Full Paper

Synthesis of Imperatorin Analogs and Their Evaluation as Acetylcholinesterase and Butyrylcholinesterase Inhibitors

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In this study, we synthesized several imperatorin analogs using imperatorin and xanthotoxin as substrates. The anti-cholinesterase activities of all compounds were evaluated in *in vitro* experiments according to the modified Ellman's method. For each synthesized compounds, IC_{50} values for both enzymes were established. Galantamine hydrobromide was used as a positive control in the enzymatic experiments. All active compounds showed selectivity toward butyrylcholinesterase (BuChE) rather than acetylcholinesterase. The most active ones were 8-(3-methylbutoxy)-psoralen and 8-hexoxypsolaren with IC_{50} values for BuChE of around 16.5 and 16.4 μM, respectively. The results of our study may be considered as the beginning of a search for potential anti-Alzheimer's disease drugs based on the structure of natural furocoumarins.

Keywords: Acetylcholinesterase / Alzheimer's disease / *Angelica archangelica* / Butyrylcholinesterase / Furocoumarins

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Introduction

Furocoumarins are naturally occurring compounds produced by a wide variety of plants. The chemical structure of furocoumarins consists of furan ring fused with coumarin (benzo-α-pyrone). The furan motif can be attached in a different manner to produce several types of furocoumarins but there are only two most common structure types. The first one possesses an angelicin-type ring arrangement and the second, even more popular group includes psolaren-type furocoumarins often called linear furocoumarins. Some psolarens, e.g., xanthotoxin and its derivatives, have been used as photosensitizing compounds for the treatment of psoriasis, vitiligo, and other skin infections [1–3]. In the last decades, a great deal of research has been carried out on the bioactivity of linear furocoumarins. Initially, antimicrobial property of psoralens was noted [4–6]. Later studies have shown anti-inflammatory [7–12], antitumor [13], antidiabetic [14], hepatoprotective [14, 15], and anticonvulsant [16–18] activity of these compounds. Imperatorin enhances the effectivity of traditional antiepileptic drugs [16, 18]. Moreover, other studies have shown that psoralens can modulate $GABA_A$ receptor function [19]. Recently, it has been proved that linear furocoumarins such as xanthotoxin, bergapten, imperatorin, isoimperatorin, and oxypeucedanin inhibit acetylcholinesterase activity *in vitro* [20, 21]. It suggests their possible use in prevention and treatment of Alzheimer's disease (AD). The use of acetylcholinesterase inhibitors (AChEI) in AD increases the level of neural acetylcholine (ACh) and improves cognitive functions [22]. Besides, acetylcholinesterase not only plays a crucial role in AD because of its catalytic function but also is claimed to be involved in the formation of β-amyloid in central nervous system (CNS) [23]. Apart from AChEI, in the past few years there has been growing interest in butyrylcholinesterase (BuChE) and its role in AD. Some studies have shown that BuChE is also responsible for a low level of ACh in CNS

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especially in the advanced phase of AD [24]. This is why the usage of double AChE–BuChE inhibitors such as rivastigmine is nowadays recommended in the treatment of AD.

Archangelica angelica L., syn. *Angelica officinalis* HOFFM or Angel's herb, as it is commonly called, is a biennial plant from *Apiacae* family, which is a popular vegetable and herb cultivated especially in Europe and Asia for medical and culinary purposes. In some countries extracts from *Angelica* are used as an appetite stimulant, an antispasmodic, and a remedy for gastrointestinal symptoms such as bloating, poor digestion, eructation, and flatulence [25]. It is well known that traditionally used roots of Angel's herb, besides volatile oil used for aromatization of liqueurs and vermouths [25], are rich source of coumarins. After cultivation of *A. angelica* L. for medical and culinary applications companies obtain vast amounts of fruits and other aerial parts that become useless. Some studies have proven that also fruits of *Angelica* contain coumarins in high quantities [26]. In our previous study we have shown that psoralens from *A. angelica* L. inhibit not only AChE activity but BuChE as well [27]. Some compounds isolated from Angel's herb in our laboratory exhibited high selectivity toward BuChE [27]. Considering this, we have decided to use fruits of *A. angelica* L. as a rich source of linear furocoumarins for chemical transformations and further anti-AChE–BuChE activity studies.

Results and discussion

In the presented study, we found furocoumarins to be selective BuChE inhibitors. Previous research has shown that some psoralens may exhibit anti-AChE and anti-BuChE activity but there are only few studies considering any relationship between the structure and the effect on inhibition of cholinesterase [20, 21, 27–30]. In these publications, authors indicated that the presence of a side chain at C-8 position of furocoumarin nucleus is crucial for the inhibitory activity toward BuChE. Moreover, the C-5 position must not be occupied [27]. In the case of AChE, the studies showed that furocoumarins should have elongated side chain in the position C-5 or C-8 in order to achieve better AChE inhibitory activity compared to the presence of single $-OCH_3$ group [30].

Initially, we isolated imperatorin (**1**), which was a necessary substrate for further chemical transformations. Using fruits of *A. angelica* L., which become useless after cultivation, we obtained imperatorin albeit in rather inadequate amounts and therefore we decided to use commercially available xanthotoxin (**2**) as a substrate for further transformations. This compound also occurs naturally in extracts from Angel's herb. The xanthotoxin was used only for the preparation of xantotoxol (**3**) as a substrate for the formation of the corresponding alkyl ethers. Most of the obtained compounds were previously described in the literature [31–35]. Compound **6** was reported as an anti-inflammatory agent but no spectroscopic data were given [8]. To our knowledge, compound **8** is a new substance. Some of the synthesized compounds possess asymmetric centers that lead to the formation of enantiomers. Since the reactions that we used for the modification of heraclenin (**9**) were not stereoselective, we obtained racemic mixtures in the case of compounds **10** and **11**.

As previously reported, the presence of single furocoumarins moiety is not enough for inhibitory activity toward AChE and BuChE [21]. The most important for cholinesterase inhibition is the structure of the side chain of the condensed furocoumarin rings. We therefore synthesized several compounds of different side chain structure. This part of the molecule is responsible for the lipophilicity, which may affect the biological profile. Consequently, we decided to check if there is a simple correlation between the structure and anti-AChE/BuChE activity for prepared compounds.

AChE and BuChE are a dyad of enzymes occurring not only in CNS but also in other tissues. Both enzymes catalyze hydrolysis of selected ester bonds in organic compounds. AChE is responsible for rapid degradation of neurotransmitter – ACh in neurons. Although BuChE may exhibit the same activity, it is also believed to have a profound toxicological role as it hydrolyzes ester bonds in drugs and other xenobiotics. AChE has characteristic narrow gorge leading to its active site while BuChE gorge is wider [36, 37]. That is probably why AChE can hydrolyze only small acyl esters in contrary to BuChE, which can accommodate larger acyl groups and hence larger substrates. Accordingly, we expected that furocoumarins with longer and more branched side chain would be more selective toward BuChE.

On the basis of the results obtained from anti-AChE/BuChE assays, we determined the IC_{50} values for majority of the compounds based on the concentration–inhibition curves as shown in Table 1.

As we expected, some of the compounds exhibited selectivity toward BuChE. The most potent were derivatives **5** and **6**, which contain simple saturated carbon chains. It may be related to the optimal size of those molecules (they are too large for AChE but, in the case of BuChE, they fit perfectly to the active site of the enzyme). It is interesting to note that compound **4** is less effective, although it has similar structure to imperatorin. Inactivity of compound **8** is probably caused by too long side chain that makes it impossible to get near the active site of both enzymes. In the case of imperatorin derivatives, it is important that compound **11** contains the ester bond in its structure that should enhance the inhibitory activity of both cholinesterases. Nevertheless, we did not notice that the presence of an extra polar groups such as

Table 1. IC_{50} values for inhibition of AChE and BuChE by synthesized furocoumarins.

	AChE		BuChE
	IC_{50} [a] (μM)	$\%_{inh}$ [b] at 50 μM	IC_{50} [a] (μM)
Imperatorin (**1**)	>50	10.2 ± 1.8	31.4 ± 11.9
Xanthotoxin (**2**)	>50	11.0 ± 4.4	>50
Xanthotoxol (**3**)	>50	NA[d]	>50
8-Butoxypsoralen (**4**)	>50	17.8 ± 5.5	41.4 ± 10.6
8-Hexoxypsoralen (**5**)	>50	25.6 ± 1.5	16.5 ± 0.5
8-(3-Methylbutoxy)-psoralen (**6**)	>50	22.1 ± 4.4	16.4 ± 7.4
8-Benzyloxypsoralen (**7**)	>50	10.3 ± 6.8	48.1 ± 5.4
8-Decyloxypsoralen (**8**)	>50	NA[d]	>50
Heraclenin (**9**)	NT[c]	NT[c]	NT[c]
tert-O-Methylheraclenol (**10**)	>50	21.9 ± 1.9	>50
tert-O-Methylheraclenol acetate (**11**)	>50	29.1 ± 3.4	50.4 ± 12.3
Galanthamine hydrobromide	1.5 ± 0.5	95.4 ± 0.2	9.4 ± 1.1

All values were calculated from three independent experiments performed in triplicate.
[a] IC_{50} – values means ± standard deviation.
[b] $\%_{inh}$ – values means ± standard deviation.
[c] NT – not tested.
[d] NA – not active at 50 μM.

$-OCH_3$, –OH, or $-OCOCH_3$ is crucial for a better selectivity toward BuChE as our last results would suggest [27].

In order to investigate the binding mode for interaction of the compounds with AChE and BuChE and to explain different binding affinities, docking studies were performed. The activity of compound **4** is lower than that of compound **1**. The binding of both compounds to AChE (Fig. 1) indicated that π–π stacking interactions with Trp84 and Phe330 are important. Additionally the double bond in prenyl group of compound **1** serves as a specific π-hydrogen bond acceptor for both enzymes [38], that may explain the lower activity of compound **4**.

The docking studies indicated that compounds **5** and **6** can form hydrogen bonds with Gly117 and with side chain of Ser198 in the binding pocket of BuChE (Fig. 2). These H-bonds are believed to contribute to the higher affinity of these compounds toward BuChE than AChE, which may account for their selectivity toward BuChE. For comparison, compounds **5** and **6** were located at the binding pocket of AChE next to large aromatic amino acids that may cause steric interactions (Fig. 3).

In the case of compound **8**, the binding to AChE may be impossible. The interactions of a long side chain of compound **8** with AChE are prevented due to the presence of aromatic amino acids in the binding site: Phe288, Phe290, Phe330, and

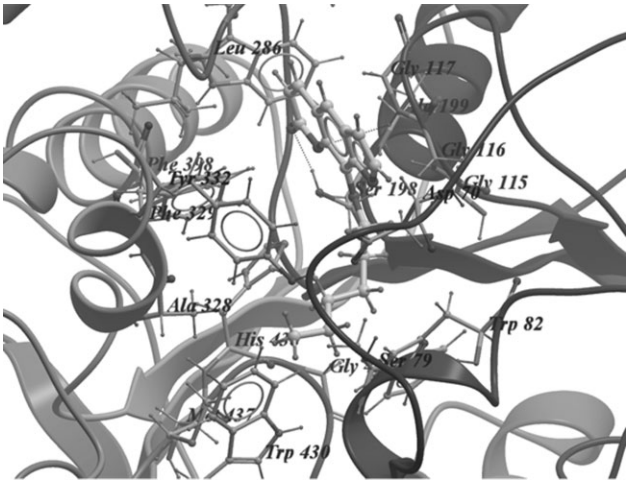


Figure 2. Binding interactions of compound **5** with selected residues of BuChE (H-bonds are indicated as black dotted lines).

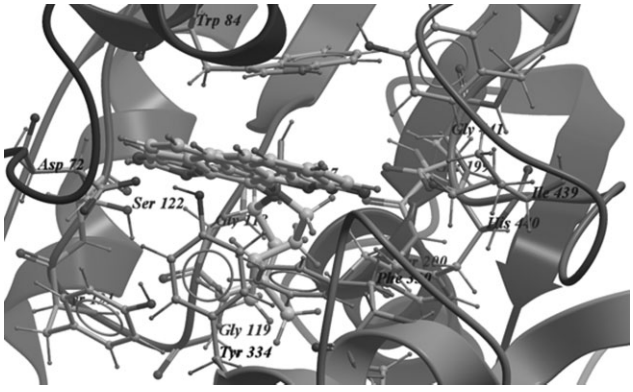


Figure 1. Binding interactions of compound **1** and **4** with selected residues of AChE.

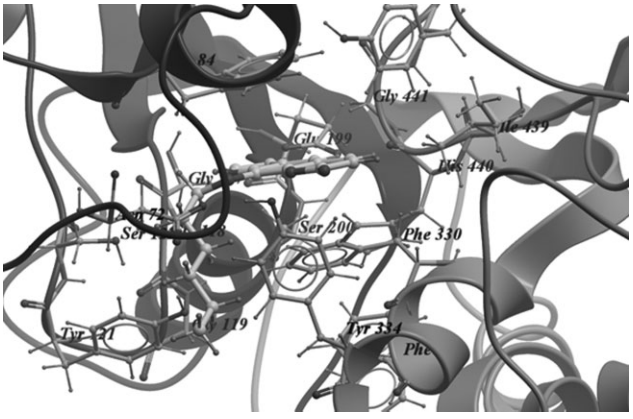


Figure 3. Binding interactions of compound **5** with selected residues of AChE.

Tyr334. Although the binding pocket in BuChE is larger than that in AChE [39], the binding of compound **8** is not possible because of the size of the molecule.

Conclusion

In conclusion, the presented study indicates that furocoumarins can be considered as selective BuChE inhibitors. Their activity depends on the structure of the molecule and can be modified by a simple chemical transformation. Therefore, plants containing significant amounts of furocoumarins should be considered as a rich source of substrates for chemical transformations leading to new and more effective compounds. Molecular docking studies were carried out to further investigate the binding modes of studied compounds with both cholinesterases.

Experimental

General procedure

^{1}H and ^{13}C NMR spectra were recorded on Varian Unityplus-200 spectrometer. MS analyses were performed using LCT (TOF) and Amazon SL Brucker apparatus. The X-ray data for a crystal structure were collected using Oxford Diffraction Xcalibur R κ-axis diffractometer equipped with CCD detector. Data were collected at room temperature using the monochromated Cu Kα radiation. Thin layer chromatography (TLC) was carried out on silica gel F_{254} plates (Merck, Darmstadt, Germany). Silica gel 60 (230–400 mesh) for column chromatography was purchased from Merck.

Chemicals

Acetylcholinesterase (AChE) (EC 3.1.1.7. type VI-S from Electric Eel), BuChE (EC 3.1.1.8. from horse serum), acetylthiocholine iodide (ATCI), *S*-butyrylthiocholine chloride (BTCCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tris–HCl was obtained from Merck. Xanthotoxin was obtained from Acros Organics (New Jersey, USA). Alkyl bromides used for modifications, anhydrous aluminum chloride, anhydrous sodium sulfate, anhydrous potassium carbonate, pyridine, acetic anhydride, and chromatographic solvents (cyclohexane, ethyl acetate, chloroform, isopropanol) were obtained from POCh (Gliwice, Poland).

Plant material extraction and imperatorin (1) isolation

Dried and powdered fruits (200 g) were extracted with hexane (3×700 mL) in a water bath at 70°C for 5 h each time. The collected extracts were evaporated giving a residue of 22 g. Hexane-soluble residue was subjected to silica gel column chromatography and eluted with $CHCl_3$ to obtain six main fractions based on their TLC profile. Fraction 2 (15 g) was rechromatographed on a silica gel column with a gradient of hexane–$CHCl_3$ from 97.5:2.5 to 85:15. One hundred ten fractions were collected and pooled into 10 main fractions based on their TLC profile. Imperatorin (270 mg) was isolated from fr. 6 by recrystallization from petroleum ether, m.p. 93–95°C (lit. m.p. 96–98°C [40]).

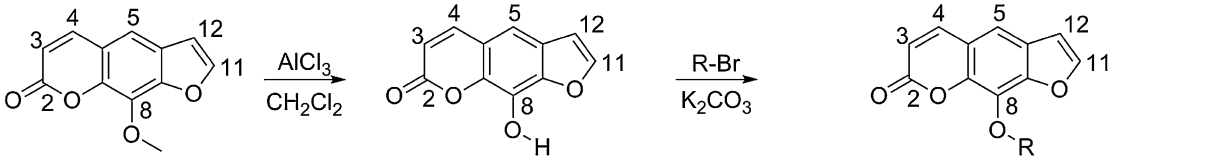
During the isolation process we obtained other less pure fractions of imperatorin, but suitable for chemical modification. Total amount of imperatorin was 990 mg (85–95% HPLC).

Synthetic approach

We used xanthotoxin supplied by Acros Organics or imperatorin (**1**) isolated in our laboratory as starting material. Xanthotoxin (**2**) was first demethylated with $AlCl_3$ to obtain 8-hydroxypsoralen (xanthotoxol) (**3**) according to the method previously described [41] with our modifications. It was then used for the preparation of the corresponding alkyl ethers using the method described in literature [34, 41–43] (Scheme 1). The epoxidation of imperatorin was carried out using MCPBA [35]. The epoxide ring was opened with oxalic acid to be next acetylated in Ac_2O/pyridine system (Scheme 2) [32].

*Preparation of 8-hydroxypsoralen (9-hydroxy-7H-furo[3,2g]chromen-7-one) (**3**)*

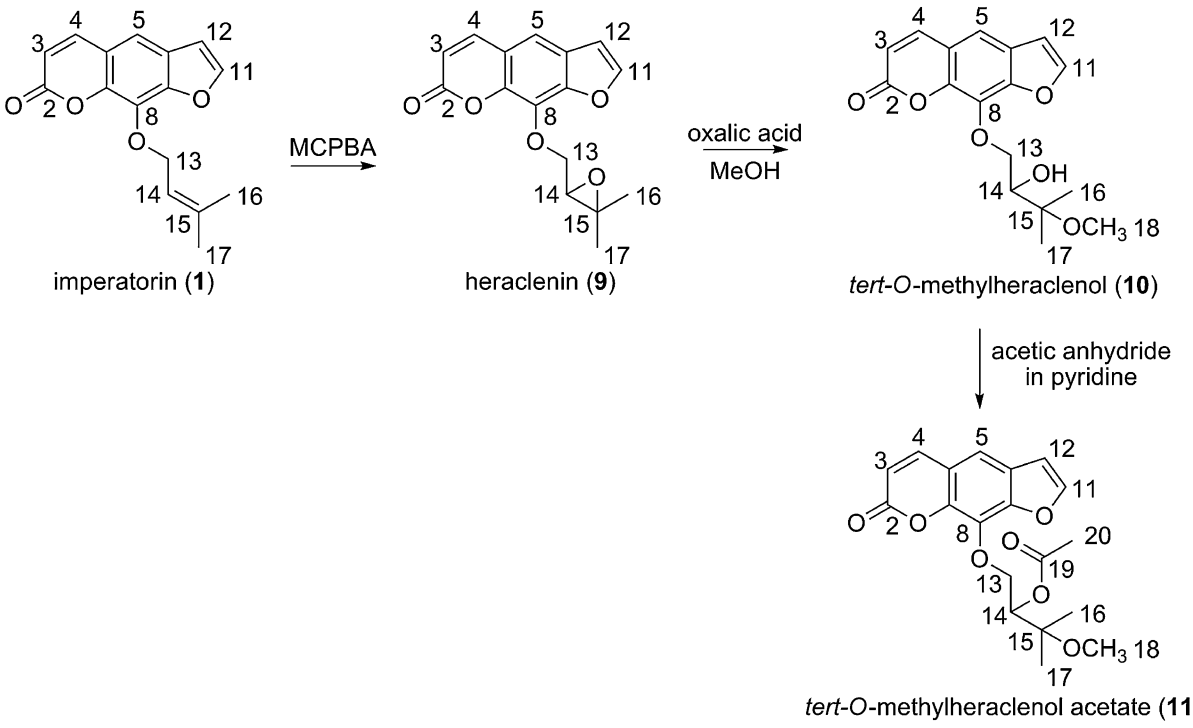
To the solution of xanthotoxin (500 mg, 2.31 mmol) in dichloromethane (25 mL) anhydrous aluminum chloride (6 g, 45 mmol) was added. The mixture was stirred for 24 h at room temperature. Then 20 mL of water was added in small portions followed by the



xanthotoxin (**2**) xanthotoxol (**3**)

	R
8-butoxypsoralen (**4**)	$CH_2CH_2CH_2CH_3$
8-hexoxypsoralen (**5**)	$CH_2(CH_2)_3CH_2CH_3$
8-(3-methylbutoxy)-psoralen (**6**)	$CH_2CH_2CH(CH_3)_2$
8-benzyloxypsoralen (**7**)	$CH_2C_6H_5$
8-decyloxypsoralen (**8**)	$CH_2(CH_2)_7CH_2CH_3$

Scheme 1. Chemical transformations of xanthotoxin.



Scheme 2. Chemical transformations of imperatorin.

addition of 5 mL of concentrated hydrochloric acid. After cooling, the mixture was extracted three times with 25 mL portions of ethyl acetate (AcOEt). Organic layers were dried over anhydrous sodium sulfate. After evaporation under reduced pressure, yellow powder was obtained (315 mg, 1.55 mmol, 67%), m.p. 247–250°C (lit. m.p. 247°C [44]).

General procedure for preparation of O-alkylated xanthotoxol derivatives

A mixture of xanthotoxol (100 mg, 0.49 mmol), anhydrous K_2CO_3 (215 mg, 1.15 mmol), alkyl or benzyl bromide (1.43 mmol), and acetone (7.5 mL) was stirred and refluxed for 24 h. Then 30 mL of water was added and the mixture was extracted three times with 25 mL portions of ethyl acetate (AcOEt). Organic layers were dried over anhydrous sodium sulfate. Removal of solvent yielded yellowish oil or solid, which was purified by column chromatography on a silica gel, eluting with mixtures of cyclohexane:ethyl acetate to give pure corresponding 8-*O*-alkyl ethers of xanthotoxol **4**–**8**.

*8-Butoxypsoralen (9-butoxy-7H-furo[3,2g]chromen-7-one) (**4**):*

An off-white powder; m.p. 79–81°C (lit. m.p. 83°C [45]); yield 30%.

*8-Hexoxypsoralen (9-hexoxy-7H-furo[3,2g]chromen-7-one) (**5**):*

An off-white powder; m.p. 54–56°C (lit. m.p. 55°C [34]); yield 35%.

*8-(3-Methylbutoxy)-psoralen (9-(3-methylbutoxy)-7H-furo-[3,2g]chromen-7-one) (**6**):*

An off-white powder; m.p. 67–72°C; yield 35%; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.77 (d, $J = 9.6$ Hz, 1H, H-4), 7.69 (d, $J = 2.3$ Hz, 1H, H-11), 7.36 (s, 1H, H-5), 6.82 (d, $J = 2.2$ Hz, 1H, H-12), 6.37 (d, $J = 9.6$ Hz, 1H, H-3), 4.52 (t, $J = 6.7$ Hz, 2H, 13-CH_2), 2.03–1.85 (m, 1H, 15-CH), 1.77 (q, $J = 6.8$ Hz, 2H, 14-CH_2), 1.01 (s, 3H, 16-CH_3), 0.98 (s, 3H, 17-CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ 160.8 C-2, 148.4 C-7, 146.8 C-11, 144.5 C-4, 143.7 C-9, 132.3 C-8, 126.2 C-6, 116.7 C-10, 114.9 C-5, 113.1 C-3, 106.9 C-12, 72.8 C-13, 39.0 C-14, 29.9 C-15, 25.0 C-16, 22.8 C-17. In the literature, no spectral data or melting point given [8].

*8-Benzyloxypsoralen (9-benzyloxy-7H-furo[3,2g]chromen-7-one) (**7**):*

An off-white powder; m.p. 118–124°C (lit. m.p. 121–122°C [31]); yield 8%.

*8-n-Decyloxypsoralen(9-n-decyloxy-7H-furo[3,2g]chromen-7-one) (**8**):*

Colorless oil; yield 32%; IR (KBr): ν cm^{-1} 3112, 2917, 1720, 1585, 1401, 1145 ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.77 (d, $J = 9.8$ Hz, 1H, H-4), 7.69 (d, $J = 2.2$ Hz, 1H, H-11), 7.35 (s, 1H, H-5), 6.82 (d, $J = 2.2$ Hz, 1H, H-12), 6.37 (d, $J = 9.4$ Hz, 1H, H-3), 4.49 (t, $J = 6.7$ Hz, 2H, 13-CH_2), 1.87 (quin, $J = 7.1$ Hz, 2H, 14-CH_2), 1.69–1.48 (m, 6H, $3 \times CH_2$), 1.46–1.18 (m, 8H, $4 \times CH_2$), 0.91–0.85 (m, 3H, 22-CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ 160.8 C-2, 148.4 C-7, 146.8 C-11, 144.5 C-4, 143.7 C-9, 132.3 C-8, 126.1 C-6, 116.7 C-10, 114.9 C-5, 113.1 C-3, 106.9 C-12, 74.4 C-13, 32.1 C-14, 30.3 C-15, 29.8 C-16, 29.6 C-17, 29.5 C-18, 27.1 C-19, 25.9 C-20, 22.9 C-21, 14.3 C-22.

LR-ESI-MS+: 365 $[M+Na]^+$, 397 $[M+MeOH+Na]^+$; HR-ESI-MS+: 365.1726 $[M+Na]^+$ (calculated for $C_{21}H_{26}O_4Na$ – 365.1729).

*Preparation of heraclenin (9-(2′,3′-epoxy-3′-methylbutoxy)-7H-furo[3,2-g]-[1]benzopyran-7-one) (**9**)*

The mixture of imperatorin (406 mg, 1.5 mmol), chloroform (25 mL), and *m*-chloroperbenzoic acid (725 mg, 3.6 mmol) was stirred at room temperature for 2 h. Then the mixture was poured into saturated solution of $NaHCO_3$ (100 mL) and extracted three times with 25 mL portions of ethyl acetate (AcOEt). Organic layers were dried over anhydrous sodium sulfate. Removal of solvent yielded a brown solid, which was purified by column chromatography on a silica gel, eluting with mixtures of cyclohexane: ethyl acetate from 100:0 to 80:20 v/v. After purification, a pale yellow powder was obtained (287 mg, 1.0 mmol, 67%), m.p. 108–111°C (lit. m.p. 112–114°C [46]).

*Preparation of tert-O-methylheraclenol (9-(3′-hydroxy-2′-methoxy-3′-methylbutoxy)-7H-furo[3,2g]chromen-7-one) (**10**)*

Heraclenin (200 mg, 0.7 mmol) and oxalic acid (80 mg, 0.6 mmol) in methanol (20 mL) were stirred and refluxed for 2 h. Removal of solvent yielded a brownish solid, which was purified by column chromatography on a silica gel, eluting with mixtures of cyclohexane:ethyl acetate from 95:5 to 85:15 v/v. After purification, a yellow solid was obtained (197 mg, 0.62 mmol, 89%), m.p. 75–80°C (lit. m.p. 84–85°C [33]).

*Preparation of tert-O-methylheraclenol acetate (9-(3′-acetoxy-2′-methoxy-3′-methylbutoxy)-7H-furo[3,2g]chromen-7-one) (**11**)*

tert-O-Methylheraclenol (50 mg, 0.16 mmol) was kept overnight in pyridine (3 mL) containing acetic anhydride (10 drops). Pyridine was then removed under reduced pressure and the residue was washed twice with toluene and 5% solution of citric acid in water. Organic layers were dried over anhydrous sodium sulfate. Removal of the solvent yielded yellow oil (35 mg, 0.1 mmol, 62%).

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.75 (d, $J = 9.6$ Hz, 1H, H-4), 7.68 (d, $J = 2.2$ Hz, 1H, H-11), 7.37 (s, 1H, H-5), 6.82 (d, $J = 2.3$ Hz, 1H, H-12), 6.36 (d, $J = 9.6$ Hz, 1H, H-3), 5.42 (dd, $J = 8.7$, 2.6 Hz, 1H, 13-CH_2), 5.00 (dd, $J = 11.1$, 2.6 Hz, 1H, 13-CH_2), 4.42 (dd, $J = 11.1$, 8.7 Hz, 1H, 14-CH), 3.26 (s, 3H, 18-CH_3), 2.16 (s, 3H, 19-CH_3), 1.27 (s, 3H, 16-CH_3), 1.23 (s, 3H, 17-CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.8 C-19, 160.2 C-2, 148.1 C-7, 146.6 C-11, 144.2 C-4, 143.5 C-9, 131.5 C-8, 125.9 C-6, 116.5 C-10, 114.9 C-5, 113.5 C-3, 106.8 C-12, 75.9 C-15, 75.5 C-14, 72.5 C-13, 49.9 C-18, 22.6 C-20, 21.2 C-16, 21.1 C-17. In the literature, no full NMR data were presented [47].

Molecular modeling

The docking study was performed using ICM suite of programs (Internal Coordinate Mechanics) [48]. 3D structures of the ligands (**1**–**11**) were built using ICM sketcher module. The crystal structures of AChE (PDB-id: 1ACJ) [49] and BuChE (PDB-id: 1P0I) [50] retrieved from the Protein Data Bank were used as the initial 3D structure. Then, the water molecules and co-crystallized ligand were removed from the PDB structures. The ICMPocketFinder was used to identify binding site. The ligands were docked using a regular rigid receptor-flexible ligand docking approach [48]. The ligand molecules were fully flexible and the protein was represented by grid interaction potentials.

Crystal structures

Monocrystals of (**5**) were obtained by slow evaporation of solvents (hexanes/ethyl acetate). The structure was solved using direct methods from SHELXS93 program [51]. After location of most non-hydrogen atoms forming condensed rings system from initial E-maps, further heavy atoms were found in subsequent difference-Fourier syntheses using SHELXL93 software [51]. The structure was refined using full-matrix least-squares procedure from the same software. Hydrogen atoms were then added using standard geometrical criteria. Crystal structure is triclinic from the centrosymmetric P-1 space group. In the independent part of the unit cell, there are two molecules of different conformation of the substituent at C8 atom (see Fig. 4).

Molecules are partly disordered at the hydrocarbon unit, which was expected for flexible fragments. The doubling of the number of molecules in the asymmetric part of the unit cell doubles the number of the refined parameters. Poor quality of the crystal taken for the experiment, large number of parameters to be refined, together with a partial disorder of the substituents resulted in a relatively high agreement factor R1. Nevertheless, all obtained geometrical parameters of the model are acceptable. Molecules in the crystal structure form two well-defined areas: hydrophilic (polar) formed by planar ring fragments connected via weak C–H···O hydrogen bonds into planes parallel to (1 0 0) plane; and hydrophobic (lipophilic) formed by alkyl substituents (see Fig. 5). The consecutive, translation related layer of molecules in the b-direction is turned with its hydrophilic part toward hydrophilic area of preceding layer of molecules. Such structure resembles that of liposome or biological membranes.

AChE inhibitory assay in 96-microtiter-well plates

The AChE assay in 96-microtiter-well plates used in this paper was based on Ellman reaction [52] with further modifications. The

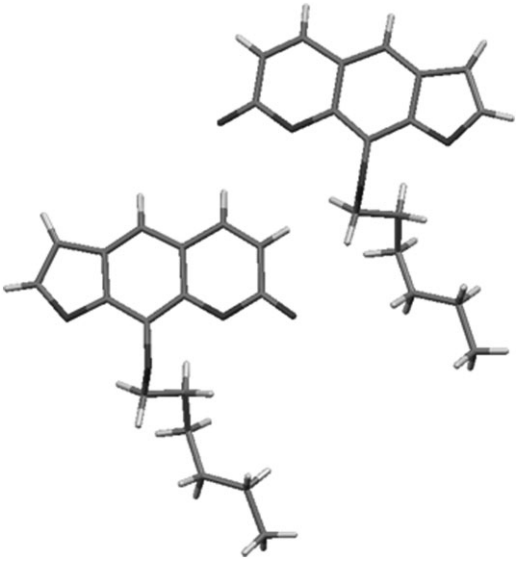


Figure 4. Structure of independent part of the unit cell.

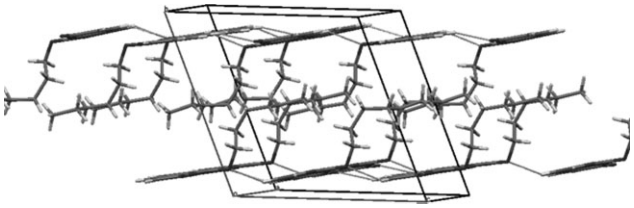


Figure 5. The crystal packing of compound **5**. The molecules are bonded into layers by weak C–H ··· O hydrogen bonds shown as the blue dashed lines.

protocol started by adding 50 μL of 50 mM Tris–HCl buffer (pH 7.8) and 25 μL of 3 mM ATCl in water to 125 μL of 0.6 mM DTNB in 50 mM Tris–HCl buffer (pH 7.8). Next 25 μL of tested furocoumarins solution in buffer containing DMSO (to obtain a final concentration in the assay of 10, 20, and 50 μM) or galantamine hydrobromide solution in 50 mM Tris–HCl buffer (pH 7.8) (as a positive control) or buffer containing DMSO (as a negative control, DMSO final concentration <0.05%) was added. Changes in absorbance were measured nine times during 5-min period to assess the spontaneous hydrolysis of the substrate (at $\lambda = 405$ nm in 37°C, Biotek Synergy 4). Then, 25 μL of AChE (0.30 U/mL in 50 mM Tris–HCl buffer, pH 7.8) was added, and immediately after that, the plates were shaken for 3 s and the measurement was repeated under the same conditions as described above. The activity of tested furocoumarins was calculated as inhibition percentage ($\%_{inh}$) of AChE in relation to maximum activity (negative control) after subtracting in all cases spontaneous hydrolysis of the substrate as shown in Eq. (1)

$$\%_{inh} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100\% \quad (1)$$

BuChE inhibitory assay in 96-microtiter-well plates

BuChE inhibitory activity of furocoumarins was measured using protocol similar to the one described for AChE activity. The difference was that 25 μL of 3 mM BTCCl in water was added as a substrate and 25 μL of BuChE (0.15 U/mL in 50 mM Tris–HCl buffer, pH 7.8) was used to start enzymatic reaction. Galantamine hydrobromide solution in 50 mM Tris–HCl buffer (pH 7.8) was used as a positive control.

All compounds used for biochemical assays were purified up to 95% (HPLC).

Statistics

The results were expressed as a mean ± standard deviation (SD) of three independent experiments performed in triplicate. IC_{50} values for examined compounds were established basing on concentration–inhibition curves.

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