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Brief Article

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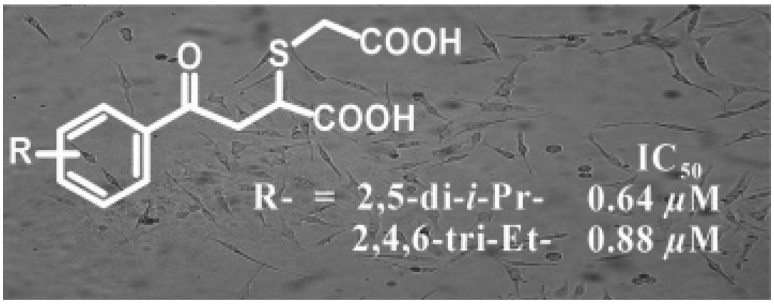

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Brief Articles

2-[(Carboxymethyl)sulfanyl]-4-oxo-4-arylbutanoic Acids Selectively Suppressed Proliferation of Neoplastic Human HeLa Cells. A SAR/QSAR Study

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A series of twenty alkyl-, halo-, and methoxy-aryl-substituted 2-[(carboxymethyl)sulfanyl]-4-oxo-4-arylbutanoic acids were synthesized. The new compounds, called CSAB, suppressed proliferation of human cervix carcinoma, HeLa cells, in vitro in a concentration range of 0.644 to 29.48 μM/L. Two compounds exhibit antiproliferative activity in sub-micromolar concentrations (**16**, **19**). Five compounds act in low micromolar concentrations (<2 μM/L). The most active compounds exert lower cytotoxicity toward healthy human peripheral blood mononuclear cells (PBMC and PBMC+PHA) (selectivity indexes > 10). A strong structure−activity relationship, using estimated log *P* values and BCUT descriptors, was observed.

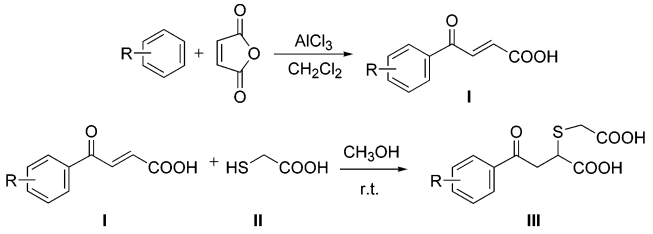
Introduction

In our previous paper[1] antiproliferative action of (*E*)-4-aryl-4-oxo-2-butenoic acids (ArC(O)CH=CHCOOH) toward human cervix carcinoma HeLa cells was reported. It was suggested that cytostatic actions of those compounds were attributed to Michael-type addition of thiol groups on the ketovinyl moiety of the molecules. Similar molecules exert high affinity toward thiol groups of biologically important molecules.[2−4] It is known that 4-aryl-4-oxobutanoic acid derivatives, having mercapto group in their structures, exert a potent immunosuppressive,[5] cytostatic,[6] and antibacterial[7,8] activity.

Results presented in this article show that 2-[(carboxymethyl)sulfanyl]-4-oxo-4-arylbutanoic acids (CSAB), obtained by the Michael addition of inactive thioglicolic acid on the ketovinyl moiety of (*E*)-4-aryl-4-oxo-2-butenoic acids, exert stronger cytostatic activity toward HeLa cells in vitro in comparison to corresponding parent (*E*)-4-aryl-4-oxo-2-butenoic acids, by approximately one order of magnitude. The most active compounds (**16**−**20**) acts selectively, i.e. exert lower cytotoxicity toward healthy human peripheral mononuclear blood cells (PBMC and PBMC+PHA).

The 2-[(carboxymethyl)sulfanyl]-4-oxo-4-arylbutanoic acids (**III**) (Scheme 1) were synthesized in Michael-type addition of the thioglycolic acid (**II**) to series of (*E*)-4-aryl-4-oxo-2-butenoic acids (**I**) (Scheme 1). (*E*)-4-Aryl-4-oxo-2-butenoic acids were prepared by Friedel−Crafts acylation using maleic acid anhydride.[1,9] All synthesized 2-[(carboxymethyl)sulfanyl]-4-oxo-4-arylbutanoic acids are the mixtures of *R* and *S* enantiomers.

Scheme 1. Syntheses of Compounds **1**-**20**[a]



[a] R = H (**1**), 3,4-di-Me (**2**), 2,4-di-Me (**3**), 2,5-di-Me (**4**), 4-Me (**5**), 4-Et (**6**), 4-*i*-Pr (**7**), 4-*n*-Bu (**8**), 4-*tert*-Bu (**9**), 4-*n*-dodecyl (**10**), 2-Cl-4-Me (**11**), 4-F (**12**), 4-Cl (**13**), 4-Br (**14**), 2,3,4-tri-MeO (**15**), 2,5-di-*i*-Pr (**16**), 2,4-di-*i*-Pr (**17**), 2,5-di- *tert*-Bu (**18**); 2,4,6-tri-Et (**19**), 2,4,6-tri-*i*-Pr (**20**).

The structures of synthesized compounds were confirmed by infrared (IR) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra (see Experimental Section). The crystal structure of the lead compound, 2-[(carboxymethyl)sulfanyl]-4-oxo-4-phenylbutanoic acid (**1**), was determined, too.[10] Crystallization solvent, melting points, yields, and analysis of compounds **1**−**20** are given in Table 1.

The NMR data are used as a basis for structure elucidation. This series contains closely related structures that share a common major structural component and have very similar spectral features. The assignment of the peaks is shown in the example of 2-[(carboxymethyl)sulfanyl]-4-oxo-4-(4-ethylphenyl)butanoic acid (**6**) in Charts 1 and 2.

Experimental Section

Substituted benzenes, thiogycolic acid, and anhydrous $AlCl_3$ were purchased from Sigma-Aldrich or Fluka. For the synthesis of **1** and for crystallization, thiophene-free benzene was used. All other solvents were p.a. grade. Melting points were determined in open capillary tubes on Büchi apparatus and are uncorrected. Infrared (IR) spectra were recorded with a Perkin-Elmer 1725X spectrophotometer, (KBr disk). ^{1}H and

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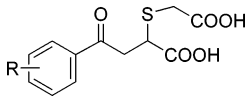
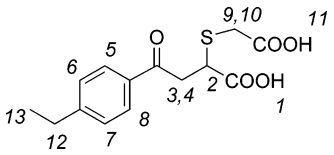
Table 1. Crystallization Solvent, Melting Points, Yields, and Analysis of Compounds **1**−**20**



	R	mp, °C	solvent	yield, %	M_w	analysis[a]
1	H	96	water	77	268.29	($C_{12}H_{12}O_5S$) C,H,S
2	3,4-di-Me	163	benzene	79	296.34	($C_{14}H_{16}O_5S$) C,H,S
3	2,4-di-Me	138	benzene	80	296.34	($C_{14}H_{16}O_5S$) C,H,S
4	2,5-di-Me	140	benzene	86	296.34	($C_{14}H_{16}O_5S$) C,H,S
5	4-Me	174	benzene	83	282.31	($C_{13}H_{14}O_5S$) C,H,S
6	4-Et	132	benzene	87	296.34	($C_{14}H_{16}O_5S$) C,H,S
7	4-*i*-Pr	106	water	78	310.37	($C_{15}H_{18}O_5S$) C,H,S
8	4-*n*-Bu	120	benzene	82	324.39	($C_{16}H_{20}O_5S$) C,H,S
9	4-*tert*-Bu	139	benzene	84	324.39	($C_{16}H_{20}O_5S$) C,H,S
10	4-*n*-dodecyl	90	cyclohexane	71	436.61	($C_{24}H_{36}O_5S$) C,H,S
11	2-Cl-4-Me	121	benzene	81	316.76	($C_{13}H_{13}ClO_5S$) C,H,S
12	4-F	148	benzene	86	286.28	($C_{12}H_{11}FO_5S$)
13	4-Cl	141	benzene	86	302.73	($C_{12}H_{11}ClO_5S$) C,H,S
14	4-Br	145	benzene	86	347.18	($C_{12}H_{11}BrO_5S$) C,H,S
15	2,3,4-tri-MeO	161	benzene	75	358.36	($C_{15}H_{18}O_8S$) C,H,S
16	2,5-di-*i*-Pr	118	cyclohexane	74	352.45	($C_{18}H_{24}O_5S$) C,H,S
17	2,4-di-*i*-Pr	126	cyclohexane	68	352.45	($C_{18}H_{24}O_5S$) C,H,S
18	2,5-di-*tert*-Bu	163	benzene	40	380.50	($C_{20}H_{28}O_5S$) C,H,S
19	2,4,6-tri-*i*-Et	125	benzene	70	352.45	($C_{18}H_{24}O_5S$) C,H,S
20	2,4,6-tri-*i*-Pr	168	benzene	75	394.53	($C_{21}H_{30}O_5S$) C,H,S

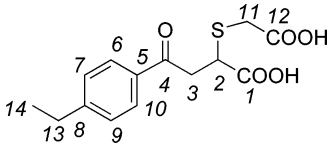
[a] Analysis for C, H, S within ±0.4% of calculated values.

Chart 1. Hydrogen Labels Correspond to the List of ^{1}H NMR Shifts[a]



[a] δ 1.20 (3H, t, J = 7.68 Hz) [*12*]; 2.68 (2H, q, J = 7.67 Hz) [*13*]; 3.34 (1H, dd, J = 17.76, 3.73) [*4*]; 3.52 (2H, s) [*9 and 10*]; 3.65 (1H, dd, J = 18.41, 10.73) [*3*]; 3.85 (1H, dd, J = 10.52, 3.73) [*2*]; 7.37 (2H, d, J = 8.11 Hz) [*5 and 8*]; 7.91 (2H, d, J = 8.11 Hz) [*6 and 7*]; 12.70 (2H, b) [*1 and 11*].

Chart 2. Carbon Labels Correspond to the List of ^{13}C NMR Shifts[a]



[a] δ 15.449 [*14*]; 28.485 [*13*]; 33.401 [*11*]; 40.665 [*3*]; 41.136 [*2*]; 128.475 [*7 and 9*]; 128.529 [*6 and 10*]; 133.991 [*5*];150.377 [8]; 171.260 [*12*]; 172.880 [*1*]; 197.004 [*4*].

^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 200/50 MHz with tetramethylsilane (TMS) as internal standard on a Varian "Gemini 200" spectrometer in DMSO-d_6. The chemical shifts are expressed as ppm downfield from TMS. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, sx = sextet, sp = septet, m = multiplet, and b = broad. The mass spectrum was taken on a Finnigan-MAT 8230 BE MS, chemical ionization (*i*-C_4H_{10}).

Typical Experimental Procedure. In a 10 mL flask equipped with a magnetic stirrer, 0.001 mol of (*E*)-4-aryl-4-oxo-2-butenoic acid and 0.0015−0.002 mol of thioglycolic acid in 5 mL of methanol was stirred 3−4 h at room temperature. Solvent was removed under reduced pressure. Addition of water precipitated crystalline 2-[(carboxymethyl)sulfanyl]-4-oxo-4-phenylbutanoic acid (**1**) and 2-[(carboxymethyl)sulfanyl]-4-oxo-4-(4-*i*-Pr-phenyl)butanoic acid (**7**), which were separated by filtration. Other 2-[(carboxymethyl)sulfanyl]-4-oxo-4-arylbutanoic acids were isolated by crystallization from the proper solvent.

Antiproliferative Action. A stock solution of examined compounds was made in dimethyl sufoxide, at 10 mM/L concentration, and then various dilutions were prepared in nutrient medium (RPMI 1640 medium without phenol red, supplemented with L-glutamine (3 mmol/L), streptomycin (100 μg/mL), and penicillin (100 IU/mL), 10% heat-inactivated fetal bovine serum, FBS, and 25 mM HEPES, adjusted to pH 7.2 (by bicarbonate solution)) to various final concentrations (between 3.12 and 50 or 100 μM/L). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemicals (St. Louis, MO). MTT was dissolved (5 mg/mL) in phosphate-buffered saline, pH 7.2, and filtered through a Millipore filter, 0.22 μm, before use. RPMI 1640 cell culture medium and FBS were products of ICN Pharmaceuticals Co. (Costa Mesa, CA).

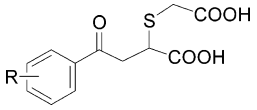
Treatment of HeLa Cells. HeLa cells were seeded into 96-well microtiter plates, 2000 cells in 0.1 mL of nutrient medium per well. After 20 h, to wells with HeLa cells, five different concentrations of investigated substituted 2-[(carboxymethyl)sulfanyl]-4-oxo-4-arylbutanoic acids derivative were applied, except to the control wells, the wells with cells grown in a nutrient medium only. All concentrations were set up in triplicate. Nutrient medium with corresponding concentrations of investigated agent, but without cells, was used as a blank, also in triplicate.

Preparation of Peripheral Blood Mononuclear Cells, PBMC. PBMC were separated from whole heparinized blood of two healthy volunteers by Lymphoprep gradient centrifugation. Interface cells, washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5. 1 mM K^+, 6. 2 mM. Ca^{2+}, 145 mM Cl^-, and 35 g/L gelatin polymers, pH 7.4) were counted and resuspended in nutrient medium.

Treatment of PBMC. PBMC were seeded (150 000 cells per well) into nutrient medium or in nutrient medium enriched with (5 μg/mL) phytohaemaglutinin (PHA) (Welcome) in 96-well microtiter plates, and 2 h later, investigated substances were added to the wells, in triplicate, to five final concentrations, except to the control wells where a nutrient medium only was added to the cells. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as blank.

Determination of Cell Survival. HeLa cell survival, or PBMC survival, was determined by MTT test, according to Mosmann,[11] with modification by Ohno and Abe,[12] 72 h upon addition of the drug, as it was described earlier.[13] Briefly, 20

Table 2. Concentrations of Examined Agents that Induced a 50% Decrease in HeLa Cell Survival (IC_{50}), Observed log(1/(IC_{50})) − Observed Activities, Predicted log(1/(IC_{50})) − Predicted Activities (derived from eq 1), and Descriptors Used in QSAR



		cell line HeLa				
	R	IC_{50} (μmol/L)	observed, log(1/(IC_{50}))	predicted, log(1/(IC_{50}))	BEHv5	log *P*
1	H	14.98	4.824	4.613	2.655	0.71
2	3,4-di-Me	3.81	5.419	5.394	2.878	1.68
3	2,4-di-Me	3.83	5.417	5.449	2.928	1.68
4	2,5-di-Me	2.68	5.572	5.416	2.898	1.68
5	4-Me	17.30	4.762	4.912	2.655	1.20
6	4-Et	4.79	5.320	5.299	2.820	1.61
7	4-*i*-Pr	3.33	5.478	5.488	2.867	1.94
8	4-*n*-Bu	1.915	5.718	5.793	2.997	2.45
9	4-*tert*-Bu	1.39	5.857	5.660	2.885	2.41
10	4-*n*-dodecyl	1.71	5.767	5.695	3.275	5.79
11	2-Cl-4-Me	4.73	5.325	5.229	2.700	1.75
12	4-F	20.69	4.684	4.717	2.655	0.87
13	4-Cl	17.24	4.763	4.950	2.655	1.27
14	4-Br	9.50	5.022	5.086	2.655	1.54
15	2,3,4-tri-MeO	29.48	4.530	4.609	2.898	0.33
16	2,5-di-*i*-Pr	0.644	6.191	6.012	3.081	3.18
17	2,4-di-*i*-Pr	1.10	5.959	6.025	3.093	3.18
18[a]	2,5-di-*tert*-Bu	4.36	5.361	6.082	3.163	4.12
19	2,4,6-tri-Et	0.88	6.056	6.079	3.129	3.42
20	2,4,6-tri-*i*-Pr	1.66	5.780	6.053	3.180	4.41
/	thioglycolic acid	>200	/	/	/	/

[a] Omitted from derivation of equation.

μL of MTT solution (5 mg/mL PBS) were added to each well. Samples were incubated for further 4 h at 37 °C in 5% CO_2 and humidified air atmosphere. Then, 100 μL of 10% SDS were added to the wells. Absorbance at 570 nm was red the next day. To get cell survival (%), absorbance *A* of a sample with cells grown in the presence of various concentration of substituted 2-[(carboxymethyl)sulfanyl]-4-oxo-4-arylbutanoic acid derivative was divided by the control absorbance A_c (the absorbance of sample with cells grown only in nutrient medium) × 100. (It was implied that *A* of blank was always subtracted from *A* of a corresponding sample with target cells.) IC_{50} concentration was defined as the concentration of a drug that inhibits cell survival by 50%, compared with vehicle-treated control.

Results and Discussion

(a) HeLa Cells. Concentrations of examined compounds (**1**−**20**) that induced the 50% decrease in HeLa cell survival (IC_{50}), determined under exactly same experimental conditions, are given in Table 2.

Our first observation that 2-[(carboxymethyl)sulfanyl]-4-oxo-4-phenylbutanoic acid (**1**) inhibited proliferation of the human cervix carcinoma HeLa cells in vitro led us to assume that the structure of **1** is a minimal scaffold enabling activity. By using **1** as a template, one to three alkyl, halo, or alkoxy substituents were introduced in the phenyl ring to explore structure−activity relationships. Increases in cytostatic activity were observed as the halo substituent changes from 4-F (**12**) to 4-Cl (**13**) to 4-Br (**14**). Only the 4-Br derivative (**14**) exerts higher activity than the lead compound (**1**). Introduction of one alkyl substituent larger than methyl group in para position moderately improves activity. Presence of one *tert*-Bu (**9**) group or *n*-alkyl chain (more than four C atoms) (**8**, **10**) in the para position caused a markedly improved activity. Introduction of two alkyl substituents moderately increase activity. Preferred positions on phenyl ring are 2 and 5, rendering compound **4** more active than compounds **2** and **3**. Observation that alkyl substitution in positions 2, 4, and 5 of the phenyl ring improves activity, and that branched alkyl chains are preferred for higher activity, prompted us to substitute positions 2, 4, and 5 with branched alkyl chains. Moreover, we observed that compounds with long alkyl chains in position 4 exert significant activity. Having in mind that one of the ortho positions should be occupied for high activity, we assumed that (because of conformational flexibility of *n*-alkyl chains) part of or the free end of the 4-*n*-alkyl chain occupies a position near the ortho position of the phenyl ring, within the receptor. Compounds **16**−**20**, with two or three alkyl substituents longer than one C, were synthesized. These derivatives have been drastically more active than all other compounds. The most active compounds have an *i*-Pr group in positions 2 and 5 (**16**), an Et group in positions 2, 4, and 6 (**19**), and an *i*-Pr group in positions 2 and 4 (**17**), in decreasing order of activity. A tri-*i*-Pr-substituted derivative (positions 2, 4, and 6) (**20**) is slightly less active. Compounds **16** and **19** are active in sub-micromolar concentrations (0.64 and 0.88 μM/L), while compounds **8**, **9**, **10**, and **20** exert activity in low micromolar concentrations (<2 μM/L). The two *tert*-Bu groups in positions 2 and 5 (**18**) reduce the activity related to other compounds substituted with branched alkyl chains. Introduction of one *o*-halo substituent in place of alkyl, in disubstituted compounds, decreased the activity (**11**). Occupation of positions 2 and 4 with methoxy substituents (**15**) makes the least active member of series.

Table 3. Concentrations of Examined Compounds (**16**−**20**) That Induced the 50% Decrease in PBMC and PBMC+PHA Cell Survival (IC_{50}) and Selectivity Indexes (S_i)

	R	IC_{50} (μM)	S_i
	PBMC+PHA		
16	2,5-di-*i*-Pr	15.67	24.33
17	2,4-di-*i*-Pr	7.14	6.49
18	2,6-di-*tert*-Bu	37.1	8.50
19	2,4,6-tri-Et	19.92	22.63
20	2,4,6-tri-*i*-Pr	4.78	2.58
	thioglycolic acid	> 200	/
	PBMC		
16	2,5-di-*i*-Pr	13.3	20.65
17	2,4-di-*i*-Pr	6.79	6.17
18	2,6-di-*tert*-Bu	43.72	10
19	2,4,6-tri-Et	18.97	21.56
20	2,4,6-tri-*i*-Pr	3.32	2.00
	thioglycolic acid	> 200	/

To quantify above-reported facts, a QSAR study was performed. Correlation between activity and lipophilicity was observed (by increasing activity in the series 4-F (**12**), 4-Cl (**13**), 4-Br (**14**) and 4-Me (**5**), 4-Et (**6**), 4-*i*-Pr (**7**), 4-*n*-Bu (**8**), 4-*n*-dodecyl (**10**)). Therefore, log *P* and number of 2D whole-molecule descriptors were calculated. Estimation of the logarithm of partition coefficient [*n*-octanol/water] log(*P*) = log(K_{OW}) was done by Crippen's fragmentation method.[14] Whole molecular descriptors that can quantitavely describe diversity between the most active compounds (structural isomers), BCUT,[15] were calculated by using Dragon[16] program. The significant parabolic (eq 1) and bilinear (eq 2) dependencies were assessed[17] using Crippen log *P* values and BEHv5

(highest eigenvalue n.5 of Burden matrix/weighted by atomic van der Waals volumes − BCUT descriptor),[18] for 19 compounds (**1**−**17**, **19**, **20**):

$$\log(1/C) = -0.117\ (\pm 0.032)\ [\log P]^2 + 0.832\ (\pm 0.22)\ \log P + 1.096\ (\pm 0.73)\ \text{BEHv5} - 1.172\ (\pm 1.91) \quad (1)$$

$$\log P_{\text{optimum}} = 3.56\ (3.10/4.10)$$

$$(n = 19;\ r = 0.965;\ s = 0.145;\ F = 67.032;\ Q^2 = 0.771;\ S_{\text{press}} = 0.264)$$

$$\log(1/C) = +\ 0.638\ (\pm 0.16)\ \log P - 0.868\ (\pm 0.21)\ \log(\beta P + 1) + 1.048\ (\pm 0.66)\ \text{BEHv5} - 1.356\ (\pm 1.72) \quad (2)$$

$$\log \beta = -2.594\ \log P_{\text{optimum}} = 3.04$$

$$(n = 19;\ r = 0.974;\ s = 0.130;\ F = 63.875)$$

Obtained models suggest a change in the rate-limiting transport step (for compounds of high log *P*) from diffusion across the membranes (cell and/or nucleus). Term BEHv5 could be attributed to ligand−receptor interaction, describing the steric demand for interaction.

2,5-Di-*tert*-Bu derivative (**18**) was omitted from correlations (eqs 1 and 2). Inclusion of compound **18** results in an inferior correlation, probably because of steric hindrance of substrate binding by the receptor. Introduction of a bulky *tert*-Bu group in position 5 (**18**) induces steric congestion on the area covered by substituents 4, 5, and 6, which cannot be accommodated by the receptor. Implicitly, proof for this is lower activity of compound **20** (two *i*-Pr groups in positions 4 and 6) than of compounds **16** (one *i*-Pr group in position 5) and **19** (two Et groups in positions 4 and 6). Compound **20** is 1.9-fold less active than compound **19**.

(b) PBMC and PBMC+PHA Cells. Concentrations of examined compounds **16**−**20** that induced 50% decrease in PBMC and PBMC+PHA (healthy human's peripheral blood cells) cells survival (IC_{50}), determined under exactly same experimental conditions, are given in Table 3.

The results were assessed by a single measurement done in triplicate. In the fourth column of the Table 3 selectivity indexes S_i are shown. The S_is were calculated as IC_{50}(toward normal cells)/IC_{50}(toward malignant cells), for the every examined compound (**16**−**20**).

Compounds **16** and **19,** which act in sub-micromolar concentrations toward human cervix carcinoma HeLa cells, exert the highest selectivity and will be further examined. Also, parabolic dependencies between activity toward healthy cells (implying selectivity) and lipophilicity were observed. Because of the small set of compounds tested on normal cells (PBMC and PBMC+PHA), this observation was given only as an indication. The above conclusions provide a basis for planning syntheses of new congeners with expected higher activity.

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Supporting Information Available: Analytical and spectral data for all synthesized compounds. Statistics of IC_{50} data toward HeLa cells. Regression data for eqs 1 and 2. Microanalysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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