Synthesis and antiproliferative activity of combretastatin derivatives with adamantane fragment

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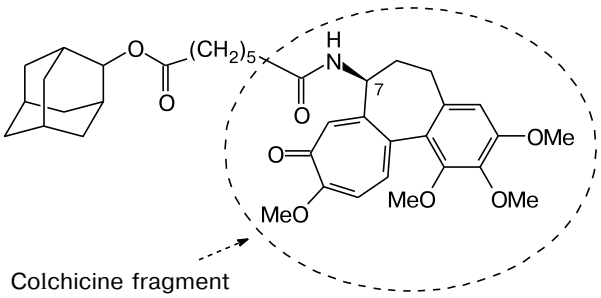
The work describes the synthesis of 2-adamantyl 7-[(2-{[(2*E*)-3-(3-hydroxy-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)prop-2-enoyl]amino}ethyl)amino]-7-oxoheptanoate and 7-[(2-{[(2*E*)-2-(3,4-dimethoxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)prop-2-enoyl]amino}ethyl)amino]-7-oxoheptanoate. The latter compound exhibits moderate cytotoxicity (EC_{50} = 4.8 μmol L^{-1}) against the human epithelial lung carcinoma cells A549.

Key words: combretastatin, adamantane, colchicine, tubuloclustin, tubulin, cytotoxicity, carcinoma A549.

Earlier,[1—3] in the search for compounds with antitumor activity we obtained C(7)-substituted derivatives of natural colchicine, among which were compounds (for example, tubuloclustin (**1**)) with very high cytotoxicity against different types of tumor cells *in vitro*.[3,4]

Attempted replacement of the colchicine moiety in compounds of the type **1** with other analogues possessing lower general toxicity, such as nocodazole and 2-methoxyextradiol, was unsuccessful because of either synthetic difficulties[5,6] or instability[7] and inactivity[8] of the compounds obtained. The purpose of the present work is the synthesis and biotesting of analogues of **1** based on the natural combretastatin A-4 (CA-4), whose antitumor activity is also related to the interaction with the colchicine binding site with its cell target protein tubulin.[9]

According to a pharmacophore model of the tubulin—colchicine site ligand complexes,[10,11] the nitrogen atom of the colchicine amide group is on a certain distance (~3—3.5 Å) from both carbon atoms of the combretastatin double bond. This necessitates the elongation of the linker connecting the adamantane core with CA-4, as compared to that of tubuloclustin (**1**). Among known CA-4 derivatives with substituents at the double bond carbon atoms appropriate for modification, the ability to cause the effect typical of colchicine,[12] *viz.*, the inhibition of tubulin polymerization approximately in the same concentrations as for colchicine, was confirmed only for compound **2**. Therefore, we decided to synthesize compounds **3a**,**b**, in which a substituted adamantane group is attached to the amide group of **2**. We assumed that the introduction of two additional (as compared to **1**) methylene groups into the structures **3a**,**b** would provide the linker elongation mentioned above. In these studies, **3b** was the target



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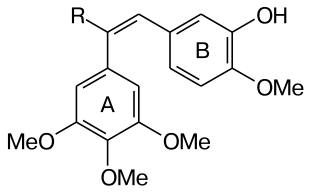

CA-4, **2**

R = H (CA-4), $C(O)NH_2$ (**2**)

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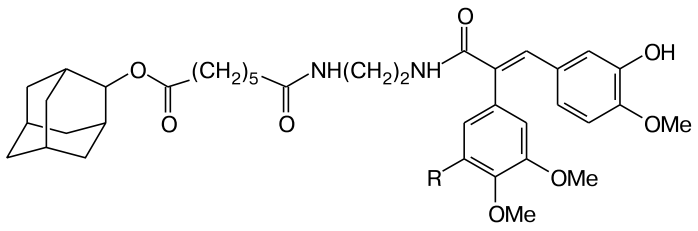


3a,b

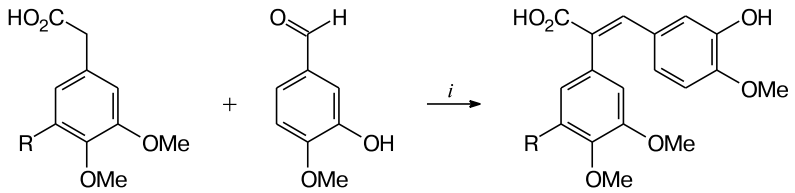
R = H (**3a**), OMe (**3b**)

compound, whereas **3a** was used as a model for the studies of the role of trimethoxyphenyl group in **3b** in exhibiting cytotoxic properties.

The starting combretastatin derivatives **4a** and **4b** were obtained by Perkin condensation[12,13] from 3,4-dimethoxy- or 3,4,5-trimethoxyphenylacetic acid and 3-hydroxy-4-methoxybenzaldehyde in the presence of acetic anhydride and triethylamine (Scheme 1).

The synthesis of target compounds **3a**,**b** was carried out according to Scheme 2. In the first step, the reaction of monoprotected *N*-Boc-ethylenediamine (**5**) and pimelic polyanhydride gave amide **6**. The ^{13}C NMR spectrum of this compound exhibits three signals characteristic of the carbonyl carbon atoms: the *tert*-butoxycarbonyl, the carboxy, and the amide ones at δ 157.02, 174.25, and 177.29, respectively. Then, pimelic acid monoamide **6** was esterified with adamantan-2-ol to ester **7**. In the ^1H NMR spectrum of compound **7**, the signal for the proton at atom C(2) of the adamantane group is downfield shifted (δ 4.89) as compared to the starting adamantan-2-ol (δ 3.83). A sim-

Scheme 1

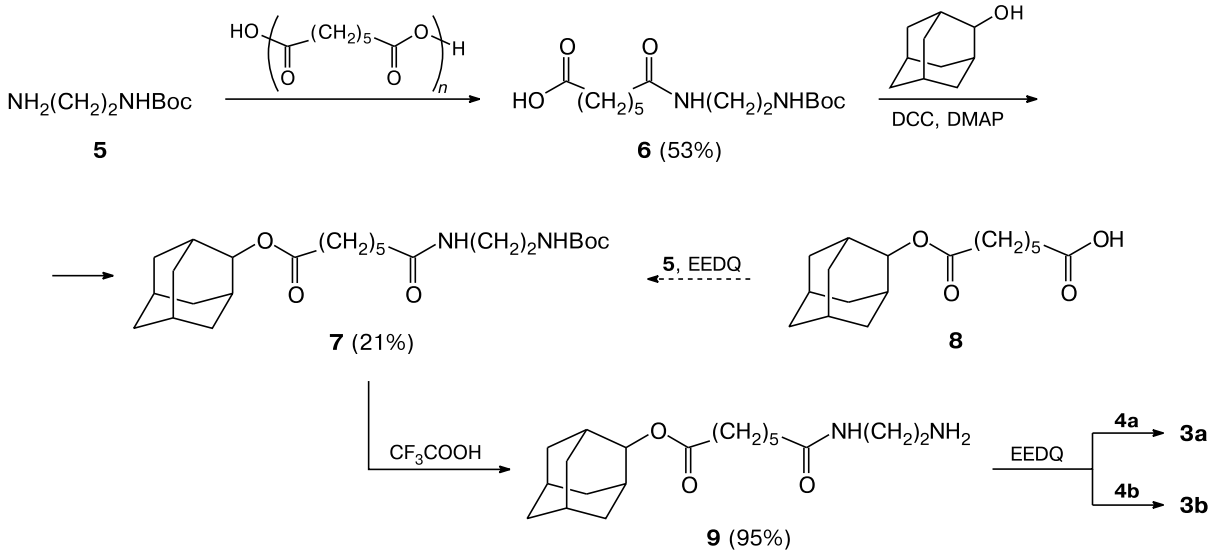


4a,b

R = H (4**a**, 34%), OMe (**4b**, 43%)

i. NEt_3, Ac_2O, heating.

Scheme 2



ilar shift is also observed for atom C(2) in the ^{13}C NMR spectrum (δ 76.82 for **7** and δ 74.52 for Ad-2-OH).

It should be noted that an alternative order of assembling molecule **7**, namely, the addition of *N*-Boc-ethylenediamine to monoester of adamantan-2-ol and pimelic acid **8** (obtained according to procedure[3]) in the presence of 2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) was preparatively unsuccessful because of very low yield of product **7** (see Experimental).

The protecting group in compound **7** was removed according to a standard procedure, the resulting amine **9** was involved in the reaction with combretastatin carboxy derivatives **4a**,**b** in the presence of EEDQ. The ^{13}C NMR spectra of the target products exhibit the signals for three carbonyl carbon atoms in the range of δ 168—174 and the signals for the aromatic atoms of combretastatin fragment within δ 106—155. The ^{1}H NMR spectroscopy and elemental analysis data also confirm the structure of compounds **3a** and **3b**.

The biotesting of conjugates **3a** and **3b** in the standard colorimetric MTT test[14] against the culture of human lung carcinoma cells A549 showed that compound **3b** possesses a noticeable, but a moderate cytotoxicity: EC_{50} = 4.8±0.1 μmol L^{-1} (for colchicine, EC_{50} = = 0.04±0.09 μmol L^{-1}). Compound **3a** is low active (EC_{50} > 50 μmol L^{-1}).

Based on the assumption that the obtained values of cytotoxicity are determined only by the binding with protein (without considering metabolism, the ease of penetration into the cell, *etc*.), we attempted to explain the results using computer molecular modeling. The results of the automatic docking of ligand **3b** into the 3D model of tubulin in the colchicine binding site[10] using the CLC Drug Discovery Workbench program showed that in one of the most favorable variants of arrangement of structure **3b** in the colchicine site (among several different variants with similar to each other and to compound **1** scoring functions), the combretastatin group of **3b** was placed into the region similar to that occupied by free CA-4 (Fig. 1).

The binding of **3b** with protein involves both methoxy groups in *meta*-positions of the aromatic ring. The oxygen atom of one of them forms a hydrogen bond with the hydrogen atom of the amide group of the main chain βLeu255, whereas the methyl group of the other one is involved in hydrophobic interactions with the side chains βAla354, βAla316, βVal318 and the alkyl fragment of the side chain βLys352. This explains a decrease in the activity for analogue **3b** without one *meta*-methoxy substituent in the aromatic ring (**3a**). A decrease in cytotoxicity of conjugate **3b** as compared to tubuloclustin (**1**) can be explained by a noticeable difference in the position of free CA-4 and that in conjugate **3b** (see Fig. 1), apparently, caused either by too high conformational "rigidity" or too long diamide chain of **3b**. Therefore, the further search for the tubulin ligands of the type under consideration should be focused on the analogues of **3b** with more "flexible" and shorter group between the CA-4 fragment and the substituted adamantane core. The approaches to the synthesis of such compounds are currently under study.

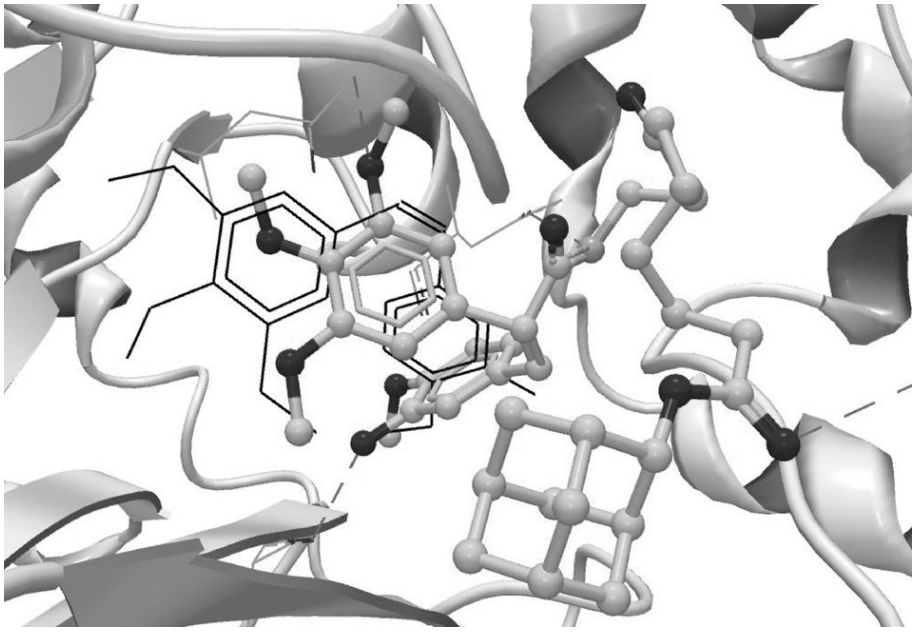


Fig. 1. One of the most energetically favorable variants of the arrangement of structure of **3b** in the tubulin dimer (the β-subunit is on the left) according to the results of automatic docking (CLC Drug Discovery Workbench). The molecule of free combretastatin A-4 is shown for comparison, hydrogen bonds are depicted in dashed lines (hydrogen atoms are omitted).

Experimental

Automatic docking into the 3D model of the tubulin complex with *N*-deacetyl-*N*-(2-mercaptoacetyl)colchicine (a predetermined radius 16 Å) was performed using the CLC Drug Discovery Workbench program (Version 1.5): Evaluation license (2014).

Reaction progress and purity of compounds were monitored by TLC on Silufol-UV254 plates. Chromatographic separation was carried out on a column with Acros silica gel (40—60 μm). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 and 100 MHz, respectively) at 28 °C. Chemical shifts are given relative to the residual signals of the solvent. Elemental analysis was performed on a Vario micro cube CHN-analyzer. IR spectra were recorded on a IR-200 ThermoNicolet spectrophotometer in KBr pellets.

(2*E*)-3-(3-Hydroxy-4-methoxyphenyl)-2-(3,4-dimethoxyphenyl)acrylic acid (4a). 3,4-Dimethoxyphenylacetic acid (1.73 g, 8.82 mmol) was added to a solution of 3-hydroxy-4-methoxybenzaldehyde (0.67 g, 4.4 mmol) in Ac_2O (4 mL, 42.4 mmol) and NEt_3 (2 mL, 14.29 mmol). The mixture was stirred for 6 h at 120 °C, poured onto ice (20 g), and acidified with concentrated HCl. A precipitate was filtered, washed with cold CH_2Cl_2, and recrystallized from EtOH to obtain compound **4a** (0.5 g, 34%), yellow crystals, m.p. 173—175 °C. ^{1}H NMR ($CDCl_3$), δ: 3.83 (s, 3 H, Me); 3.88 (s, 3 H, Me); 3.94 (s, 3 H, Me); 6.69 (s, 1 H, C_{Ar}(2)H); 6.70 (m, 2 H, C_{Ar}(5)H and C_{Ar}(6)H); 6.77 (d, 1 H, C_{Ar}(2)H, J = 1.7 Hz); 6.83 (dd, 1 H, C_{Ar}(5)H, J = 8.4 Hz, J = 1.7 Hz); 6.93 (d, 1 H, C_{Ar}(6)H, J = 8.4 Hz); 7.83 (s, 1 H, C=CH). ^{13}C NMR (DMSO-d_6), δ: 55.87 (Me); 55.93 (Me); 55.98 (Me); 111.99, 112.31, 113.68, 117.61, 122.23 (C_{Ar}(6)); 123.26 (C_{Ar}(6)); 127.74 (C(2)); 129.34 (C_{Ar}(1)); 130.77 (C_{Ar}(1)); 139.39 (C(3)); 146.29 (C_{Ar}(3)); 146.78 (C_{Ar}(3)); 148.63 (C_{Ar}(4)); 149.18 (C_{Ar}(4)); 169.29 (C=O). IR (KBr), ν/cm^{-1}: 808, 1024, 1136—1144, 1255, 1439, 1514, 1603, 1666 (C=O), 2837, 2953, 3417 (OH). Found (%): C, 65.52; H, 5.51. $C_{18}H_{18}O_6$. Calculated (%): C, 65.45; H, 5.49.

(2*E*)-3-(3-Hydroxy-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (4b) was obtained according to the procedure described earlier.[12] The yield was 43%, yellow crystals, m.p. 238—240 °C (*cf.* Ref. 13: m.p. 237—239 °C). Spectral data agree with those given in the literature.[12]

7-[2-(*tert*-Butoxycarbonylamino)ethylamino]-7-oxoheptanoic acid (6). Amine **5** (0.64 g, 4 mmol) was added to a solution of pimelic anhydride (0.715 g, 5 mmol) in CH_2Cl_2 (10 mL). After stirring the mixture for 24 h at 20 °C, it was diluted with Et_2O (15 mL) and washed with water. The organic fraction was dried with $MgSO_4$ and concentrated, the residue was subjected to chromatography (eluent ethyl acetate—light petroleum ether (40—60 °C), 1 : 1; then methanol—dichloromethane, 1 : 10) to obtain compound **6** (0.638 g, 53%), a colorless oily liquid. ^{1}H NMR ($CDCl_3$), δ: 1.35 (m, 2 H, $C_\gamma H_2$); 1.41 (s, 9 H, Bu^t); 1.62 (m, 4 H, $C_\beta H_2$); 2.19 (m, 2 H, $C_\alpha H_2$); 2.32 (m, 2 H, $C_\alpha H_2$); 3.29 (m, 2 H, C$\underline{H_2}$NHCO); 3.32 (m, 2 H, C$\underline{H_2}$NHBoc); 5.38 (br.s, 1 H, NH); 6.80 (br.s, 1 H, NH). ^{13}C NMR ($CDCl_3$), δ: 24.37, 25.25, 28.36 (Me); 28.52, 33.83 (C_α); 36.02 ($\underline{C}H_2$CONH); 36.21 ($\underline{C}H_2$NHCO); 40.16 ($\underline{C}H_2$NHBoc); 79.62 ($\underline{C}Me_3$); 157.02 (CO_2Bu^t); 174.25 (CO_2H); 177.29 (CONH). Found (%): C, 55.60; H, 8.69; N, 9.25. $C_{14}H_{26}N_2O_5$. Calculated (%): C, 55.61; H, 8.67; N, 9.26.

2-Adamantyl 7-[2-(*tert*-butoxycarbonylamino)ethylamino]-7-oxoheptanoate (7). Method *A*. 1,3-Dicyclohexylcarbodiimide (0.494 g, 2.4 mmol), adamantan-2-ol (0.357 g, 2.3 mmol), and a catalytic amount of DMAP were added to a solution of acid **6** (0.638 g, 2.1 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 20 h at 20 °C, concentrated, diluted with ethyl acetate (10 mL), and allowed to stand for 3 h at 0—5 °C. The crystals formed were filtered off, the solution was concentrated, the residue was subjected to chromatography (eluent ethyl acetate—light petroleum ether (40—60 °C), 1 : 1) to obtain compound **7** (0.187 g, 21%), a colorless oily liquid.

Method *B*. A solution of 7-(2-adamantyloxy)-7-oxoheptanoic acid **8** (0.298 g, 0.001 mol), amine **5** (0.131 mL, 0.83 mmol), EEDQ (0.329 g, 1.33 mmol) in CH_2Cl_2 (5 mL) was stirred for 24 h at 20 °C; the mixture was concentrated, the residue was subjected to chromatography (eluent ethyl acetate—light petroleum ether (40—60 °C), 1 : 2; then methanol—dichloromethane, 1 : 15) to obtain compound **7** (0.020 g, 5%). ^{1}H NMR ($CDCl_3$), δ: 1.38 (m, 2 H, $C_\gamma H_2$); 1.46 (s, 9 H, Bu^t); 1.56—1.59 (m, 2 H, Ad); 1.64—1.72 (m, 4 H, $C_\beta H_2$); 1.75—1.80 (m, 4 H, Ad); 1.85 (m, 4 H, Ad); 1.89—2.03 (m, 4 H, Ad); 2.16 (t, 2 H, $C_\alpha H_2$, $^3J_{a,\beta}$ = 7.8 Hz); 2.31 (t, 2 H, $C_\alpha H_2$, $^3J_{\alpha,\beta}$ = 7.4 Hz); 3.25 (m, 2 H, BocNH—C$\underline{H_2}$); 3.33 (m, 2 H, C$\underline{H_2}$NHC=O); 4.89 (m, 1 H, C_{Ad}(2)H); 5.21 (br.s, 1 H, NH); 6.49 (br.s, 1 H, NH). ^{13}C NMR ($CDCl_3$), δ: 24.76, 25.28, 26.98, 27.21, 28.35 (Me); 28.71 (γ-CH_2); 31.77 (C_{Ad}); 31.86 (C_{Ad}); 34.61 ($\underline{C}H_2CO_2$); 36.31 (C_{Ad}); 36.39 ($\underline{C}H_2$CONH); 37.36, 40.29 ($\underline{C}H_2$NHCO); 40.68 ($\underline{C}H_2$NHBoc); 76.82 (C_{Ad}(2)); 79.60 ($\underline{C}Me_3$); 158.18 (CO_2Bu^t); 173.10 (C=O); 173.63 (C=O). Found (%): C, 66.09; H, 9.19; N, 6.40. $C_{24}H_{40}N_2O_5$. Calculated (%): C, 66.03; H, 9.23; N, 6.42.

2-Adamantyl 7-(2-aminoethylamino)-7-oxoheptanoate (9). Trifluoroacetic acid (0.5 mL, 6.52 mmol) was added to protected amine **7** (0.187 g, 0.429 mmol) in CH_2Cl_2 (2.5 mL). The mixture was stirred for 3 h at 20 °C and concentrated. The residue was diluted with CH_2Cl_2 (10 mL), washed with saturated aqueous $NaHCO_3$, dried with $MgSO_4$, and concentrated to obtain compound **9** (0.155 g, 95%), a colorless oily liquid. ^{1}H NMR ($CDCl_3$), δ: 1.36 (m, 2 H, $C_\gamma H_2$); 1.38—1.48 (br.s, 2 H, NH_2); 1.55 (m, 2 H, Ad); 1.65 (m, 4 H, 2 $C_\beta H_2$); 1.72—1.76 (m, 4 H, Ad); 1.82 (m, 4 H, Ad); 1.96—2.00 (m, 4 H, Ad); 2.19 (t, 2 H, C$\underline{H_2}$CONH, $^3J_{\alpha,\beta}$ = 7.6 Hz); 2.33 (m, 2 H, CH_2CO_2); 2.84 (t, 2 H, C$\underline{H_2}$NH$_2$, ^3J = 5.5 Hz); 3.30 (m, 2 H, C$\underline{H_2}$NHCO, ^3J = 5.5 Hz); 4.90 (m, 1 H, C_{Ad}(2)H); 6.09 (br.s, 1 H, NH). ^{13}C NMR ($CDCl_3$), δ: 24.77, 25.53, 26.97, 27.20, 28.70, 31.76 (Ad); 31.87 (Ad); 34.6, 36.31 (Ad); 36.41, 37.36, 41.24 (CH_2NH_2); 41.55 (CH_2NH); 76.83 (C_{Ad}(2)H); 173.14 (C=O); 173.39 (C=O). Found (%): C, 67.76; H, 9.64; N, 8.39. $C_{19}H_{32}N_2O_3$. Calculated (%): C, 67.82; H, 9.59; N, 8.33.

2-Adamantyl 7-{2-[(2*E*)-2-(3,4-dimethoxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)prop-2-enoylamino]ethylamino}-7-oxoheptanoate (3a) Acid **4a** (0.030 g, 0.09 mmol) and EEDQ (0.15 g, 0.607 mmol) were added to a solution of amine **9** (0.123 g, 0.366 mmol) in CH_2Cl_2. The mixture was stirred for 24 h at 20 °C and concentrated, the residue was subjected to chromatography (eluent ethyl acetate—light petroleum ether (40—60 °C), 1 : 1; then methanol—dichloromethane, 1 : 10) to obtain compound **3a** (0.050 g, 52%), a white solid substance, m.p. 130—135 °C. ^{1}H NMR ($CDCl_3$), δ: 1.35 (m, 2 H, $C_\gamma H_2$); 1.53—1.56 (m, 2 H, Ad); 1.62—1.68 (m, 4 H, 2 $C_\beta H_2$); 1.73—1.77 (m, 4 H, Ad); 1.82 (m, 4 H, Ad); 1.97—2.00 (m, 4 H, Ad); 2.17 (t, 2 H, $C_\alpha H_2$, $^3J_{\alpha,\beta}$ = 7.6 Hz); 2.32 (t, 2 H, $C_\alpha H_2$, $^3J_{\alpha,\beta}$ = 7.6 Hz); 3.34 (m, 2 H, BocNHC$\underline{H_2}$); 3.43 (m, 2 H, C$\underline{H_2}$NHC=O); 3.83 (s, 6 H, 2 OMe); 3.95 (s, 3 H, OMe); 4.90 (m, 1 H, C_{Ad}(2)H); 5.99 (s, 1 H,

OH); 6.02 (br.s, 1 H, NH); 6.53 (br.s, 1 H, NH); 6.57 (dd, 1 H, Ar, J = 8.7 Hz, J = 1.7 Hz); 6.65 (d, 1 H, Ar, J = 8.7 Hz); 6.67 (d, 1 H, Ar, J = 1.8 Hz); 6.72 (d, 1 H, Ar, J = 1.7 Hz); 6.80 (dd, 1 H, Ar, J = 8.2 Hz, J = 1.7 Hz); 6.96 (d, 1 H, Ar, J = 8.2 Hz); 7.72 (s, 1 H, C=CHAr). ^{13}C NMR ($CDCl_3$), δ: 24.81, 25.31, 26.95, 27.19, 28.73, 31.75, 31.83, 34.61, 36.29, 36.36, 37.35, 39.83 (NH—$\underline{C}H_2$), 40.41 (NH—$\underline{C}H_2$), 55.78 (Me); 55.87 (Me), 55.99 (Me), 76.79 (C_{Ad}(2)), 110.36, 112.12, 112.60; 116.82, 122.14, 123.26, 127.74, 128.10, 131.63, 137.24, 145.24, 147.51, 149.18, 149.91, 169.12 (C=O), 173.11 (C=O), 173.6 (C=O). Found (%): C, 68.45; 7.38; N, 4.35. $C_{37}H_{48}N_2O_8$. Calculated (%): C, 68.50; H, 7.46; N, 4.32.

2-Adamantyl 7-{2-[(2*E*)-3-(3-hydroxy-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)prop-2-enoylamino]ethylamino}-7-oxoheptanoate (3b) was obtained similarly to compound **3a** from amine **9** (0.123 g, 0.366 mmol), acid **4b** (0.16 g, 0.444 mmol), and EEDQ (0.15 g, 0.607 mmol). The yield was 0.037 g (16%), a white wax-like mass. ^{1}H NMR ($CDCl_3$), δ: 1.33—1.38 (m, 2 H, $C_\gamma H_2$); 1.53—1.56 (m, 2 H, Ad); 1.62—1.65 (m, 4 H, $C_\beta H_2$); 1.73—1.77 (m, 4 H, Ad); 1.82 (m, 4 H, Ad); 1.92—2.0 (m, 4 H, Ad); 2.15—2.18 (t, 2 H, $C_\alpha H_2$, $^3J_{\alpha,\beta}$ = 7.5 Hz); 2.31 (t, 2 H, $C_\alpha H_2$, $^3J_{\alpha,\beta}$ = 7.1 Hz); 3.34 (m, 2 H, BocNH—$C\underline{H}_2$); 3.44 (m, 2 H, $C\underline{H}_2$—NHC=O); 3.81 (s, 6 H, OMe); 3.84 (s, 3 H, OMe); 3.93 (s, 3 H, OMe); 4.90 (m, 1 H, C_{Ad}(2)H); 6.05 (br.s, 2 H, 2 NH); 6.45 (s, 2 H); 6.52 (m, 1 H); 6.55 (dd, 1 H, Ar, J = 8.4 Hz, J = 1.9 Hz); 6.65 (d, 1 H, Ar, J = 8.4 Hz); 6.69 (d, 1 H, Ar, J = 1.9 Hz); 7.7 (s, 1 H, C=CHAr). ^{13}C NMR ($CDCl_3$), δ: 24.8, 25.3, 26.97, 27.2, 28.74, 31.76, 36.3, 37.37, 39.9 (NH—$\underline{C}H_2$); 40.45 (NH—$\underline{C}H_2$); 55.81 (Me); 56.27 (2 Me), 61.03 (Me); 76.81 (C_{Ad}(2)); 106.56, 110.37, 116.81, 123.26, 127.92, 131.13, 131.80, 137.22, 138.18, 145.26, 147.54, 154.33, 168.72 (C=O); 173.09 (C=O); 173.57 (C=O). IR (KBr), ν/cm^{-1}: 1128, 1240, 1257, 1412, 1452, 1512, 1581, 1651, 1725 (C=O); 2854, 2927, 3369 (OH and NH). Found (%): C, 67.20; H, 7.38; N, 4.07. $C_{38}H_{50}N_2O_9$. Calculated (%): C, 67.24; H, 7.42; N, 4.13.

MTT-test on cytotoxicity. The human epithelial lung carcinoma cells (line A-549, CCL-185) were seeded in 96-well plates (~3000 cells per well) in DMEM culture medium (200 μL) at 37 °C. Solutions of tested compounds (or colchicine as a positive control) in DMSO were added into the wells in the concentration range 0.005—50 μmol L^{-1} (eight wells for each concentration) and this was matured for 24 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg mL^{-1}) was prepared in a phosphate buffer, filtered through a 0.22-mm filter, and 2 h before the end of maturing the tested compound, the solution of MTT (20 μL) was added into each well so that the final concentration would be 0.45 mg mL^{-1}. After removal of the cell medium, a solution of DMSO containing 10% of sodium dodecylsulfate and 0.6% of acetic acid (100 μL) was added into each well, the crystals of formazane formed were solubilized by stirring, using a shaker. Optical density was measured at 590 nm with a 690-nm reference filter on a EL808 Ultra Microplate Reader instrument (Bio-Tek Instruments, USA).

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