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Original article

Synthesis, computational study and cytotoxic activity of new 4-hydroxycoumarin derivatives

Stancho Stanchev [a,c], Georgi Momekov [b], Frank Jensen [c], Ilia Manolov [a,*]

[a] Department of Organic Chemistry, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav St., BG-1000 Sofia, Bulgaria
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav St., BG-1000 Sofia, Bulgaria
[c] Department of Physics and Chemistry, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

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Abstract

Six new 4-hydroxycoumarin derivatives have been synthesized. They were characterized by UV–vis, IR, ^{1}H NMR, ^{13}C NMR, mass spectral data, elemental analysis, TLC and melting point determination. The new 4-hydroxycoumarin derivatives are studied by computational methods – DFT (B3LYP) and force field methods (MM2 and OPLS), in order to optimize their geometry and calculate quantum-chemical properties and conformational analysis. Five new 4-hydroxycoumarin derivatives were tested for cytotoxic activity in two tumor cell lines – HL-60 and EJ. The obtained results are compared with the utilized anticancer drug melphalan. Two of these compounds – ethyl 2-[(3,4-dihydroxyphenyl)-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)methyl]-3-oxobutanoate (**SS-16**) and ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (**SS-21**) show comparatively good cytotoxic properties. Their activity is weaker than melphalan. **SS-16** seems to be more active than **SS-21**.

Keywords: 4-Hydroxycoumarins; Knoevenagel reaction; Michael reaction; DFT calculations; Cytotoxic activity; MTT

1. Introduction

Coumarins are highly active biological substances. They have spasmolytic, antioxidant, anticoagulant, antibacterial, antiviral and antifungal activities. There are a lot of data about antineoplastic action of coumarin derivatives. They are acting at different stages of cancer formation. Some of them have cytostatic properties and the others have cytotoxic activity. It has been found that coumarin and 7-hydroxycoumarin (7-HC) have antitumor activity [1]. 7-Hydroxycoumarin is the active metabolite of coumarin. Both coumarin and 7-HC were found to be growth-inhibitory (cytostatic) for the following human malignant cell lines: A549, ACHN, Caki-2, Dakiki, HS-Sultan, H727, HCT-15, HL-60, K562, LNCaP, PC-3, Du 145 COLO-232, MCF-7 and RP-1788. In studies on the antiproliferative actions of coumarin compounds, we discovered that dicoumarol (a coumarin anticoagulant, 3,3′-methylenebis[4-hydroxycoumarin]) inhibits the first cleavage of *Strongylocentrotus purpuratus* (sea urchin) embryos in a concentration-dependent manner with 50% inhibition occurring at a concentration of 10 μM [2]. It was found that dicoumarol binds to bovine brain tubulin with a $K(d)$ of 22 μM and that 0.1 μM dicoumarol strongly stabilizes the growing and shortening dynamics at the plus ends of the microtubules in vitro. Dicoumarol and taxol in combination gave results in a synergistic inhibition of cell division of sea urchin embryos. These two compounds can be used in combination, in order to reduce the high toxicity of taxol. There are data about cytotoxic action of some coumarin derivatives and their metal complexes established by brine shrimp bioassay [3]. Some substituted benzopyranobenzothiazinones are synthesized by the interaction of 4-hydroxycoumarin and 2-aminothiophenol.

* Corresponding author. Fax: +359 2 9879874.
E-mail address: imanolov@gmx.net (I. Manolov).

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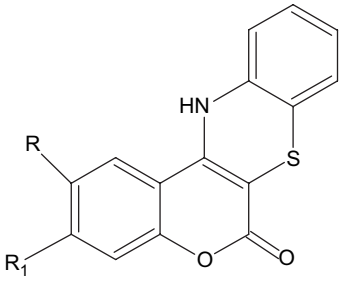
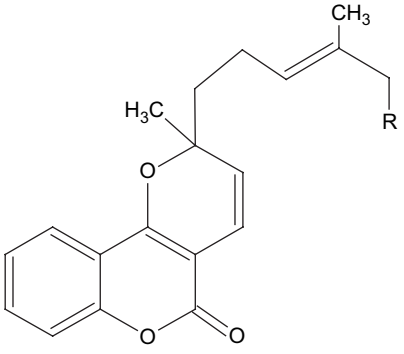
These compounds express estrogenic activity on MCF-7 breast carcinoma cells [4]. Their structure can be expressed as follows:



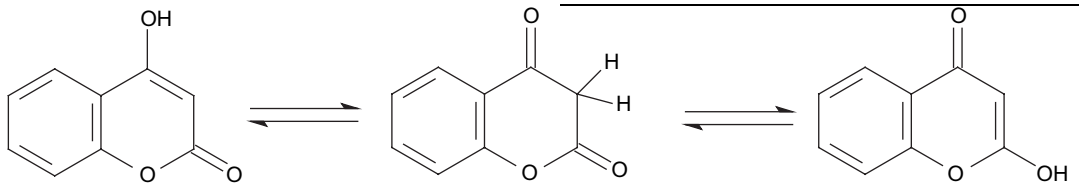


2-methyl-2-[4-methylhex-3-enyl]-2*H*,5*H*-pyrano[3,2-*c*]chromen-5-one

The most active compound is when R = H and R_1 = OH. The results are compared with 17β-estradiol. Their structural properties and structure–activity relationship were also studied by molecular modeling. These compounds may contribute for building a new chemical library, used for treatment of diseases, which are estrogenic-dependent.

The third position in 4-hydroxycoumarin ring is highly activated, because of the influence of hydroxyl group with electron-donating properties and electron-withdrawing effects of carbonyl oxygen atom at the second place. There is a conjugation of π-electrons from the double bond and lone p-electron pairs from oxygen atom. These factors make the third position in the coumarin ring very convenient for Michael addition to the compounds with activated double bond (Michael acceptors). The electronic and molecular properties of some substituted di(4-hydroxycoumarins) were calculated by the hybrid DFT method – B3LYP [5]. Different basis sets were tested in the course of the calculations: 6-31G*, 6-31 + G** and 6-311G*. Electron density distribution, molecular electrostatic potential, hardness, electrophilicity index and reactive sites of the compounds are also calculated and discussed. The possible places of electrophylic attack and hydrogen bond formation were predicted. The different tautomeric forms of 4-hydroxycoumarin have been established [6].

Also such tri- or tetracyclic 4-hydroxycoumarin derivatives were obtained by asymmetric Michael reaction [7]. This reaction was performed as interaction of 4-hydroxycoumarin and 4-substituted-2-oxo-3-butenoate esters in the presence of (*S*)-*t*Bu-BOX–$Cu(OTf)_2$ (*t*Bu-bisoxazolines–$Cu(OTf)_2$ complexes) as catalyst in Et_2O. The first product of this reaction is 3-substituted 4-hydroxycoumarin, which contains 4-substituted-2-oxo-3-butanoate fragment in the third place. There is equilibrium between this 3-substitued 4-hydroxycoumarin and its cyclic form in which one more pyran ring is formed. The interaction of 6-substituted 4-hydroxycoumarins and 1-(3-oxo-1,4-benzoxazin-6-yl)-3-allylpropen-1-ones was implemented according to the Michael reaction [8]. The product is substituted 4-hydroxycoumarin in the third place, containing propyonyl-2*H*-[1,4]-benzoxazin-3(4*H*)-ones. The reaction was carried out with pyridine as a basic agent and also as a medium. The Michael addition to benzylydenecyclohexanones and 4-hydroxycoumarins was carried out in order to obtain new derivatives with better anticoagulant activity [9]. The products of the reaction are different 4-hydroxy-3-[(2-oxo)-cyclohexyl]-benzylcoumarins. The reaction was accomplished in dioxane medium with piperidine as a basic agent. Enantioselective synthesis of warfarin was performed via Michael reaction [10]. This reaction was carried out via interaction of 4-hydroxycoumarin



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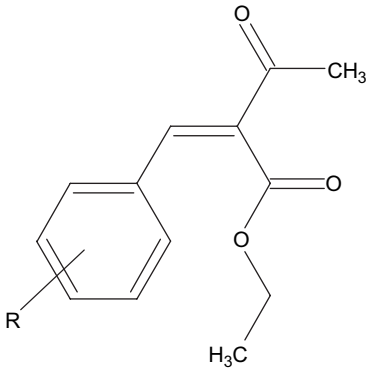
The nature of these three forms was elucidated by using different spectral methods. There are also some reactions with the compounds with activated double bond, which are giving very interesting polycyclic structures [6]. For example, when 4-hydroxycoumarin is reacting with farnesal the product is:

and optically active amines in the presence of *p*-toluenesulfonic acid. The different enamines were the product of that reaction. After that two reactions were performed. The first reaction is between the obtained enamine with benzalacetone in the presence of *p*-toluenesulfonic acid in methanol media at room temperature, which gives very low yield of (*S*) warfarin and (*R*)

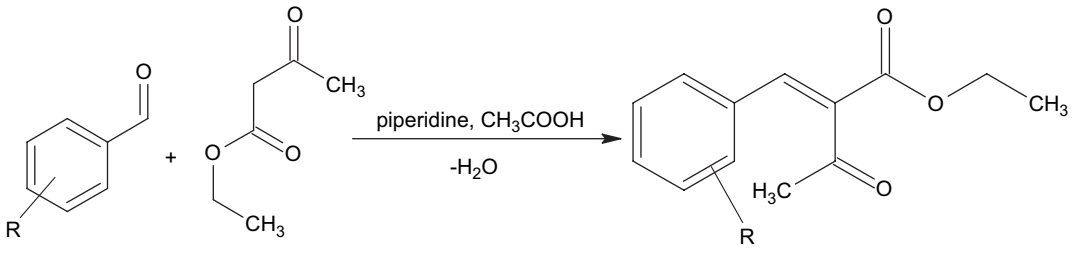
warfarin. Another reaction is in the presence of LDA and Lewis acids (TMSCl) in THF media at −78 °C which gives the high yield of optically active warfarin. The reaction of acylation at the third place of 4-hydroxycoumarin has been performed with acetic acid and phosphoroxychloride [11]. The product is 3-acetylcoumarin, which is used in further reaction of aldol condensation with aromatic aldehydes for obtaining antibacterial 4-hydroxycoumarins. Long 3-acyl derivatives of 4-hydroxycoumarin were obtained [12] via acylation with undec-10-enoylchloride. The reaction was performed in nitrogen atmosphere in sonochemical reactor with dry pyridine as a solvent and piperidine as a catalyst. The reaction mixture is sonicated at 21 kHz for 1.5 h at 38 °C.

2. Chemistry

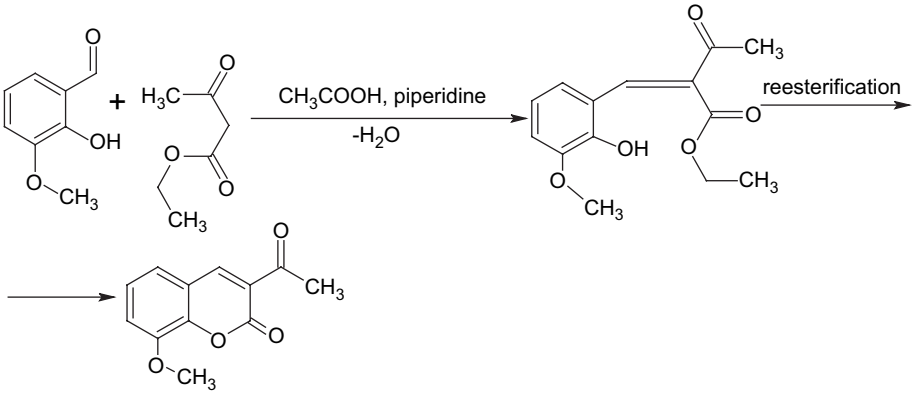
Differently substituted aromatic aldehydes are used for the synthesis of arylydene-β-ketoesters via Knoevenagel reaction with ethylacetoacetate in the presence of piperidine as a basic agent and a glacial acetic acid. These arylydene-β-ketoesters may be presented as:



where R = *m*-NO_2 (ethyl 2-(3-nitrobenzylidene)-3-oxobutanoate) (**SS-19**), R = *p*-NO_2 (ethyl 2-(4-nitrobenzylidene)-3-oxobutanoate) (**SS-1**), R = *p*-OH (ethyl 2-(4-hydroxybenzylidene)-3-oxobutanoate) (**SS-4**), R = *m*,*p*-diOH (ethyl 2-(3,4-dihydroxybenzylidene)-3-oxobutanoate) (**SS-8**), R = *p*-COOH (ethyl 2-(4-carboxybenzylidene)-3-oxobutanoate) (**SS-6**).

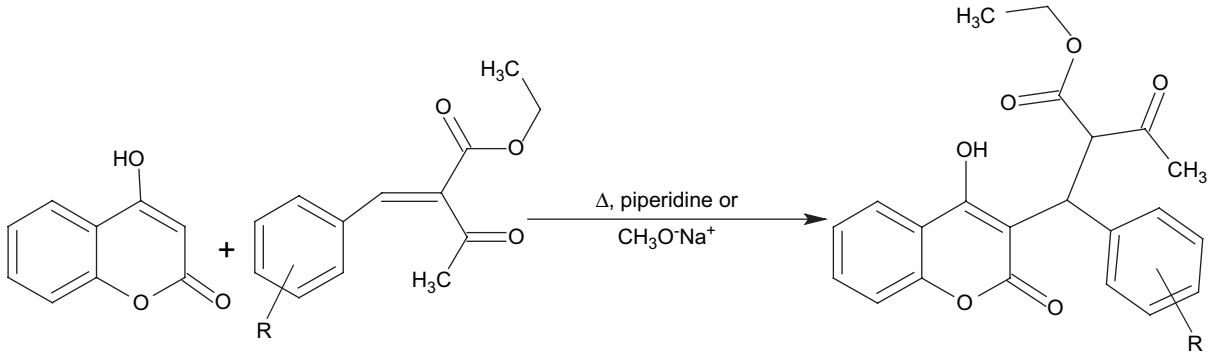


3-Acetyl-8-methoxycoumarin (**SS-5**) is synthesized via Knoevenagel reaction of 2-hydroxy-3-methoxybenzaldehyde and ethylacetoacetate with above-mentioned conditions.



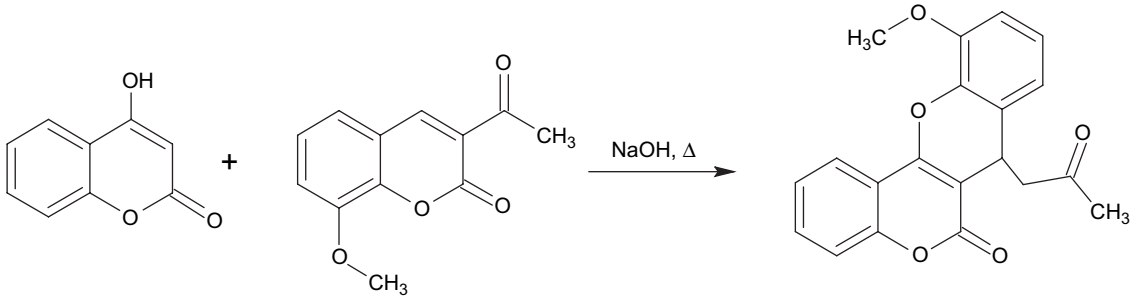
This process of reesterification is carried out spontaneously.

The second step of the reaction is a condensation of the obtained arylydene-β-ketoesters with 4-hydroxycoumarin through Michael reaction, by using sodium methoxide or piperidine as a basic agent. This reaction can be expressed as follows:

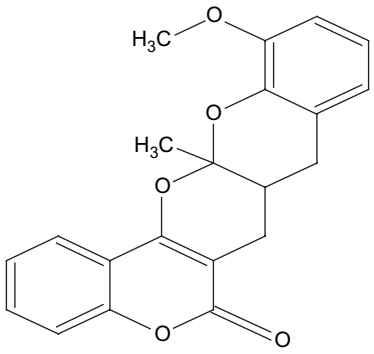


where R = *m*-NO_2 ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (**SS-21**), R = *p*-NO_2 ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-nitrophenyl)methyl]-3-oxobutanoate (**SS-3**), R = *p*-OH ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)methyl]-3-oxobutanoate (**SS-14**), R = *m*,*p*-diOH ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3,4-dihydroxyphenyl)methyl]-3-oxobutanoate (**SS-16**), R = *p*-COOH ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-carboxyphenyl)-methyl]-3-oxobutanoate (**SS-17**).

Compound **SS-5** interacts with 4-hydroxycoumarin (according to Michael reaction), giving polycyclic compound – oxabicyclononane (chromanocoumarin) derivative (**SS-20**). The reaction scheme can be written as follows:



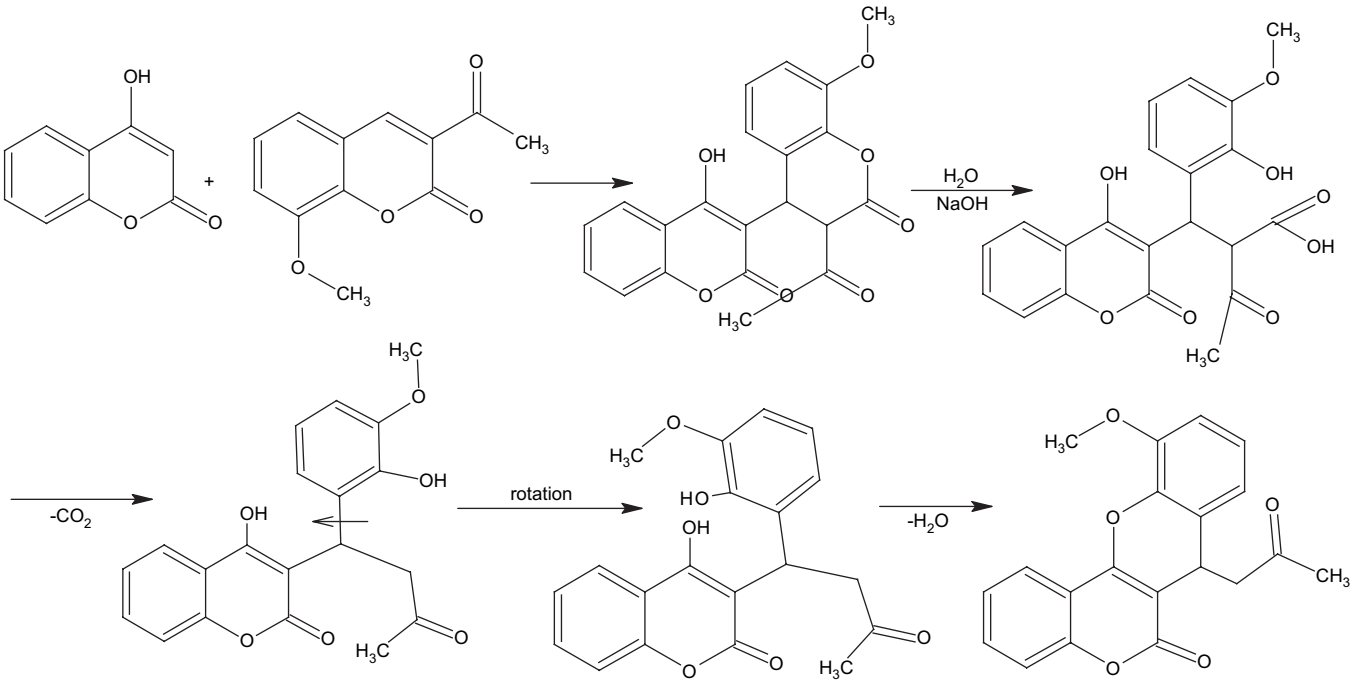
It can be proposed that the real product of reaction is:



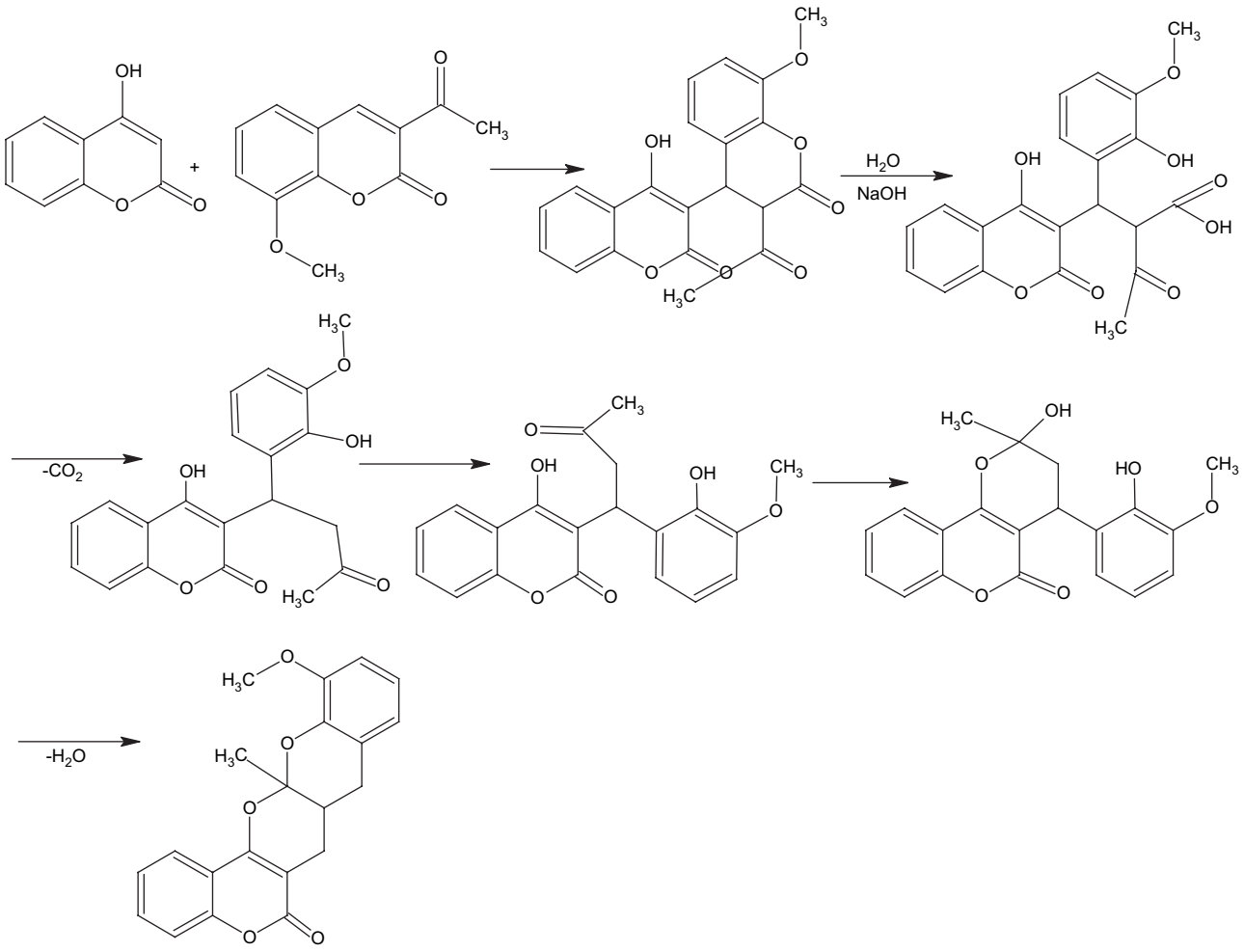
which is isomer of **SS-20**.

The probable mechanism is expressed in Scheme 1 and for the isomeric product, the reaction mechanism is as follows (Scheme 2).

All of the synthesized compounds are characterized by ^{1}H NMR, ^{13}C NMR, UV–vis, IR, mass spectrometry and also by TLC, elemental analysis and melting point determination.



Scheme 1. Probable mechanism of the interaction of 3-acetyl-8-methoxycoumarin with 4-hydroxycoumarin.



Scheme 2. Alternative mechanism of the interaction of 3-acetyl-8-methoxycoumarin with 4-hydroxycoumarin.

3. Computational studies

The purpose of this investigation is to study the silico conformational behavior of the newly synthesized 4-hydroxycoumarin compounds and determine physicochemical parameters like dipole moment, Log *P*, HOMO and LUMO energies, molecular volume and charges, which will be useful for future QSAR analysis.

4. Pharmacology

These five 4-hydroxycoumarin derivatives were tested for cytotoxic activity on urinary bladder carcinoma-derived EJ cells and promyelocyte leukemia-derived HL-60 cells using MTT test – [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as described by Mosmann [13] with some modifications [14]. Absorption of the samples was measured by an ELISA reader at 580 nm. Survival fraction was calculated as percentage of the untreated control. The experimental data were processed and were fitted to sigmoidal concentration–response curves via non-linear regression.

The cytotoxic activity of the newly synthesized coumarin derivatives was evaluated in vitro against HL-60 and EJ cell-lines, after 72 h of continuous exposure. The cell viability was assessed using MTT-dye reduction assay and the corresponding IC_{50} values were calculated as the concentrations of tested compounds causing 50% decrease of cell survival. The clinically utilized antineoplastic drug melphalan – (2-amino-3-[4-bis(2-chloroethyl)amino]phenylpropanoic acid), was exploited as positive control.

5. Results and discussion

5.1. Chemistry

Six 4-hydroxycoumarin derivatives are synthesized – ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)-methyl]-3-oxobutanoate (**SS-14**), ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-nitrophenyl)methyl]-3-oxobutanoate (**SS-3**), ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)-methyl]-3-oxobutanoate (**SS-21**), 4-[1-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl]benzoic acid (**SS-17**), ethyl 2-[(3,4-dihydroxyphenyl)(4-hydroxy-2-oxo-2*H*-chromen-3-yl)methyl]-3-oxobutanoate (**SS-16**) and 7-acetonyl-11-methoxy-6-oxo-6*H*,7*H*-(1)-benzopyrano[4,3b]-(1)-benzopyran (**SS-20**). These compounds are synthesized by two steps: Knoevenagel reaction of aromatic aldehyde and ethylacetoacetate as the first step. The second step is Michael reaction of the obtained arylydene-β-ketoester with 4-hydroxycoumarin. They are characterized by spectral methods – ^{1}H NMR, ^{13}C NMR, UV–vis, IR, mass spectrometry and also by TLC, elemental analysis and melting point determination.

5.2. Computational studies

Computational studies of these new compounds were made by DFT (B3LYP) and force field methods (MM2 and OPLS2005). Different quantum-chemical parameters were calculated by B3LYP/6-31G** methods such as ESP charges, Log *P*, energy of HOMO and LUMO orbitals, dipole moment and molecular volume. These descriptors can be used in future QSAR analysis. These data have significance for the biological activity – for interaction with the receptor/enzyme, penetration through the cell membrane, chemical properties of the molecule during drug metabolism. The generation of the conformations was made by using force field (MM2 and OPLS2005) and Monte Carlo simulation method. DFT (B3LYP/6-31G*) methods are used for calculating their single point energy. All of the studied compounds have many conformations in the bioactive energy range 0–15 kJ/mol, which means that entropy factor may be important for the interaction with protein receptor. Many conformers are stabilized by intramolecular hydrogen bonding between hydroxyl group from 4-hydroxycoumarin fragment and acetyl group from side chain. Cluster analysis of one **SS-3** as an example was made in order to generate representative conformations. Nine representative conformations are found and more significant difference is in the ethyl-2-acetyl-propionate chain.

Table 1 contains atomic charges, calculated by the ESP procedure, which might participate in hydrogen bonding with the

Table 1
ESP charges for more important atoms

R	*q*1(O)	*q*2(O)	*q*3(OH)	*q*4(O)	*q*5(O)	*q*6(O)	*q*8(C)	*q*9(C)	*q*(NO_2)	*q*(OH)	*q*(COOH)
3-NO_2-(**SS-21**)	−0.45	−0.51	O −0.51; H 0.41	−0.45	−0.44	−0.5	−0.05	−0.56	N 0.61; O1 −0.39; O2 −0.38	–	–
3,4-diOH-(**SS-16**)	−0.44	−0.51	O −0.52; H 0.41	−0.46	−0.43	−0.51	0.20	−0.5	–	O1 −0.55; O2 −0.58; H1 0.43; H2 0.44	–
4-COOH-(**SS-17**)	−0.41	−0.54	O −0.48; H 0.42	−0.48	−0.39	−0.48	0.21	−0.30	–	–	C 0.49; O1 −0.519; O2 −0.522; H 0.42
4-NO_2-(**SS-3**)	−0.43	−0.52	O −0.52; H 0.43	−0.54	−0.37	−0.54	0.25	−0.57	N 0.62; O1 −0.390; O2 −0.386	–	–
4-OH-(**SS-14**)	−0.45	−0.53	O −0.55; H 0.42	−0.47	−0.42	−0.56	0.48	−0.75	–	O −0.56; H 0.41	–

enzyme and/or receptor. The Log *P* is a measurement for hydrophobicity and is important for evaluating the distribution of the compound between lipid/water phases and for penetration through a cell membrane, metabolism of the drug molecule, hydrophobic interactions with proteins and toxicity. The dipole moment is a measure of the polarity of the molecule. This is useful also for determining the penetration through cell membrane (solubility in lipid bilayer) and for the speed of excretion. The HOMO and LUMO energies are important for evaluating the reduction and oxidation potentials of the compound. Information about these orbitals may be significant for drug metabolism, because many drugs are metabolized by oxidation and reduction reactions. The molecular volume may provide further information, regarding the interaction between drug molecule and receptor.

Conformational analysis is useful for searching the probable bioactive conformation. Usually, the bioactive conformation is in the range between 0 kJ/mol and 15 kJ/mol of the global minimum. A conformational analysis can also be used for predicting the Boltzmann distribution and entropy of interaction between conformation family and receptor.

The B3LYP/6-31G** method was used for calculating the above molecular properties and a conformational search was performed with the aid of MM2 and OPLS 2005 force field (for **SS-20**). Monte Carlo simulation method at 20 000 iterations is used in order to generate well minimized conformations. Refined estimates of relative energies were done by using B3LYP/6-31G* method. This hybrid DFT method (B3LYP) was chosen due to its high accuracy and reasonable computational cost for systems of the present size (4-hydroxycoumarin derivatives have up to 60 atoms).

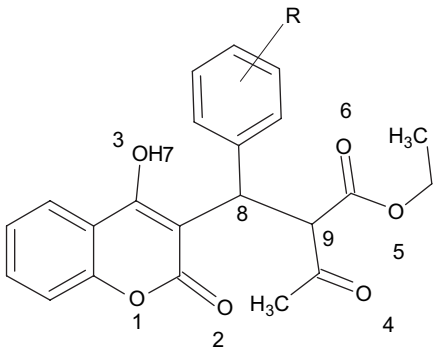
The optimized geometries of the compounds are shown in Fig. 1. The geometry of 4-[1-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl]benzoic acid (**SS-17**) is taken for example.

The atomic charges derived from the electrostatic potential for some of the more significant atoms are presented in Table 1, for which the labeling is indicated in the structure below.

The results for quantum-chemical parameters are presented in Table 2.

The geometry of the compound **SS-20** was optimized and the charges are calculated in the same way as above-mentioned compounds (Fig. 2)

This chromanocoumarin derivative has the values of quantum-chemical parameters as follows:



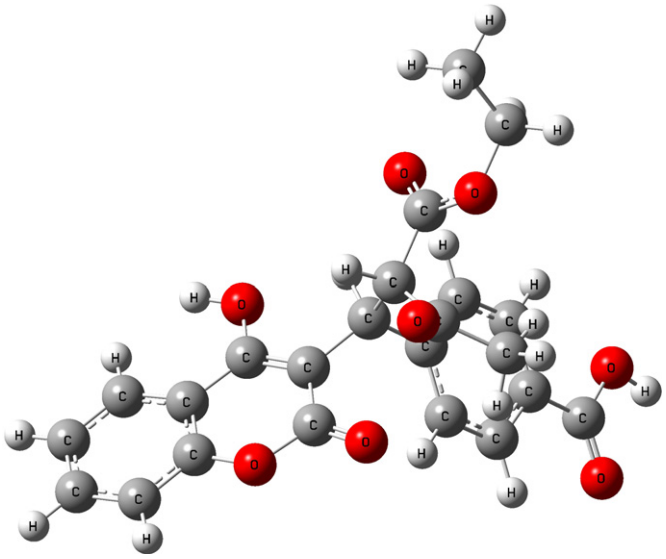


Fig. 1. Optimized geometry of **SS-17** obtained by B3LYP/6-31G** method.

Log *P* = −0.18, μ = 4.48 D, *E* (HOMO) = −6.29 eV, *E* (LUMO) = −1.87 eV, molecular volume = 331.3 Å^3.

It can be inferred that these compounds possess hydrophobic properties, from their Log *P* values. Hydrophobicity contributes for good penetration through cell membrane and for resorption. The compound **SS-20** is an exception of that order, as it has hydrophilic properties. All the compounds are polar molecules, as judged from the dipole moments. These compounds are relatively small molecules, according to their molecular volume data and they have several negative ESP charges and mobile hydrogen atoms for hydrogen bond formation and van der Waals interaction with protein fragments. The energies of HOMO orbitals are between −6.00 eV and −6.53 eV, depending on the nature of the substituent (electron-withdrawing or electron-donating), for 3,4-dihydroxy these values are between −5.00 eV and −6.00 eV. The same relationship is visible for LUMO orbitals. Their energy is increasing of electron-donating substituents (between −1.29 eV and −2.63 eV).

The results from the conformational analysis are presented as a graphical relationship between the relative energy of the conformers (in kJ/mol) and the number of conformers. From these charts it can be seen that how many conformations exist in the energy range 0–15 kJ/mol, i.e. the conformations,

Table 2
Main quantum-chemical properties

	R	Log *P* (Ghose–Crippen)	μ [D]	*E* (HOMO) [eV]	*E* (LUMO) [eV]	Molecular volume [Å^3]
1	3-NO2-	2.82	5.65	−6.47	−2.63	401.2
2	3,4-diOH-	2.01	6.99	−5.90	−1.56	392.1
3	4-COOH-	2.34	2.70	−6.36	−1.75	406.0
4	4-NO_2-	2.82	4.90	−6.44	−2.41	399.3
5	4-OH-	2.4	4.92	−6.07	−1.59	385.3

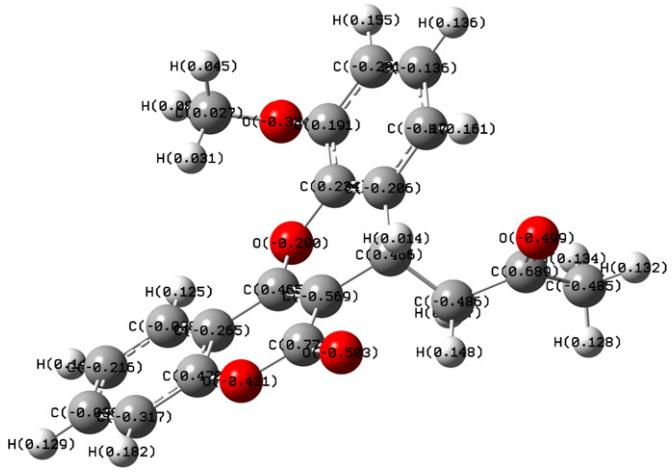


Fig. 2. Optimized geometry and ESP charges of **SS-20** obtained by B3LYP/6-31G** method.

which are within an acceptable energy for binding to the receptor or enzyme.

These charts are presented as Figs. 3–8.

It is seen that all of the compounds have many low-energy conformations and the number of conformations is connected with the entropy factor for the interaction with a protein receptor. Some of the bioactive conformations are stabilized by intramolecular hydrogen bonds between OH group from 4-hydroxycoumarin fragment and acetyl group (carbonyl oxygen atom) from the side chain.

Based on a cluster analysis, we have selected some representative structures of the compounds and Fig. 9 shows the overlaid representative structures of compound **SS-3**. From 49 found conformations, after cluster analysis 9 representative structures were found in cluster level 41. The main differences are in the position of ethyl-2-acetyl-propionate chain, where the bigger part of the rotatable bonds is located.

5.3. Pharmacology

These compounds are tested for cytotoxic activity with MTT-dye reduction assay on urinary bladder carcinoma cells (EJ-60) and leukemia-derived HL-60 cells. The data about new compounds are compared with the clinically utilized antineoplastic drug melphalan – (2-amino-3-[4-bis(2-chloroethyl)amino] phenylpropanoic acid).

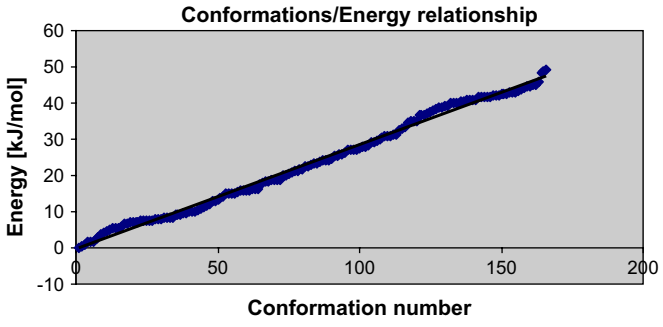


Fig. 3. Graphical relationship between number of conformations and their energy for compound **SS-16**.

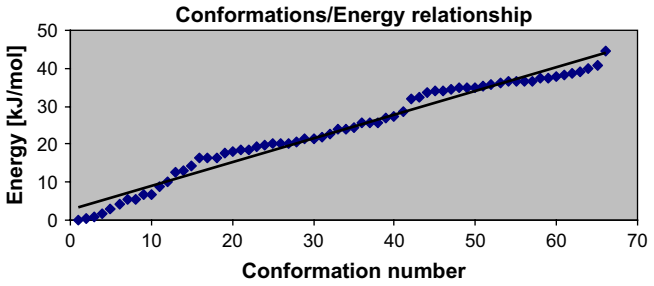


Fig. 4. Graphical relationship between number of conformations and their energy for compound **SS-21**.

Among the five coumarin derivatives only compounds **SS-16** and **SS-21** displayed significant cytotoxic activity causing 50% inhibition of cell viability within the investigated concentration range, whereas the remaining analogues exerted only marginal effects. On the basis of the IC_{50} values obtained **SS-16** was found to be more active than **SS-21** in both cell lines. All of the tested coumarin analogues are less active than melphalan, which is utilized as neoplastic drug (see Table 3).

On the basis of the established micromolar cytotoxicity of **SS-16** in both the cells this compound could be considered as lead for further development of cytotoxic coumarin derivatives.

6. Conclusion

Six 4-hydroxycoumarin derivatives are synthesized – ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)-methyl]-3-oxobutanoate (**SS-14**), ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-nitrophenyl)methyl]-3-oxobutanoate (**SS-3**), ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)-methyl]-3-oxobutanoate (**SS-21**), 4-[1-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl]benzoic acid (**SS-17**), ethyl 2-[(3,4-dihydroxyphenyl)(4-hydroxy-2-oxo-2*H*-chromen-3-yl)methyl]-3-oxobutanoate (**SS-16**) and 7-acetonyl-11-methoxy-6-oxo-6*H*,7*H*-(1)-benzopyrano[4,3b]-(1)-benzopyran (**SS-20**). These compounds are synthesized by two steps: Knoevenagel reaction of aromatic aldehyde and ethylacetoacetate as the first step. The second step is Michael reaction of the obtained arylydene-β-ketoester with 4-hydroxycoumarin. They are characterized by spectral

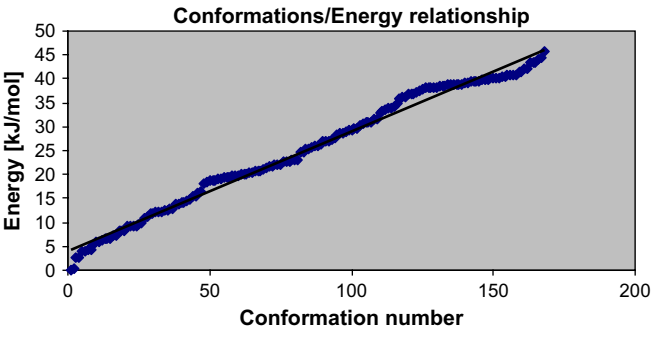


Fig. 5. Graphical relationship between number of conformations and their energy for compound **SS-17**.

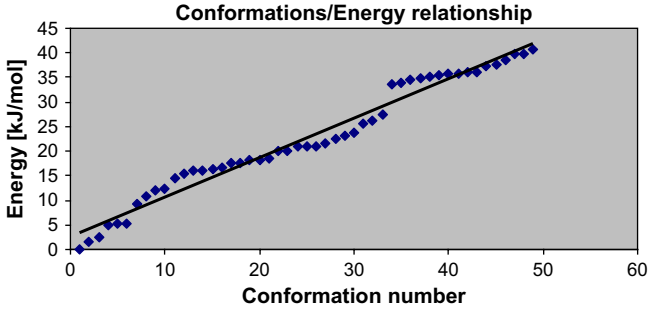


Fig. 6. Graphical relationship between number of conformations and their energy for compound **SS-3**.

methods – ^{1}H NMR, ^{13}C NMR, UV–vis, IR, mass spectrometry and also by TLC, elemental analysis and melting point determination.

Computational studies of these new compounds were made by DFT (B3LYP) and force field methods (MM2 and OPLS2005). Different quantum-chemical parameters were calculated by B3LYP/6-31G** methods such as ESP charges, Log *P*, energy of HOMO and LUMO orbitals, dipole moment and molecular volume. These descriptors can be used in future QSAR analysis. These data have significance for the biological activity – for interaction with the receptor/enzyme, penetration through the cell membrane, chemical properties of the molecule during drug metabolism. The generation of the conformations was made by using force field (MM2 and OPLS2005) and Monte Carlo simulation method. DFT (B3LYP/6-31G*) methods are used for calculating their single point energy. All of the studied compounds have many conformations in the bioactive energy range 0–15 kJ/mol, which means that entropy factor may be important for the interaction with protein receptor. Many conformers are stabilized by intramolecular hydrogen bonding between hydroxyl group from 4-hydroxycoumarin fragment and acetyl group from side chain. Cluster analysis of one representative compound was made in order to generate representative conformations. Nine representative conformations are found and more significant difference is in the ethyl-2-acetyl-propionate chain.

These compounds are tested for cytotoxic activity with MTT-dye reduction assay on urinary bladder carcinoma cells (EJ-60) and leukemia-derived HL-60 cells. The data about new compounds are compared with the clinically utilized antineoplastic drug melphalan – (2-amino-3-[4-bis(2-chloroethyl)amino] phenylpropanoic acid). All the compounds are

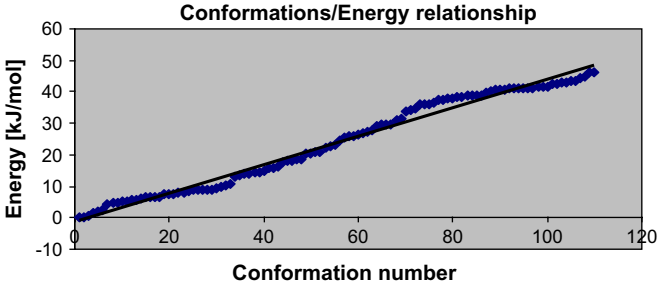


Fig. 7. Graphical relationship between number of conformations and their energy for compound **SS-14**.

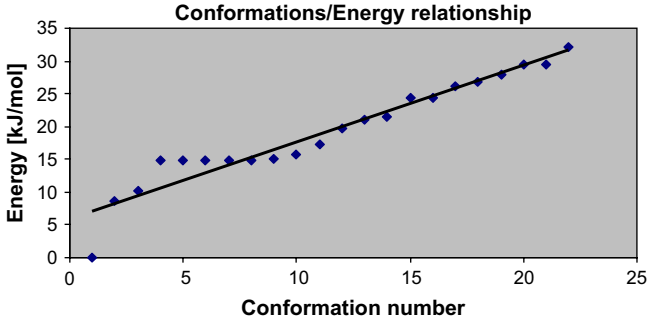


Fig. 8. Graphical relationship between number of conformations and their energy for compound **SS-20**.

less active than melphalan. The compound (**SS-16**) expresses the best cytotoxic activity, according to the EJ-60 and HL-60 cell lines. The compound (**SS-21**) also possesses significant cytotoxic activity. Oxabicyclononane derivative (**SS-20**) is in hand to be tested for cytotoxic activity.

7. Experimental protocols

7.1. Synthesis of 4-hydroxycoumarin derivatives

7.1.1. Materials and methods

All starting materials were purchased from Merck, Sigma–Aldrich and Fluka. They are used without further purification. Melting points are measured in open capillary tubes on a Büchi 535 melting point apparatus. The IR spectra were recorded at Shimadzu FTIR 8101M spectrometer in nujol and frequencies are expressed in cm^{-1}. The ^{1}H NMR spectra were recorded in

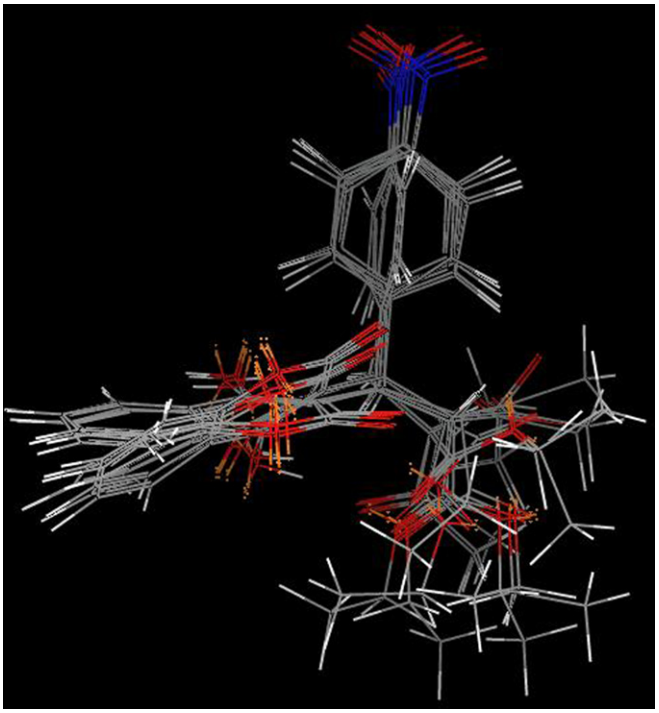


Fig. 9. Overlaid representative conformations of the compound **SS-3**.

Table 3
Cytotoxic activity of the newly synthesized compounds and melphalan in a panel of tumor cell lines after 72 h exposure (MTT-dye reduction assay)

Compound	IC_{50} (μM)[a]	
	HL-60[b]	EJ[c]
SS-14	>200	>200
SS-16	35.1 ± 2.4	115.8 ± 7.9
SS-17	>200	>200
SS-21	90.8 ± 8.1	172.5 ± 7.3
SS-3	>200	>200
Melphalan	11.4 ± 3.2	22.1 ± 2.7

[a] Arithmetic mean ± sd of eight independent experiments.
[b] Human acute promyelocyte leukemia.
[c] Human urinary bladder carcinoma.

Brucker 250 MHz in DMSO-d_6 or acetone using TMS as an internal standard (chemical shifts are reported in ppm units, coupling constants (J) in Hz). Abbreviations used are as follows: s − singlet, d − doublet, dd − double doublet, t − triplet, m − multiplet, br − broad.

Mass spectral analysis was performed by electron ionization on mass spectrometer Hewlett−Packard 5973 at 70 eV.

7.1.2. General procedure for the preparation of arylydene-β-ketoesters

Aromatic aldehyde and ethylacetoacetate in equimolar quantities are mixed in round-bottomed flask. Piperidine (0.03 mol) and glacial acetic acid (0.04 mol) are also added to the reaction mixture. The latter is stirred at room temperature for 90 min. After that 20 ml of ether and/or 150 ml distilled water are added to the reaction mixture and crystals with different colors are formed. These crystals are filtered and washed. Then they are dried at room temperature and recrystallized in appropriate solvents − mainly alcohols (ethanol, propanol, 2-propanol) and water (for **SS-8**).

*7.1.2.1. Ethyl 2-(4-nitrobenzylidene)-3-oxobutanoate (**SS-1**).* White crystals, m.p. 160−161 °C. The substance crystallizes from ether and purified after recrystallization from isopropyl alcohol. Yield: 66%; UV−vis: $\lambda_{max} = 204$, 270 nm; FTIR (nujol): 1732.3, 1711.1, 1608.8, 1529.7, 1464.1, 844.9 cm^{-1}; ^1H NMR (acetone, 200 MHz): $\delta = 0.9$ (t, 3H, aliphatic), 1.3 (s, 3H, aliphatic), 2.8 (q, 2H, aliphatic), 6.1 (s, 1H, aliphatic), 7.5 (d, 2H, aromatic), 8.2 (d, 2H, aromatic); ^{13}C NMR (acetone, 67 MHz): $\delta = 15$, 30, 45, 125, 130, 145, 165, 175, 200; TLC: $R_f = 0.75$ (hexane/chloroform/acetone/methanol = 5:3:2:1). Anal.: $C_{13}H_{13}NO_5$ (263) (C,H) = (calcd/found): %C 59.31/58.28, %H 4.98/5.92, %N 5,32/3,52.

*7.1.2.2. Ethyl 2-(4-hydroxybenzylidene)-3-oxobutanoate (**SS-4**) [15–18].* Yellow crystals, m.p. 141−143 °C. The substance crystallizes from ether and purified after recrystallization from isopropyl alcohol. Yield: 51%; UV−vis: $\lambda_{max} = 206$, 224, 286 nm; FTIR (nujol): 3325.7, 1732.3, 1641.6, 1597.3, 1462.2, 1205.7, 819.8 cm^{-1}; ^1H NMR (acetone, 200 MHz): $\delta = 1.3$ (t, 3H, aliphatic), 2.3 (s, 3H, aliphatic), 4.3 (q, 2H, aliphatic), 6.9 (dd, 2H, aromatic), 7.4 (dd, 2H, aromatic), 7.6 (s, 1H, aliphatic), 10.5 (s, 1H, hydroxyl); ^{13}C NMR (acetone, 67 MHz): $\delta = 30$, 110, 135, 140, 160, 190; EIMS: m/z (%) = 234 (100, M^+), 233 (57), 220 (10), 219 (69), 217 (17), 205 (15), 191 (25.4), 189 (38.25), 187 (11.3), 175 (8.7), 163 (11.3), 161 (11.3), 160 (28.7), 151 (28.7), 147 (68.7), 146 (11.3), 145 (37.4), 131 (7), 123 (30.4), 120 (9.6), 119 (20), 118 (19.1), 115 (2.6), 107 (6), 91 (20), 89 (19.1), 77 (7), 65 (11.3), 63 (12.1), 53 (5), 45 (0.9); TLC: $R_f = 0.39$ (hexane/acetone = 2:1). Anal.: $C_{13}H_{14}O_4$ (234) (C,H) = (calcd/found): %C 66.66/66.50, %H 6.02/5.94.

*7.1.2.3. 3-Acetyl-8-methoxy-2H-chromen-2-one (**SS-5**).* Yellow crystals, m.p. 171−172 °C. The substance crystallizes from ether and purified after recrystallization from ethanol. Yield: 28%; UV−vis: $\lambda_{max} = 220$, 254, 316 nm; FTIR (nujol): 1728.4, 1689.8, 1601.1, 1465, 1207.6, 1093.8, 765.8 cm^{-1}; ^1H NMR (acetone, 200 MHz): $\delta = 2.7$ (s, 3H, aliphatic), 4.0 (s, 3H, aliphatic), 7.4 (m, 3H, aromatic), 8.4 (s, 1H, aliphatic); ^{13}C NMR (acetone, 67 MHz): $\delta = 30$, 60, 115, 125, 130, 150, 205; TLC: $R_f = 0.51$ (hexane/acetone = 2:1). Anal.: $C_{12}H_{10}O_5$ (218) (C,H) (calcd/found): %C 66.05/66.04, %H 4.62/4.38.

*7.1.2.4. 4-[2-(Ethoxycarbonyl)-3-oxobut-1-en-1-yl]benzoic acid (**SS-6**).* Yellow crystals, m.p. 148−150 °C. The substance crystallizes from water and purified after recrystallization from ethanol. Yield: 57%; UV−vis: $\lambda_{max} = 204$, 292 nm; FTIR (nujol): 3300−2400, 1736.1, 1689.8, 1608.8, 1460.3, 848 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H, aliphatic), 2.4 (s, 3H, aliphatic), 4.2 (q, 2H, aliphatic), 7.4 (s, 1H, aliphatic), 7.6 (s, 1H, aromatic), 7.8 (s, 1H, aromatic), 8.0 (d, 2H, aromatic), 13.23 (s, 1H, carboxyl group); EIMS: m/z (%) = 262 (64, M^+), 261 (16.7), 247 (18.4), 233 (8), 218 (18.4), 217 (100), 191 (10), 189 (14.9), 179 (9.6), 175 (26.3), 173 (27.2), 171 (11.4), 155 (14.9), 151 (16.7), 147 (7.9), 131 (9.6), 129 (17.5), 115 (7.9), 103 (18.4), 101 (14.9), 91 (5.3), 77 (11.4), 75 (8.7), 63 (3.5), 51 (2.6), 45 (1.8); TLC: $R_f = 0.33$ (hexane/chloroform/acetone/methanol = 5:3:2:1). Anal.: $C_{14}H_{14}O_5$ (262) (C,H) (calcd/found): %C 64.12/64.44, %H 5.38/5.26.

*7.1.2.5. Ethyl 2-(3,4-dihydroxybenzylidene)-3-oxobutanoate (**SS-8**) [19].* Brown-yellow crystals, m.p. 147.8−151 °C. The substance crystallizes from water and purified after recrystallization from water. Yield: 19%; UV−vis: $\lambda_{max} = 206$, 252, 344 nm; FTIR (nujol): 3540, 1714.9, 1643.6, 1603, 1464.1, 1197 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H, aliphatic), 2.3 (s, 3H, aliphatic), 4.2 (q, 2H, aliphatic), 6.9 (d, 2H, aromatic), 7.0 (s, 1H, aromatic), 7.6 (s, 1H, aliphatic), 8.4 (s, 2H, hydroxyl); EIMS: m/z (%) = 250 (100, M^+), 249 (40), 235 (20), 233 (29.6), 222 (17.4), 205 (31), 189 (76.5), 177 (10.4), 176 (31.3), 163 (42.6), 161 (73.9), 147 (4.3), 134 (19.1), 117 (9.6), 103 (5.2), 89 (15.7), 88 (11.3), 77 (11.3), 69 (4.3), 62 (8.7), 51 (8.7); TLC: $R_f = 0.4$ (hexane/chloroform/acetone/methanol = 5:3:2:1). Anal.: $C_{13}H_{14}O_5$ (250) (C,H) (calcd/found): %C 62.39/62.31, %H 5.64/5.64.

*7.1.2.6. Ethyl 2-(3-nitrobenzylidene)-3-oxobutanoate (**SS-19**).* White crystals, m.p. 100−103 °C. The substance crystallizes from water and purified after recrystallization from isopropyl

alcohol. Yield: 17%. UV–vis: $\lambda_{max} = 210$, 266 nm; FTIR (nujol): 1728.4, 1660.9, 1628.1, 1529.7, 780, 735 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 0.9$ (t, 3H, aliphatic), 1.6 (s, 3H, aliphatic), 4.8 (q, 2H, aliphatic), 7.4 (s, 1H, aliphatic), 7.6 (t, 1H, aromatic), 7.9 (d, 1H, aromatic), 8.1 (d, 1H, aromatic), 8.3 (s, 1H, aromatic); EIMS: *m/z* (%) = 263 (65.2, M^+), 262 (20), 248 (99.1), 246 (100), 234 (19.1), 220 (32.1), 218 (51.8), 216 (15.7), 202 (35.7), 200 (24.3), 192 (13), 180 (18.3), 176 (66.09), 174 (27), 160 (10.4), 152 (21.7), 146 (13), 130 (20.9), 129 (36.5), 120 (17.4), 115 (19.1), 102 (35.7), 101 (47.8), 89 (13.9), 75 (29.6), 63 (9.6), 51 (13), 45 (2.6); TLC: $R_f = 0.5$ (hexane/acetone = 2:1). Anal.: $C_{13}H_{13}NO_5$ (263) (C,H) (calcd/found): %C 59.31/59.54, %H 4.98/H 5.13.

7.1.3. General procedure for the preparation of condensation products with 4-hydroxycoumarin

Arylydene-β-ketoester, obtained in previous reaction, and 4-hydroxycoumarin are mixed in equimolar quantities in 25–30 ml methanol (used as a solvent). Sodium methoxide (0.003 mol) as a basic agent is also added to the reagents. The reaction mixture is boiled and stirred for 60 h under reflux. The reaction is controlled by TLC (hexane:acetone = 2:1 or hexane:acetone:chloroform:methanol = 5:3:2:1). When the quantities of reagents are depleted the heating was stopped. The residue from the reaction mixture was filtered off and washed with hot water, in order to remove the 4-hydroxicoumarin, which was not reacted. After that the residue is dried at room temperature and recrystallized in appropriate solvent (methanol, ethanol or 2-propanol).

*7.1.3.1. Ethyl 2-[(4-hydroxy-2-oxo-2H-chromen-3-yl)(4-nitrophenyl)methyl]-3-oxobutanoate (**SS-3**).* White crystals, m.p. 250–254 °C. Purified after recrystallization from ethanol. Yield: 33%; UV–vis: $\lambda_{max} = 206$, 272 nm; FTIR (nujol): 3362.3, 1732.3, 1651.3, 1616.5, 1601.1, 833.3, 765.1 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H, aliphatic), 2.0 (s, 3H, aliphatic), 3.9 (q, 2H, aliphatic), 4.2 (m, 1H, aliphatic), 5.0 (m, 1H, aliphatic), 7.2 (m, 3H, aromatic), 7.5 (m, 2H, aromatic), 7.8 (m, 1H, aromatic), 7.9 (m, 2H, aromatic), 10 (s, 1H, hydroxyl); EIMS: *m/z* (%) = 426 (0.8, M^+), 380 (0.8), 368 (0.4), 343 (0.8), 327 (5.3), 317 (3.5), 302 (1.8), 284 (0.9), 274 (0.8), 256 (7), 242 (2.6), 230 (6.1), 213 (27.2), 202 (4.4), 187 (4.4), 176 (5.3), 163 (10), 162 (80.7), 149 (7.9), 134 (1.8), 121 (48.2), 120 (100), 105 (4.4), 92 (57), 77 (7.9), 63 (18.4), 51 (6.1), 46 (1.8); TLC: $R_f = 0.48$ (hexane/acetone = 2:1). Anal.: $C_{22}H_{19}NO_8$ (426) (C,H) (calcd/found): %C 62.12/62.02, %H 4.5/4.38, %N 3.29/3.21.

*7.1.3.2. Ethyl 2-[(4-hydroxy-2-oxo-2H-chromen-3-yl)(4-hydroxyphenyl)methyl]-3-oxobutanoate (**SS-14**).* White crystals, m.p. 195–197 °C. Purified after recrystallization from ethanol. Yield: 21%; UV–vis: $\lambda_{max} = 214$, 280, 308 nm; FTIR (nujol): 3391.3, 1699.5, 1622.3, 1599.2, 1464.1, 821, 760 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H, aliphatic), 2.0 (s, 3H, aliphatic), 4.1 (q, 2H, aliphatic), 4.4 (m, 1H, aliphatic), 4.6 (m, 1H, aliphatic), 6.9 (m, 2H, aromatic), 7.3 (m, 1H, aromatic), 7.4 (m, 1H, aromatic), 7.6 (m, 2H, aromatic), 7.9 (m, 1H, aromatic), 8.1 (m, 1H, hydroxyl), 8.6 (s, 1H, hydroxyl); EIMS: *m/z* (%) = 396 (0.09, M^+), 364 (0.09), 350 (0.09), 321 (0.09), 307 (0.9), 279 (0.4), 266 (56.1), 265 (100), 249 (31.6), 237 (10.5), 221 (2.6), 210 (2.6), 181 (1.8), 165 (1.8), 153 (2.6), 146 (7), 130 (7), 121 (19.3), 118 (17.5), 102 (4.4), 92 (15.8), 85 (12.3), 76 (2.6), 63 (10.5), 53 (2.6), 46 (0.09); TLC: $R_f = 0.48$ (hexane/chloroform/acetone/methanol). Anal.: $C_{22}H_{20}O_7$ (396) (C,H) (calcd/found): %C 66.66/66.36, %H 5.09/5.13.

*7.1.3.3. Ethyl 2-[(3,4-dihydroxyphenyl)(4-hydroxy-2-oxo-2H-chromen-3-yl)methyl]-3-oxobutanoate (**SS-16**).* Tiled-red crystals, m.p. 243.4–247 °C. Purified after recrystallization from isopropyl alcohol. Yield: 5%; UV–vis: $\lambda_{max} = 208$, 280 nm; FTIR (nujol): 3451, 1732.3, 1662.8, 1608.8, 1460.3, 1180.6, 1109.2, 825.6, 756.2 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.2$ (t, 3H, aliphatic), 2.1 (s, 3H, aliphatic), 4.1 (q, 2H, aliphatic), 4.4 (m, 1H, aliphatic), 4.6 (m, 1H, aliphatic), 6.8 (m, 1H, aromatic), 7.1 (m, 1H, aromatic), 7.2 (m, 2H, aromatic), 7.4 (m, 1H, aromatic), 7.8 (m, 1H, aromatic), 7.9 (s, 1H, aromatic), 8.1 (s, 3H, hydroxyl); EIMS: M^+ is probably very unstable and it goes to a fragmentation spontaneously. *m/z* (%) = 396 (0.09), 374 (0.9), 348 (0.5), 331 (0.9), 317 (5.3), 282 (8.8), 281 (12.3), 265 (9.6), 241 (1.8), 228 (26.3), 213 (0.9), 200 (48.2), 189 (1.8), 171 (8.8), 162 (93), 144 (7), 134 (5.3), 120 (100), 110 (58.8), 92 (74.6), 81 (8.8), 64 (35), 51 (11.4), 45 (3.5); TLC: $R_f = 0.12$ (hexane/chloroform/acetone/methanol = 5:3:2:1). Anal.: $C_{22}H_{20}O_8$ (412) (C,H) (calcd/found): %C 64.07/64.44, %H 4.89/4.52.

*7.1.3.4. 4-[1-(4-Hydroxy-2-oxo-2H-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl]benzoic acid (**SS-17**).* White crystals, m.p. 150–155 °C. Purified after recrystallization from methanol. Yield: 28%; UV–vis: $\lambda_{max} = 208$, 282, 308 nm; FTIR (nujol): 3442, 3300–2400, 1732.3, 1693.7, 1612.7, 1462.4, 1109, 846.3, 756.2 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H, aliphatic), 2.1 (s, 3H, aliphatic), 3,9 (q, 2H, aliphatic), 4.2 (m, 1H, aliphatic), 4.6 (m, 1H, aliphatic), 7.2 (m, 1H, aromatic), 7.4 (m, 1H, aromatic), 7.5 (m, 2H, aromatic), 7.6 (m, 2H, aromatic), 7.8 (m, 1H, aromatic), 8.0 (m, 1H, hydroxyl), 12.83 (s, 1H, carboxyl); EIMS: *m/z* (%) = 424 (1.3, M^+), 392 (0.4), 378 (17.5), 360 (1.8), 335 (48.2), 317 (19.3), 307 (9.6), 294 (44.7), 293 (34.2), 265 (6.1), 257 (50.9), 250 (22.8), 249 (100), 239 (5.3), 229 (1.8), 215 (12.3), 205 (1.8), 187 (2.6), 173 (2.6), 165 (4.4), 146 (2.6), 130 (6.1), 120 (19.3), 102 (6.1), 92 (30.7), 75 (6.1), 64 (8.8), 51 (2.6); TLC: $R_f = 0.62$ (hexane:chloroform:glacial acetic acid = 10:10:4). Anal.: $C_{23}H_{20}O_8$ (424) (C,H) (calcd/found): %C 65.09/65.07, %H 4.75/4.90.

*7.1.3.5. 7-acetonyl-11-methoxy-6-oxo-6H,7H-(1)-benzopyrano[4,3b]-(1)-benzopyran (**SS-20**).* Beige crystals, m.p. 149–152 °C. Yield: 7%; ^1H NMR (DMSO, 250 MHz): $\delta = 1.9$ (s, 3H, aliphatic), 3.3 (m, 2H, aliphatic), 3.9 (s, 3H, aliphatic), 5.0 (s, 1H, aliphatic), 6.9 (m, 3H, aromatic), 7.3 (m, 2H, aromatic), 7.6 (m, 1H, aromatic), 7.8 (m, 1H, aromatic); EIMS:

m/z (%) = 336 (100, M^+), 321 (77.2), 305 (38.6), 293 (17.5), 279 (21), 265 (3.5), 238 (3.5), 227 (4.4), 213 (53.5), 201 (7.9), 188 (2.6), 175 (24.6), 160 (9.6), 152 (9.6), 132 (7.9), 121 (15.8), 103 (2.6), 77 (6.1), 65 (5.3), 51 (2.6); TLC: $R_f = 0.76$ (hexane/ether = 1:2).

*7.1.3.6. Ethyl 2-[(4-hydroxy-2-oxo-2H-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (**SS-21**).* White crystals, m.p. 135–140 °C. Purified after recrystallization from ethanol. Yield: 37%; UV–vis: $\lambda_{max} = 210$ nm; FTIR (nujol): 3335, 1732.3, 1674.4, 1620.4, 1529.7, 1068.7, 763, 736 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H, aliphatic), 1.9 (s, 3H, aliphatic), 3.9 (q, 2H, aliphatic), 4.4 (m, 1H, aliphatic), 4.6 (m, 1H, aliphatic), 7.2 (m, 1H, aromatic), 7.4 (m, 1H, aromatic), 7.5 (m, 1H, aromatic), 7.7 (m, 3H, aromatic), 7.9 (m, 2H, aromatic), 9.8 (s, 1H, hydroxyl); EIMS: *m/z* (%) = 425 (4.4, M^+), 382 (4.4), 361 (12.3), 336 (38.6), 320 (1.8), 308 (2.6), 294 (58.8), 278 (100), 266 (8.8), 257 (14.9), 249 (48.2), 248 (91.2), 239 (1.8), 220 (8.8), 205 (1.8), 176 (3.5), 165 (10.5), 139 (5.3), 130 (15.8), 120 (71.9), 101 (13.2), 92 (68.4), 85 (18.4), 75 (14.9), 64 (17.5), 51 (6); TLC: $R_f = 0.34$ (hexane/acetone = 2:1).

7.2. Computational study

All of the calculations are made with the aid of Horseshoe computer cluster (University of Southern Denmark). The DFT calculations about geometry optimization and ESP charges are implemented by Gaussian 03 program [20]. The other properties like Log *P*, dipole moment, energies of HOMO and LUMO orbitals and molecular volume are calculated by Spartan 04 program [21]. B3LYP/6-31G** method is used for geometry optimization and for calculation of some properties. Gauss View program is used to visualize the results from calculations, which are made by Gaussian 03.

All the initial optimizations and conformational search are implemented by Maestro Macromodel program from Schrødinger package [22], using the force field MM2 or OPLS2005. Conformational search is made by Monte Carlo simulation method at 20 000 iterations. Single point energies of different conformations are calculated by Gaussian 03 with B3LYP/6-31G* method.

XCluster program from Maestro Schrødinger package is used in order to obtain representative conformations.

7.3. Pharmacological study

The urinary bladder carcinoma-derived EJ cells were obtained from the American Type Cell Cultures; the promyelocyte leukemia-derived HL-60 cells were purchased from the German Collection of Microorganisms and Cell Cultures. Cells were kept in controlled environment – RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine, at 37 °C in a 'Heraeus' incubator with 5% CO_2 humidified atmosphere.

The cytotoxicity of the compounds was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay. Exponentially growing cells were seeded in 96-well microplates (100 μl/well at a density of 3.5×10^5 cell/ml for the adherent and 1×10^5 cells/ml for the suspension cell lines) and allowed to grow for 24 h prior to the exposure to the studied compounds. Stock solutions of the tested compounds were freshly prepared in DMSO and then diluted with growth medium. At the final dilutions the solvent concentration never exceeded 0.5%. Cells were exposed to the tested agents for 72 h, whereby for each concentration a set of eight separate wells were used. After the exposure period MTT solution (10 mg/ml in PBS) aliquots were added to each well. The plates were further incubated for 4 h at 37 °C and the formazan crystals formed were dissolved by adding 110 μl of 5% HCOOH in 2-propanol. Absorption of the samples was measured by an ELISA reader (Uniscan Titertec) at 580 nm. Survival fraction was calculated as percentage of the untreated control. The experimental data were processed by means of GraphPad Prism software and were fitted to sigmoidal concentration–response curves via non-linear regression.

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