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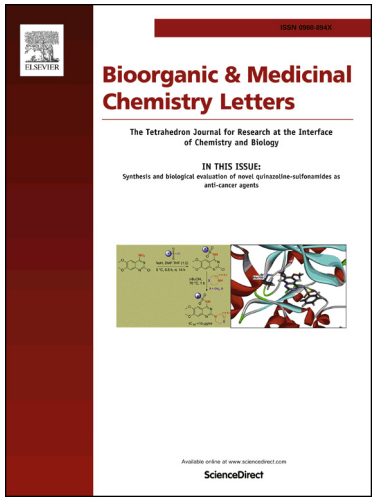

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Anti-influenza activity of monoterpene-containing substituted coumarins

Tatyana M. Khomenko,[a,c] Vladimir V. Zarubaev,[b] Iana R. Orshanskaya,[b] Renata A. Kadyrova,[b] Victoria A. Sannikova,[a,c] Dina V. Korchagina,[a] Konstantin P. Volcho,[a,c] Nariman F. Salakhutdinov[a,c]

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Abstract: Compounds simultaneously carrying the monoterpene and coumarin moieties have been tested for cytotoxicity and inhibition of activity against influenza virus A/California/07/09 (H1N1)pdm09. The structure of substituents in the coumarin framework, as well as the structure and the absolute configuration of the monoterpenoid moiety, are shown to significantly influence the anti-influenza activity and cytotoxicity of the compounds under study. The compounds with a bicyclic pinane framework exhibit the highest selectivity indices (the ratios between the cytotoxicity and the active dose). The derivative of (-)-myrtenol **15c**, which is characterized by promising activity, low cytotoxicity, and synthetic accessibility, has the greatest potential among this group of compounds. It exhibited the highest activity when added to the infected cell culture at early stages of viral reproduction.

Keywords: terpene, coumarin, anti-influenza activity, antivirals, cytotoxicity, influenza A virus, aurapten, myrtenal

Influenza A virus is the major cause of seasonal or pandemic influenza worldwide. These annual epidemics are estimated to result in about 3 to 5 million cases of severe illness, and about 250,000 to 500,000 deaths.[1] New influenza viruses are constantly evolving by mutation or by reassortment, giving rise to new strains that can infect people who are immune only to the pre-existing influenza strains.[2] Although vaccination against the virus is quite effective, low-molecular anti-influenza drugs are the first line of protection against the virus during an epidemic outbreak, since an effective vaccine for the circulating strains usually takes at least 6 months to be developed.[3] The ability of the influenza virus to develop resistance to the available

drugs is a serious problem[4,5] that necessitates designing new structural types of drugs with novel targets, improved antiviral effects, higher safety, and increased tolerability.

An important area in searching for novel antiviral agents is using natural compounds, including marine natural products,[6] monoterpenoid derivatives,[7-13] phenylethanoid glycosides,[14] etc., as starting compounds. Natural coumarins and their derivatives are attracting significant attention as lead structures to search for orally bioavailable antiviral agents.[15] Thus, it has recently been suggested using the pharmacophore-based virtual screening of the library of natural compounds taken from the Princeton database that some coumarin derivatives, for example **1** (Fig. 1), may act as novel neuraminidase inhibitors.[16] Coumarin derivative **2** was identified as a promising anti-influenza agent by cell-based high-throughput screening of 20,000 compounds.[17] The detailed studies focused on structure–activity relationship revealed that BPR2-D2 (Fig. 1) exhibits an excellent antiviral efficacy against the oseltamivir-resistant virus.[18] virus. BPR2-D2 may target viral ribonucleoproteins that are responsible for viral RNA synthesis. A promising group of sesquiterpene coumarins with anti-influenza activity was isolated from *Ferula assafoetida*.[19] The structure of one of these compounds (**3**) is presented in Fig. 1. This compound contains a set of functional groups with fixed stereoconfiguration; synthesizing this compound is very challenging. We supposed that replacement of the sesquiterpene moiety with a monoterpene might give rise to new synthetically accessible compounds with anti-influenza activity. Hence, this study was aimed at searching for novel agents that would possess activity against H1N1 influenza virus among synthetic coumarin derivatives containing a monoterpenoid moiety.

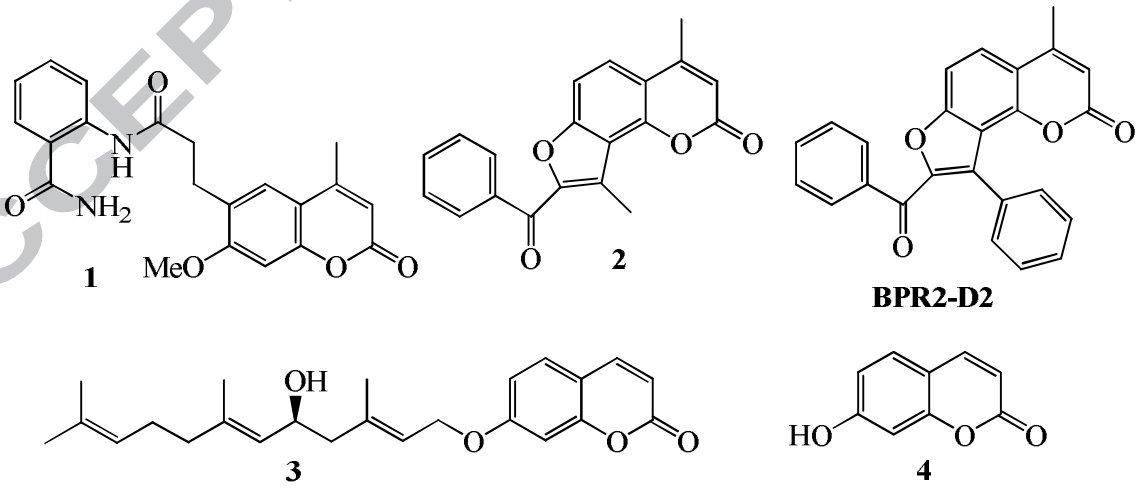
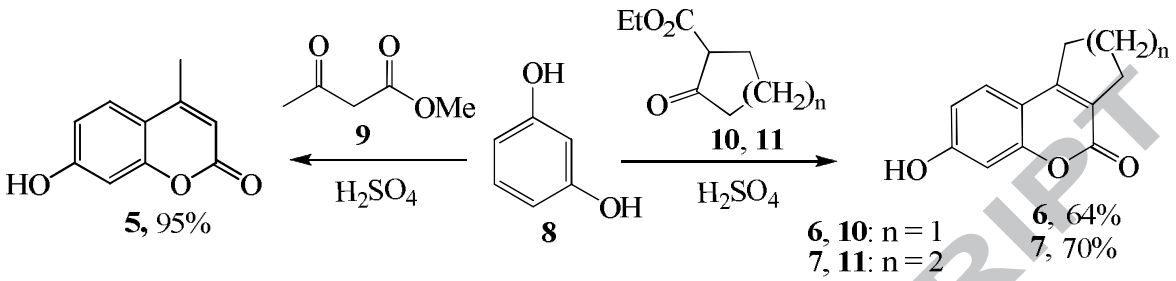


Fig. 1. Structures of anti-influenza active coumarin derivatives and umbelliferone.

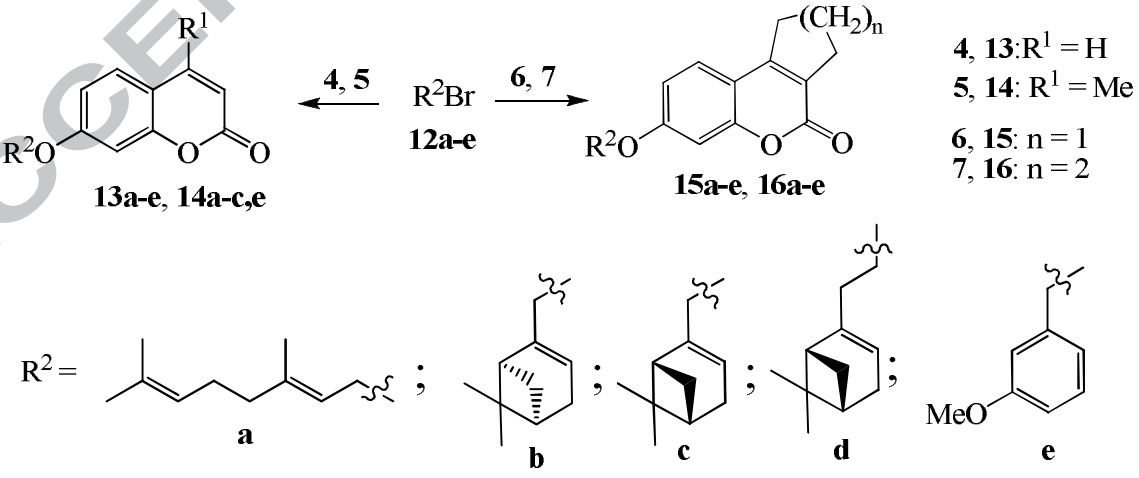
The commercially available umbelliferone **4** (Fig. 1) and its analogues **5-7** synthesized *via* interaction between resorcin **8** and esters of the corresponding β-keto acids **9-11** (Scheme 1) as described previously[20,21] were used as a coumarin component.



Scheme 1. Synthesis of coumarins **5-7** and their yields.

Aurapten **13a** and its analogues **13b-e** (Scheme 2) were obtained by interaction of umbelliferone **4** with monoterpenoid bromides **12a-d** and, for the sake of comparison, with aryl bromide **12e** using DBU in DMF.[22] Bromides **12a**, **12b**, **12c**, **12d** and **12e** were synthesized by interaction between the corresponding alcohols and PBr_3 with the yields of 91%, 55%, 60%, 24% and 65%, respectively.[23] Compound **12d** obtained by interaction between nopol and PBr_3 was insufficiently pure, thus making it necessary to use column chromatography for purification and abruptly decreasing its yield.

In a similar manner, compounds **14a-c**,**e**, **15a-e**, and **16a-e** were prepared as described previously[21] using K_2CO_3, ethanol, and coumarins **5-7** as phenol components.[24] The products were purified by recrystallization or column chromatography (the yields of 29-56%). the reaction of nopyl bromide **12d** with methylcoumarin **14** was not successful due to the formation of a complex reaction mixture with high level of resinification.



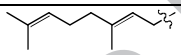
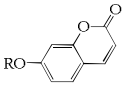
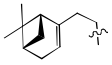
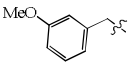
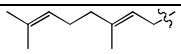
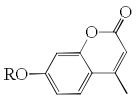
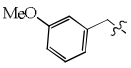
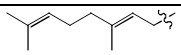
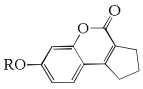
Scheme 2. Synthesis of substituted coumarins.

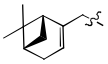
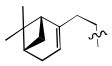
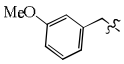
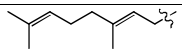
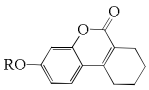
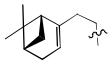
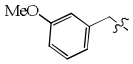
The water/octanol partition coefficient (Log P)) is often considered to be an important molecular descriptor as it is linked to toxicity issues and oral bioavailability. The Calculated Log P (cLog P) data are presented in Table 1. Although cLog P of all the compounds carrying the monoterpene and coumarin moieties exceeds the Lipinski's cLog P = 5 limit,[25] most of them are within the known drug chemical space according to this criterion (cLog P ≤ 6.5).[26]

The antiviral activity of the synthesized compounds was studied[27] against the pandemic influenza virus A/California/07/09 (H1N1)pdm09 cultivated in cell culture by the technique described earlier.[28] Cytotoxicity of the compounds was evaluated[29] in uninfected MDCK cells as described previously.[30]The selectivity index was calculated for each derivative based on the data obtained. The compounds with SI = 10 and higher were regarded as active. The test results are summarized in Table 1. Rimantadine was taken as a reference drug due to its polycyclic structure being close to the pinane scaffold used in the study.

Table 1.

Antiviral activity and cytotoxicity of compounds **13a-e, 14a-c,e, 15a-e, and 16a-e** against influenza virus A/California/07/09 (H1N1)pdm09 in MDCK cells

Compound	R	cLogP[a]	CC_{50}[b], μM	IC_{50}[b], μM	SI[d]
13a		5.69	7±1	7±2	1
13b		5.04	7±0	>10	1
13c		5.04	310±22	>101	3
13d		5.52	10±1	0.5±0	20
13e		3.35	>1170	>1170	1
14a		6.28	28±2	>10	3
14b		5.63	130±9	10±2	13
14c		5.63	30±2	4±1	9
14e		3.94	>1115	1014±113	1
15a		6.75	>888	>888	1
15b		6.09	133±8	33±5	4

15c		6.09	982±47	36±5	28
15d		6.57	>857	21±3	41
15e		4.40	>932	>932	1
16a		7.31	100±6	>85	1
16b		6.66	415±29	149±18	3
16c		6.66	857±71	64±5	13
16d		7.14	18±1	3±1	6
16e		4.97	>982	>982	1
Rimantadine			363 ± 20	41 ± 7	9

[a] cLog P Calculated using the ACD/LogP ChemSketch 12 software

[b] CC_{50} is the median cytotoxic concentration; i.e., the concentration causing 50% cell death.

[c] IC_{50} is the 50% inhibiting concentration; i.e., the concentration causing a 50% decrease in virus replication.

[d] SI is the selectivity index, which is the CC_{50}/IC_{50} ratio.

First, we studied the antiviral activity of aurapten **13a** that is the most abundant natural prenyloxycoumarin. Aurapten **13a** exhibits versatile biological activities, but to the best of our knowledge, no data on its antiviral activity have been reported.[31] We found that aurapten **13a**, identically to its analogue **13b** containing a (+)-myrtenol fragment, exhibits high cytotoxicity against MDCK cells that is comparable to its antiviral activity and, therefore, possesses zero selectivity (SI = 1). Compound **13c** synthesized from (-)-myrtenol turned out to be both less toxic and less active than its enantiomer **13b**. The methylene moiety inserted between the monoterpene and coumarin moieties when proceeding to compound **13d** drastically increased its activity: IC_{50} of this compound lay in the nanomolar concentration range, which made it possible to achieve a good selectivity index (SI = 20) despite the significant cytotoxicity. The use of an aromatic moiety (**13e**) resulted in complete disappearance of antiviral activity. Hence, compound **13d** is the most promising umbelliferone derivative.

When proceeding to type **14** compounds, insertion of methyl group into the coumarin framework decreased toxicity and increased the selectivity index in all compounds excluding **14c**, while the level of activity remained the same. Compound **14b** showed the greatest

selectivity index (SI = 13). Unfortunately, we did not succeed in obtaining an analogue of compound **13d** with an acceptable purity for compounds of this structural type.

Compound **15a**, which has the cyclopentane ring annulated with the coumarin framework and containing a geraniol moiety, was inactive. Meanwhile, compounds **15b-d** containing the bicyclic monoterpene moiety exhibited a significant antiviral activity. Due to their low cytotoxicity, compounds **15c** and **15d** synthesized using (-)-myrtenol and nopol, respectively, exhibited a selectivity index as high as 41.

After proceeding to type **16** compounds that contained the cyclohexane ring annulated with the coumarin framework, we found that the selectivity index was higher than 10 only for compound **16c**. Although compound **16d** exhibited good antiviral activity, its selectivity index was low because of high cytotoxicity.

The compounds containing an aromatic moiety (**13e**, **14e**, **15e**, and **16e**) exhibited no anti-influenza activity regardless of the structure of the coumarin component in the molecule.

Hence, compounds **15c** and **15d**, which exhibit both high activity and low cytotoxicity with the highest selectivity indices, are the most promising ones among the compounds under study. Taking into account the availability of the starting compound ((-)-myrtenal) and simplicity of its synthesis procedure, **15c** shows greater potential among these two compounds. Compound **15c** was earlier found[21] to be a low-toxicity inhibitor of Tdp1, one of the enzymes of DNA repair system.[32] These inhibitors can increase the potency of some anti-tumor agents whose activity is based on inhibition of topoisomerase 1 (Top1), even though they exhibit no intrinsic cytotoxicity.[33] It should be mentioned that inhibition of Tdp1 does not cause any problems associated with toxicity in the absence of Top1 inhibitors and, therefore, is expected to cause no adverse events when inhibitors are used in anti-influenza therapy. Indeed, it is known that Tdp1-/- mice were fertile and had a normal life expectancy.[34]

As can be seen from the data presented, 5 of 19 (26%) tested compounds have demonstrated good selectivity (SI>10). All 3-metoxybenzene derivatives of coumarin (**13e, 14e, 15e, 16e**) possessed low toxicity but no virus-inhibiting activity. Elongation of the linker between the coumarine and pinane moieties in groups of **13**'s and **16**'s dramatically increased cytotoxicity (**13c** and **13d**, **16c** and **16d**).

Pinane stereoisomers conferred different toxicity levels to the compounds. In most cases, (+)-isomers were more toxic than (-)-isomers, although to different degrees.

Compounds **15c** and **15d** possessed the highest selectivity among all the compounds tested. This selectivity was provided by low toxicity rather than by very high anti-influenza activity. Compound **13d** had the best IC_{50} value but its high cytotoxicity resulted in moderate SI (20).

In order to assess what stage of the viral life cycle is affected by compound **15c**, we carried out a series of experiments that differed in time when the compound was added.[35] It turned out (Fig. 2) that **15c** exhibits the highest activity when added to the infected cell culture at early stages of viral reproduction (1-2 hours after infection). The potency of the agent decreased with time. Viral infectivity statistically did not differ from the control values starting with 6 hours after infection.

Fig. 2. Time-of-addition activity of **15c** against influenza virus A/PR/8/34 (H1N1). A/Puerto Rico/8/34 (H1N1) was absorbed to MDCK cells (m.o.i. 10) for 1 h at 4 ^{o}C. After removal of the non-absorbed virions, the plates were incubated for 8 h at 36^{o}C in 5% CO_2. The starting point of incubation was denoted as 0. Cells were treated with **15c** (500 μmol) for the following time: (-2) – (-1) (before being infected); (-1) – 0 (simultaneously to absorption); 0 – 2; 2 – 4; 4 – 6; 6 – 8 and (-2) – 8 h. After 8 h of growth, the virus titer was determined in the culture medium according to $TCID_{50}$ in the MTT assay.

Hence, the specific target of **15c** is critical for viral cycle at its early stages. The most probable targets are viral hemagglutinin or proton channel M2. It should be mentioned that the virus used for testing is resistant to adamantane derivatives due to S31N substitution in M2 protein. This results in low activity of rimantadine against this virus (SI=9, Table 1). Several attempts have been made to develop the "universal" M2 inhibitor; *i.e.*, a compound that would inhibit both wild-type (with serine at position 31, M2wt) and resistant (bearing substitution S31N, V27A, L26I or G34E) M2 channels.[36] Thus far, no compound has been identified with equal activity against M2wt and S31N viruses[37], although a spiroadamantane amine derivative has recently been shown to inhibit M2wt and V27A resistant mutant in *in vitro* and *in vivo* models.[38]

Most of the compounds for inhibiting M2 ion channel are polycyclic amines.[37] The Coumarine derivatives studied here are of different chemical structures and should therefore be considered as having different viral target(s). Computer simulation of the interaction between the two most active compounds, **15c** and **15d**, suggests that they may bind to viral hemagglutinin between the head and the stalk in the region of fusion peptide. Their bulky polycyclic moiety is inserted into a pocket formed by amino acids 556-IEMNI-561 (**15c**), probably via hydrophobic interaction with I556 and I561, or into a wide pocket formed by L48-K49-S81 and S118 (**15d**). The tricyclic coumarine-derived moiety is stabilized by either serines 291 and 292 (**15c**) or T264

and F563 (**15d**) on one side and either E305 and K281 (**15c**) or F264 (**15d**) on another side. Using this mechanism, compounds might suppress the conformational changes in an HA molecule, thus interfering with membrane fusion.

On the other hand, one cannot rule out that these compounds are directed against cellular target(s) important for viral life cycle at early stages, such as the components of cytoskeleton, transport proteins, lysosomal components, etc. Further studies are therefore needed to identify the exact mechanism of activity of coumarin derivatives, as well as their range of activity against influenza viruses.

Hence, we have studied the anti-influenza activity of a series of compounds containing both the coumarin and monoterpenoid moieties. The compounds having the bicyclic pinane framework were shown to exhibit the highest selectivity index (the ratio between cytotoxicity and the active dose). Both the structure of substituents in the coumarin framework and the absolute configuration of the monoterpene moiety have a significant effect on the selectivity index. The (-)-myrtenol derivative **15c**, which is simultaneously characterized by promising activity, low cytotoxicity, and synthetic accessibility, shows the greatest potential among this group of compounds. It is found to exhibit the highest activity when added to the infected cell culture at the early stages of viral reproduction.

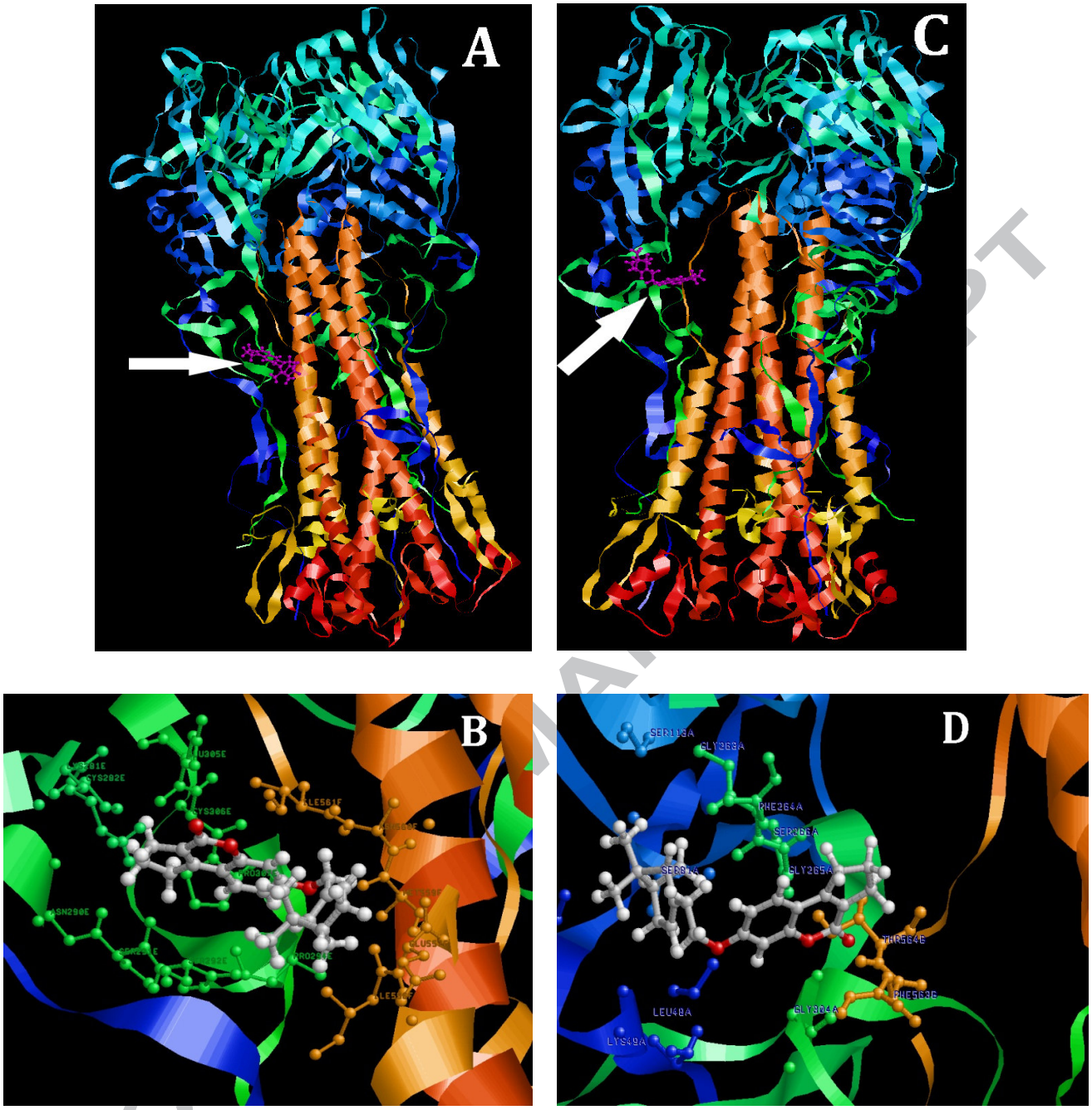


Fig. 3. Model of binding of coumarine derivatives **15c** (A, B) and **15d** (C, D) to trimeric hemagglutinin of influenza virus A/Puerto Rico/8/34 (H1N1). A, C – general view. Ligands are shown in magenta and indicated with arrows. B, D – a close view with specific amino acids.

Acknowledgments

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20. *General procedure for the synthesis of coumarins* ***5-7***. Conc. H_2SO_4 (5 mL, 94 mmol) was added dropwise to cooled (0-5 °C) solution of resorcin **8** (45 mmol) and appropriate β-keto esters (**9-11**) (45 mmol) in dry ethanol (15 mL) with vigorously stirring. The mixture was stirred until to be congealed, left overnight at r.t., and poured into ice water (150 mL). The resulting solid was filtered off and crystallized from ethanol-water (75%). The yields of **5**-**7** were 95%, 64% and 70% respectively.
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22. *Synthesis of compounds **13a-e***. DBU (1.0 mmol) and corresponding bromide **12a-e** (0.75 mmol) were added to compound **4** (0.5 mmol) in dry DMF (5 mL) at r.t. under stirring. The reaction mixture was stirred at r.t. for 15 minutes, and then heated at 60 °C for 5 hours. H_2O (15 ml) was added and the product was extracted with ethyl acetate. The extracts were washed with brine, dried with Na_2SO_4 and evaporated.
23. *Synthesis of compounds **12a-e***. Geranyl bromide **12a** was synthesized from geraniol *via* the reaction with PBr_3. PBr_3 (8.9 mmol) was added to cooled (0-5°C) solution of geraniol (26.7 mmol) in dry ether (30 ml) and the reaction mixture was stirred for 2 h at r.t. Saturated aqueous $NaHCO_3$ was added and the product was extracted with ether. The extracts were washed with brine, dried with Na_2SO_4 and evaporated. Other used bromides **12b-e** were synthesized as described above. Compounds **12a**, **12b**, **12c** and **12e** (the yields 91%, 55%, 60% and 65%, respectively) were sufficiently pure and used for the next step without purification. The compound **12d** was purified by column chromatography on SiO_2, eluent - hexane (yield 24%).
24. *Synthesis of compounds **13a-e, 14a-c,e, 15a-e** and **16a-e.*** K_2CO_3 (1.0 mmol) and corresponding bromides **12a-e** (0.75 mmol) were added to corresponding compound **5-7** (0.5 mmol) in dry ethanol (5 mL) at r.t. under stirring. The reaction mixture was stirred at r.t. for 15 minutes, and then heated at 60 °C for 5 hours. The hot solution was filtered; the filtrate was kept at -18 °C for 48 hours. The products were isolated in the individual form by recrystallization from ethanol by column chromatography on silica gel, eluent - hexane, solution containing from 25 to 100% chloroform in hexane, ethanol.
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27. The compounds in appropriate concentrations (300 - 3 μg/ml) were dissolved in 0.1 ml DMSO and final solutions were prepared by adding MEM with 1 μg/ml trypsin. Compounds were incubated with MDCK cells for 1 h at 36^0C. Each concentration of the compounds was tested in triplicate. The cell culture was then infected with influenza virus A/California/07/09 (H1N1)pdm09 (moi 0.01) for 24 h at 36^0C in the presence of 5% CO_2. A virus titer in the supernatant was determined by hemagglutination test after cultivating of the virus in MDCK cells for 48 h at 36^0C in the presence of 5% CO_2. For

calculations, virus titer was expressed as per cent of the titer in control wells without compounds. The 50% inhibiting concentrations (IC_{50}) and the selectivity index (SI, the ratio of CC_{50} to IC_{50}) were calculated from the data obtained.

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29. The microtetrazolium test (MTT) was used to study the cytotoxicity of the compounds. Briefly, series of three-fold dilutions of each compound in MEM were prepared. MDCK cells were incubated for 48 h at 36°C in 5% CO_2 in the presence of the dissolved substances. The cells were washed twice with phosphate-buffered saline (PBS), and a solution of 3-(4,5-dimethylthiazolyl-2) 2,5-diphenyltetrazolium bromide (ICN Biochemicals Inc. Aurora, Ohio) (0.5 μg/ml) in PBS was added to the wells. After 1h incubation, the wells were washed and the formazan residue was dissolved in DMSO (0.1 ml per well). The optical density of cells was then measured on a Victor 2 1440 multifunctional reader (Perkin Elmer, Finland) at wavelength of 535 nm and plotted against the concentration of compounds. Each concentration was tested in three parallels. The 50% cytotoxic concentration (CC_{50}) of each compound was calculated from the data obtained.
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35. To determine the stage of the viral life cycle that is affected with the compound, cells were seeded into 24-wells plates and incubated with influenza virus A/Puerto Rico/8/34 (H1N1) (m.o.i. 10) for 1 hour at 4°C. After washing of non-absorbed virions for 5 min with MEM, fresh portion of compound-containing medium was added and plates were

incubated for 8 hours at 36°C at 5% CO_2. The starting point of this incubation was referred as 0 hours. **15c** (final concentration 500 μM) was dissolved in MEM and cells were treated with **15c** for the time periods as following: (-2) – (-1) (before infecting); (-1) – 0 (simultaneously to absorption); 0 – 2; 2 – 4; 4 – 6; 6 – 8 hours post infection (hpi). The treatment (-2) – 8 hpi was considered as a positive control. In each case after incubation the medium was removed and cells were washed for 5 min with MEM. After 8 hours of growth, the infectious titer of the virus was determined in culture medium and cells as described above.

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39. For computer modeling, the crystal structure of hemagglutinin trimer was taken from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The molecules of 15c and 15d were structurally optimized by Hyperchem software (Hypercube Inc., Gainesville, FL, USA) followed by computer docking with Hex 8.0.0 (accessible at http://hex.loria.fr/dist/index.php).