT-MMLV**
13	48.08 ± 9.26	[b]
14	57.88 ± 1.26	[b]
20	No activity	38.62 ± 3.25
28	No activity	50.98 ± 1.79

[a] Concentration that inhibits enzyme activity by 50%.

[b] It cannot be determinate

In summary, we have isolated four natural coumarins from *P. virgatum* and *P. alopecuroides* and prepare twenty semi-synthetic analogues besides commercial drugs. Their inhibitory activity against *Taq* DNA polymerase and MMLV-RT was evaluated and two molecules (compound **13** and **14**) with similar structures showed inhibitory activity against *Taq* DNA polymerase. Also, coumarin (**20**) and compound **28** were able to inhibit the reverse transcriptase with interesting values of activity. Thus, novel leads from these coumarins can be further developed into potential chemotherapeutic agents in antiviral treatment.

Acknowledgements

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18. Experimental: **PCR Assays.** The assayed compounds were all dissolved in DMSO. The PCR master mixture consisted of 40 mM Trisacetate pH 8.3, 15 mM MgCl2, 2.5 U of *Taq* DNA polymerase (Sigma-Aldrich), 20 mM each oligonucleotide primer, and 2.5 mM each desoxynucleotide triphosphate (dNTP). Inhibition studies were carried out with varying compound concentrations. All PCRs were done in 20 μL reaction volumes. The sequence of the sense primer was 5´-TAG AGC GTG AGG TCG ACA C-3´, and the antisense primer, 5´ TCA AGT TAG ACG TGG CCG TC 3´. Thermocycling conditions consisted of 35 cycles of denaturation at 95 °C for 1 min followed by primer annealing at 56 °C and primer extension at 72 °C for 90 seg.

RT-PCR Assays. The inverse transcription process consisted of 1μg RNA dissolved in 4 μL of commercial buffer and 2.0 U of MMLV-RT (Sigma-Aldrich), 20 μM Random Primers, and 1.0 mM each desoxynucleotide triphosphate (dNTP). Inhibition studies were carried out with varying compound concentrations and adding compounds in this stage. All RT- PCRs were done in 6.8 μL reaction volumes. Reaction conditions consisted of heating at 70 °C for 10 min and incubating 1h at 56 °C.

Analysis of PCR Products. Relative intensities of ethidium bromide stained PCR products were analyzed by using the optical scanner and the image program. The image of stained agarose gel was captured using a photography camera Kodak 2320 and then was scanned (Hewlett-Packard 3200 C). The digitized band images were processed using the Image processing program (Scion Image, public domain program); IC_{50} values were determined by the GraphPad Prism program.
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21. Spectroscopy data for natural compounds **1-4** and active tested compounds **13**, **14**, **20**, **28**.

Sabandinine (**1**). ^{1}H NMR (200 MHz, Cl_3CD) 4.14 (s, 3H), 6.01 (s, 2H), 6.18 (d, 1H, *J*= 9.4 Hz), 6.53 (brm, 1H) 7.94 (d, 1H, *J*= 9.4 Hz). ^{13}C NMR from HSQC (50.6 MHz, Cl_3CD) 59.00, 92.12, 101.80, 111.96, 138.70.

5-(3',3'-dimethylallyloxy)-6,7-methylendioxycoumarin (**2**). ^{1}H NMR (200 MHz, Cl_3CD) 1.78-1.73 (brm, 6H), 4.83 (brm, 1H), 4.87 (d, 1H, *J*= 7.2 Hz), 5.47 (brm, 1H), 6.01 (s, 2H), 6.19 (d, 1H, *J*= 9.6 Hz), 6.52 (s, 1H), 7.96 (d, 1H, *J*= 9.6 Hz); ^{13}C NMR from HSQC (50.6 MHz, Cl_3CD) 17.00, 29.30, 69.00, 92.52, 101.60, 111.88, 119.53, 139.30.

7-(3'-methyl-2',3'-dihydroxybutoxy)-6,8-dimethoxycoumarin (**3**). ^{1}H NMR (200 MHz, Cl_3CD) 1.40-1.20 (brm, 6H), 3.82 (brm, 1H), 3.84 (s, 3H), 4.01 (s, 3H), 4.23-4.11 (brm, 2H), 6.24 (d, 1H, *J*= 9.4 Hz), 6.62 (s, 1H), 7.86 (d, 1H, *J*= 9.4 Hz); ^{13}C NMR from HSQC (50.6 MHz, Cl_3CD) 25.00, 26.50, 61.57, 71.11, 75.46, 96.90, 117.99, 138.93.

7-(3'-methyl-2',3'-dihydroxybutoxy)-6-methoxycoumarin (**4**). ^{1}H NMR (200 MHz, Cl_3CD) 1.34-1.26 (brm, 6H), 3.89 (brm, 1H), 3.92 (s, 3H), 4.30-4.15 (brm, 2H), 6.25 (d, 1H, *J*= 9.8 Hz), 6.85 (s, 1H), 6.91 (brm, 1H), 7.62 (d, 1H, *J*= 9.8 Hz); ^{13}C NMR from HSQC (50.6 MHz, Cl_3CD) 26.00, 27.50, 68.40, 70.60, 74.80, 97.60, 103.40, 110.99, 142.30.

5-(3´-methyl-4´-hydroxy-2´-butenoxy)-6,7-methylendioxy coumarin (**13**). ^{1}H NMR (200 MHz, Cl_3CD) 1.82 (s, 3H), 4.11 (s, 2H), 4.95 (d, 2H, *J*= 6.8 Hz), 5,77 (t, 1H, *J*= 4.4 Hz) 6.02 (s, 2H), 6.18 (d, 1H, *J*= 9.6 Hz), 6.54 (s, 1H), 7.96 (d, 1H, *J*= 9.6 Hz); ^{13}C NMR from HSQC (50.6 MHz, Cl_3CD) 10.00, 68.00, 69.10, 93.02, 101.80, 112.50, 120.76, 141.15.

5-(2´-bromine-3´-hydroxy-3'-methyl-butiloxy)-6,7-methylendioxycoumarin (**14**). ^{1}H NMR (200 MHz, Cl_3CD) 1.44-1.25 (brm, 6H), 3.34 (brm, 1H), 4.22 (dd, 1H, *J*= 3.2; 5.6 Hz), 4.50 (dd, 1H, *J*= 8.8; 2.8 Hz), 5.02 (dd, 1H, *J*= 3.2; 8.0 Hz) 6.05 (s, 2H), 6.22 (d, 1H, *J*= 9.8 Hz), 6.56 (s, 1H), 8.12 (d, 1H, *J*= 9.8 Hz); ^{13}C NMR from HSQC (50.6 MHz, Cl_3CD) 30.00, 64.20, 74.10, 93.22, 101.90, 111.92, 139.80.

2-H-1-Benzopiran-2-ona (**20**). ^{1}H NMR (200 MHz, Cl_3CD) 6.45 (d, 1H, *J*= 9.4 Hz), 7.31 (brm, 1H), 7.35 (d, 1H, *J*= 8.1 Hz), 7.52 (d, 1H, *J*= 7.8 Hz), 7.56 (brm, 1H), 7.73 (d, 1H, *J*= 9.4 Hz); ^{13}C NMR from HSQC (50.6 MHz, Cl_3CD) 115.99, 116.18, 123.72, 127.17, 131.12, 142.73.

4-hydroxy-3-allyloxy-coumarin (**28**). ^{1}H NMR (200 MHz, Cl_3CD) 3.50 (d, 2H *J*= 6.8 Hz), 5.27 (brm, 2H), 6.03 (brm, 1H), 7.27 (brm, 2H), 7.51 (dd, 1H, *J*= 1.6; 7.2 Hz) 7.81 (d, 1H *J*= 8.0 Hz); ^{13}C NMR from HSQC (50.6 MHz, Cl_3CD) 29.00, 116.15, 117.20, 121.89, 123.05, 134.23, 135.76.

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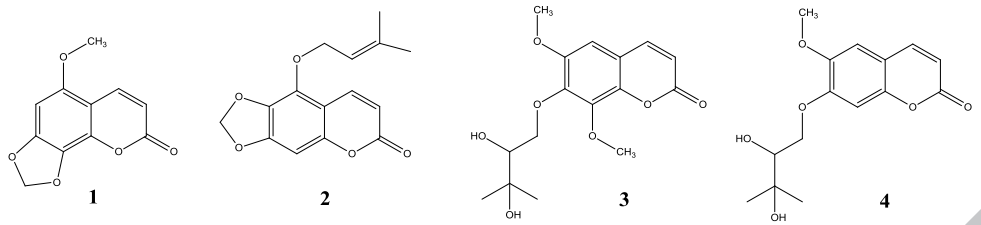
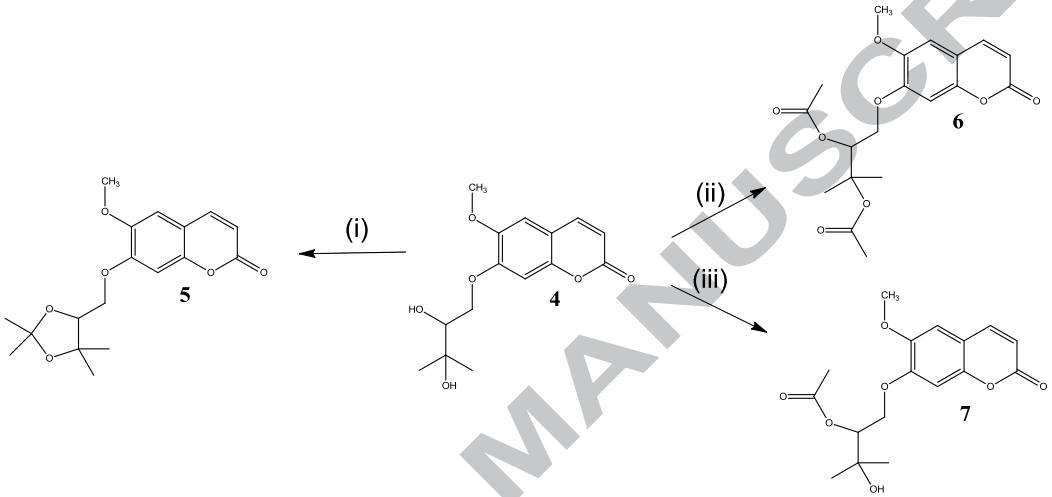
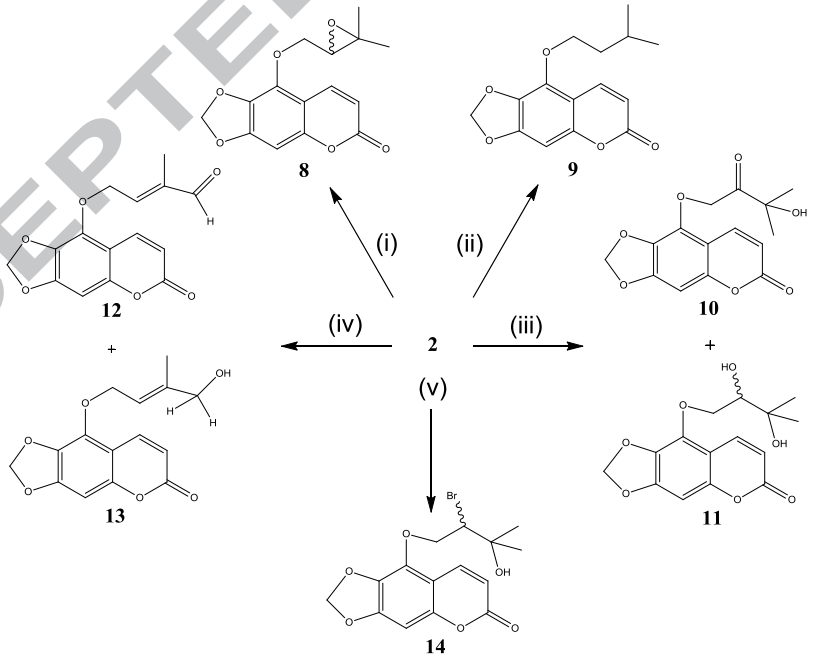


Figure 1. Compounds isolated from *P. virgatum* and *P. alopecuroides*.



Scheme 1. (i) H_2SO_4, acetone, rt. (ii) 2.3 equiv Ac_2O, Py, DMAP, rt. (iii) 1.0 equiv Ac_2O, Py, 0° C to rt.



Scheme 2. (i) *m*-CPBA, K_2CO_3, Cl_2CH_2, 0° C and dark conditions. (ii) H_2, Pd/C, Cl_2CH_2, rt. (iii) 1.1 equiv $KMnO_4$, THF/H_2O (7:3), diethyl-ammonium fluoride, rt. (iv) 0.75 equiv SeO_2, Cl_2CH_2, 2.0 equiv TBHP, 0°-4° C. (v) 2.2 equiv NBS, Cl_2CH_2, 0°-4° C.

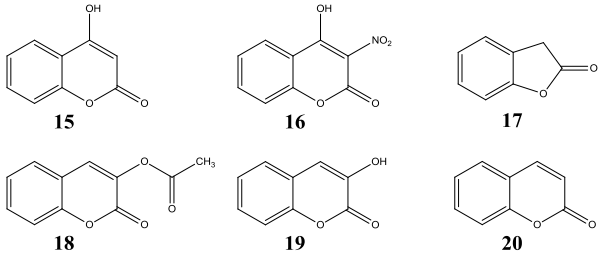
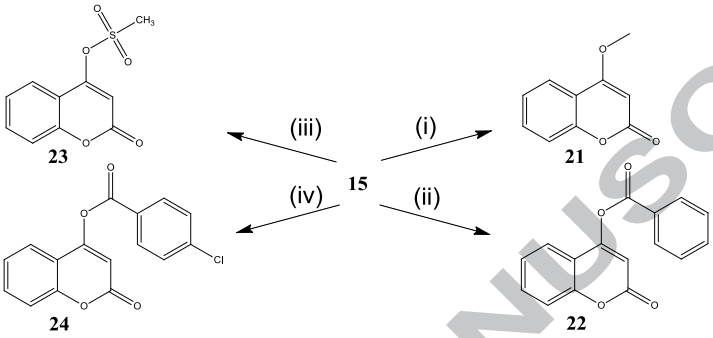
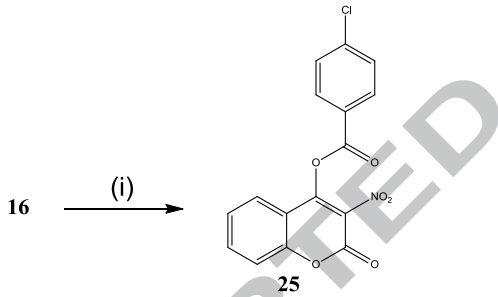


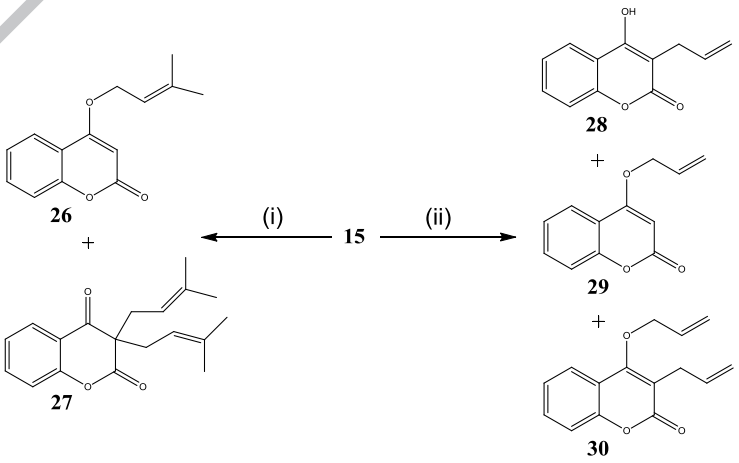
Figure 2. Commercial coumarins used.



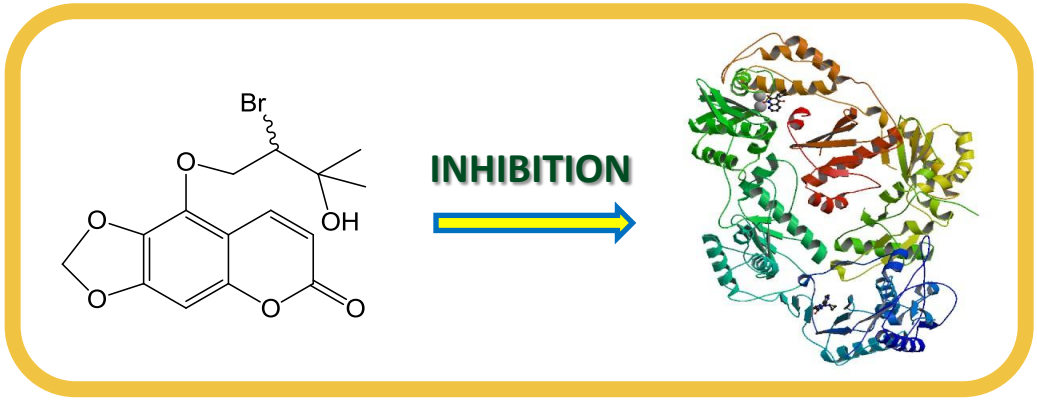
Scheme 3. (i) Me_2SO_4, K_2CO_3, acetone, 56° C. (ii) BzOCl, TEA, Cl_2CH_2, rt. (iii) 1.3 equiv MsCl, 1.2 equiv Py, 0.6 equiv DMAP, Cl_2CH_2, rt. (iv) 4-Chlorobenzoyl chloride, TEA, Cl_2CH_2, rt.



Scheme 4. (i) 4-Chlorobenzoyl chloride, TEA, Cl_2CH_2, rt.



Scheme 5. (i) 2.0 equiv *γ,γ*-dimethylallyl bromine, 1.5 equiv NaH, DMF, rt. (ii) 2.0 equiv allyl bromine, 1.5 equiv NaH, DMF, -20° C.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