Synthesis and biological testing of tubuloclustin analogs containing alicyclic groups and 2-methoxyestradiol moiety*

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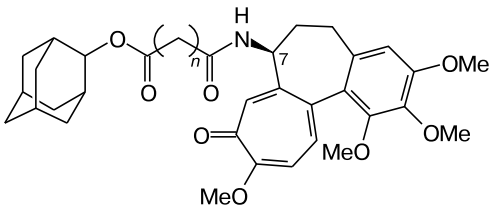
A number of analogs of tubuloclustin, *N*-[7-(2-adamantyloxy)-7-oxoheptanoyl]-*N*-deacetylcolchicine, were obtained. In these analogs, the colchicine moiety is formally replaced by the cyclohexane, adamantane, and 2-methoxyestradiol moieties (the steroid is attached through the hydroxy group at the C(17) atom). MTT assays revealed that the conjugates obtained are much less cytotoxic against A549 lung carcinoma cells than the lead compound.

Key words: adamantane, colchicine, tubuloclustin, alicyclic compounds, 2-methoxyestradiol, cytotoxicity.

The antitumor activity of natural colchicine is due to its interaction with the cellular protein tubulin, which inhibits tubulin polymerization into microtubules[1] (this process is essential in cell division). Many compounds able to interact with the colchicine binding site in tubulin are currently available. However, the overwhelming majority of them is not used in cancer therapy because of their high general toxicity or inadequate efficiency. This gives impetus to various structural modifications of colchicine and its analogs with the aim of making them more active and less toxic.[2]



1a,b

1: n = 5 (**a**), 6 (**b**)

Earlier, we have obtained a colchicine analog (**1a**), which is more cytotoxic *in vitro* to various cancer cell strains than is the parent molecule.[3,4] Because of its ability to form unusual tubulin clusters, compound **1a** was named *tubuloclustin*.[4] Early structure—activity investigations of analogs of compound **1a** revealed that the adamantane fragment and the linker of strictly specified length (five (**1a**) or six methylene units (**1b**)) are both critical for these compounds to be highly cytotoxic and capable of forming clusters.[3] In the present work, we modified compounds **1a** and **1b** in different ways involving replacement of the colchicine moiety and studied the biological activity of the resulting derivatives.

To prove the important contribution of colchicine to the cytotoxic properties of conjugates **1**, first we replaced colchicine by alicyclic cyclohexane and adamantane groups (Scheme 1). For this purpose, we esterified dicarboxylic polyanhydrides with adamantan-2-ol and isolated not only monoesters **2a**,**b** (see Ref. 3) but also diesters **3a**,**b** in the individual state. Ester **2a** was used in a reaction with cyclohexylamine in the presence of ethyl 2-ethoxy-1,2-dihydroquinoline-1-carboxylate (EEDQ), which affords target compound **4** in high yield.

Compounds **3a**,**b** and **4** failed in a standard colorimetric assay involving 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) as a dye[5] against A549 human lung carcinoma cells. Their low cytotoxicity (IC_{50} > 10 μmol L^{-1}) provides evidence for the decisive role of colchicine in binding conjugates **1a**,**b** to tubulin, although their mechanisms of action differ from that of free colchicine.[4]

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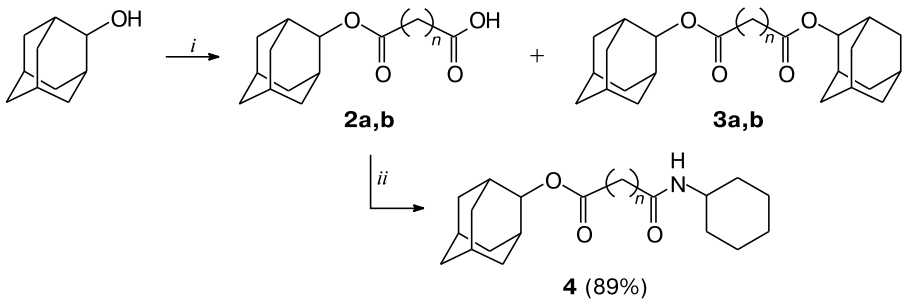
Scheme 1



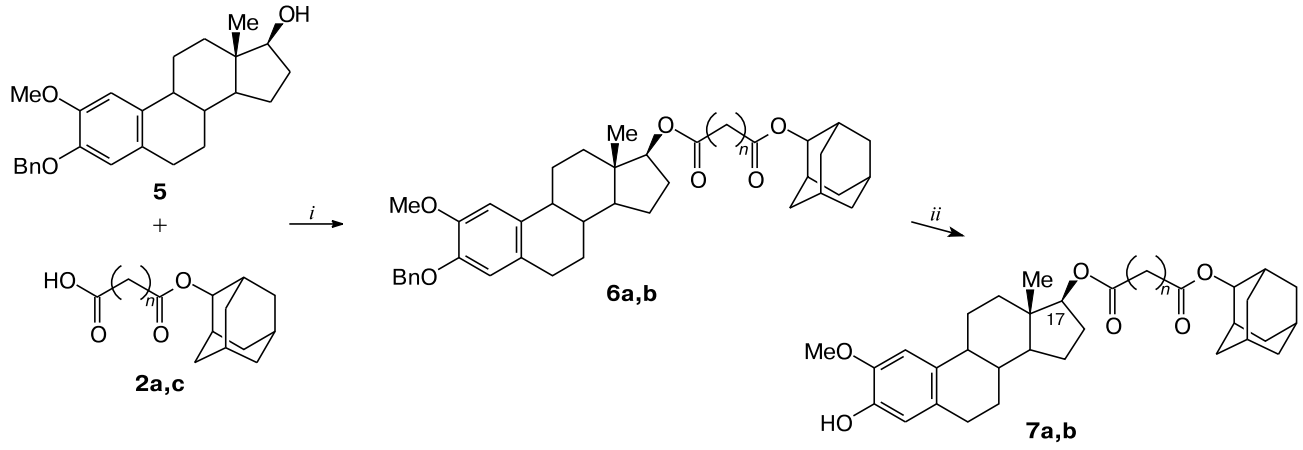
Reagents: *i*. $[OC(O)(CH_2)_nC(O)]_m$, DMAP, CH_2Cl_2; *ii*. **2a**, $C_6H_{11}NH_2$, EEDQ, CH_2Cl_2.

Compound	*n*	Yield (%)	Compound	*n*	Yield (%)
2a	5	70	**3a**	5	6
2b	6	42	**3b**	6	9

Then we studied the possibility of replacing colchicine in lead compounds by 2-methoxyestradiol, another known ligand to the colchicine-binding site of tubulin with a lower general toxicity. Since the exact location of 2-methoxyestradiol at the colchicine-binding site of the protein remains unknown and has been only hypothesized in a model,[1] when selecting a position for an adamantane substituent in the steroid, we used the literature data on the activity of 2-methoxyestradiol derivatives containing bulky groups. Specifically, the position at the C(17) atom has been reported[6,7] to be suitable for introduction of such substituents without changing the cytotoxicity of the starting molecule. As a result, we proposed analogs of lead compounds in which ester linkers are attached through the hydroxy group at the C(17) atom of the steroid (Scheme 2). To examine how the cytotoxicity of such conjugates depends on the linker length, we obtained a compound containing the same chain as in **1a** and another compound with a longer chain consisting of seven methylene units.

In the first step, 7-(2-adamantyloxy)-7-oxoheptanoic acid (**2a**) and 9-(2-adamantyloxy)-9-oxononanoic acid (**2c**) (prepared[3] by reactions of adamantan-2-one with pimelic and azelaic polyanhydrides, respectively) were esterified with 2-methoxyestradiol **5** containing the Bn-protected phenolic OH group in the presence of DCC and DMAP. The ^{1}H NMR spectra of the resulting esters **6a**,**b** show characteristic signals for the hydrogen atom at the C(17) atom (C(17)HOC(O)) of the steroid at δ 4.72 and

Scheme 2



n = 5 (**2a**, **6a**, **7a**), 7 (**2c**, **6b**, **7b**)

Reagents: *i*. DCC, DMAP, CH_2Cl_2; *ii*. 5% Pd/C, H_2, EtOH.

Compound	Yield (%)	Compound	Yield (%)
6a	80	**7a**	79
6b	78	**7b**	65

4.70, respectively. In the ^{13}C NMR spectra of compounds **6a**,**b**, the signal for the C(17) atom appears at δ 82, while the signals for two carbonyl C atoms of the linker appear at δ 173. Debenzylation of esters **6a**,**b** by hydrogenation on 5% Pd/C gave the target conjugates **7a**,**b**. The molecular formulas and structures of products **7a**,**b** were confirmed by data from NMR spectroscopy, elemental analysis, and MALDI-TOF mass spectrometry (*m/z* 578 $[M]^+$ and 606 $[M]^+$, respectively). The IR spectrum of compound **7a** features absorption bands at 1729 (C=O) and 3463—3538 cm^{-1} (O—H).

In MTT assays against the A549 cell culture, steroid conjugates **7a** and **7b** proved to be substantially less cytotoxic (EC_{50} > 50 μmol L^{-1}) than tubuloclustin **1a**. Since both compounds **7** exhibit nearly equal poor cytotoxicity regardless of the linker length, this failure may be attributed to a wrong position the substituted adamantane is attached to (the hydroxy group at the C(17) atom). This calls for further investigations aimed at introducing this fragment into other positions of 2-methoxyestradiol. Approaches to the synthesis of such derivatives are currently under study.

Experimental

The course of the reactions was monitored and the purity of the compounds obtained was checked by TLC on Silufol-UV254 plates. Chromatographic separation was carried out on Acros columns packed with silica gel (40—60 μm). ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 and 100 MHz, respectively) in $CDCl_3$ at 28 °C. Chemical shifts δ are referenced to a residual signal of $CDCl_3$ at δ 7.28 and 77.0. Elemental analysis was carried out on a Vario micro cube CHN-analyzer. IR spectra were recorded on an IR-200 spectrophotometer (ThermoNicolet) in KBr pellets. MALDI-TOF mass spectra were measured on a VISION-2000 instrument.

Di(2-adamantyl) pimelate (3a). Pimelic polyanhydride (0.190 g, 1.33 mmol) and 4-dimethylaminopyridine (DMAP, 0.01 g) were added to a solution of adamantan-2-ol (0.200 g, 1.32 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was refluxed for 12 h and concentrated. The residue was chromatographed with ethyl acetate—light petroleum (b.p. 40—70 °C, 1 : 10) as an eluent. The yield of compound **3a** was 0.072 g (6%), white crystals, m.p. 69—73 °C. ^1H NMR ($CDCl_3$), δ: 1.32—1.39 (m, 2 H, H(γ)); 1.51—1.54 (m, 4 H, Ad); 1.65 (quintet, 4 H, H(β), J = 7.6 Hz, J = 7.4 Hz); 1.70—1.75 (m, 8 H); 1.80—1.87 (m, 8 H); 1.95—1.98 (m, 8 H, Ad); 2.32 (t, 4 H, H(α), J = 7.4 Hz); 4.90 (m, 2 H, $C(2)_{Ad}H$). ^{13}C NMR ($CDCl_3$), δ: 24.69, 26.88, 27.11, 31.65, 31.75, 34.52, 36.19, 37.26, 76.86 ($C(2)_{Ad}$); 172.82 (C=O). Found (%): C, 75.56; H, 9.38. $C_{27}H_{40}O_4$. Calculated (%): C, 75.66; H, 9.41. Further elution gave monoester **2a**.

Di(2-adamantyl) suberate (3b) was obtained from adamantan-2-ol (0.250 g, 1.64 mmol) and suberic polyanhydride (0.260 g, 1.67 mmol) as described for compound **3a**. The solvent was removed, and the residue was chromatographed with ethyl acetate—light petroleum (b.p. 40—70 °C, 1 : 10) as an eluent. The yield of compound **3b** was 0.124 g (9%), white crystals, m.p. 54—56 °C. ^1H NMR ($CDCl_3$), δ: 1.36—1.39 (m, 4 H, H(γ)); 1.55—1.58 (m, 4 H, Ad); 1.65—1.68 (m, 4 H, H(β)); 1.75—1.79 (m, 8 H); 1.84—1.87 (m, 8 H); 2.00—2.03 (m, 8 H, Ad); 2.34 (t, 4 H, H(α), J = 7.4 Hz); 4.93 (m, 2 H, $C(2)_{Ad}H$). ^{13}C NMR ($CDCl_3$), δ: 24.62, 26.95, 27.20, 31.75, 31.84, 34.46, 36.29, 37.35, 76.83 ($C(2)_{Ad}$); 172.72 (C=O). Found (%): C, 76.12; H, 9.39. $C_{28}H_{42}O_4$. Calculated (%): C, 75.98; H, 9.56. Further elution gave monoester **2b**.

2-Adamantyl 7-cyclohexylamino-7-oxoheptanoate (4). Cyclohexylamine (0.050 g, 0.51 mmol) and ethyl 2-ethoxy-1,2-dihydroquinoline-1-carboxylate (0.090 g, 0.36 mmol) were added to a solution of acid **2a** (0.100 g, 0.34 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was stirred at 25 °C for 24 h and concentrated. The residue was chromatographed with ethyl acetate—light petroleum (b.p. 40—70 °C, 1 : 3) as an eluent. The yield of compound **4** was 0.114 g (89%), clear oily liquid. ^1H NMR ($CDCl_3$), δ: 1.00—1.18 (m, 2 H); 1.35—1.42 (m, 4 H); 1.56—1.59 (m, 2 H, Ad); 1.63—1.70 (m, 8 H); 1.75—1.80 (m, 4 H); 1.85—1.88 (m, 4 H); 1.90—1.94 (m, 4 H, Cy, J = 3.4 Hz, J = 12.4 Hz); 1.99—2.03 (m, 4 H, Ad); 2.15 (t, 2 H, H(α), J = 7.5 Hz); 2.35 (t, 2 H, H(α), J = 7.4 Hz); 3.78 (m, 1 H, CHN); 4.94 (m, 1 H, $C(2)_{Ad}H$); 5.33 (d, 1 H, NH, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$), δ: 24.95, 25.14, 25.31, 26.37, 26.99, 28.38, 28.54, 31.87, 31.98, 32.85, 33.67, 34.63, 37.39, 43.60 (CHN); 76.85 ($C(2)_{Ad}$); 173.41 (C=O); 175.08 (C=O). Found (%): C, 75.42; H, 9.64; N, 3.70. $C_{23}H_{37}NO_3$. Calculated (%): C, 75.56; H, 9.93; N, 3.73.

2-Adamantyl 3-benzyloxy-2-methoxyestra-1,3,5(10)-trien-17β-yl pimelate (6a). 7-(2-Adamantyloxy)-7-oxoheptanoic acid (**2a**) (0.068 g, 0.23 mmol), DCC (0.060 g, 0.29 mmol), and a catalytic amount of DMAP (0.01 g) were added to a solution of 3-benzyloxy-2-methoxyestra-1,3,5(10)-trien-17β-ol (**5**) (0.075 g, 0.19 mmol; prepared in three steps[8,9] from estradiol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred at 25 °C for 24 h and concentrated. Then EtOAc (20 mL) was added, and the resulting mixture was kept at −5 °C for 2—3 h. The crystals of *N*,*N*′-dicyclohexylurea that formed were filtered off and washed with cooled EtOAc. The solvent was removed, and the residue was chromatographed with ethyl acetate—light petroleum (b.p. 40—60 °C, 1 : 9) as an eluent. The yield of compound **6a** was 0.100 g (80%), clear oily liquid. ^1H NMR ($CDCl_3$), δ: 0.85 (s, 3 H, $C(18)H_3$); 1.26—1.59 (m, 12 H); 1.65—1.85 (m, 15 H); 2.01—2.04 (m, 4 H, Ad); 2.22 (m, 2 H); 2.31—2.38 (m, 4 H, $CH_2C(O)O$, J = 7.4 Hz, J = 7.6 Hz); 2.68—2.80 (m, 2 H, $C(6)H_2$); 3.86 (s, 3 H, OMe); 4.72 (dd, 1 H, C(17)H, J = 8.0 Hz, J = 8.8 Hz); 4.95 (m, 1 H, $C(2)_{Ad}H$); 5.12 (s, 2 H, $OC\underline{H}_2Ph$); 6.64 (s, 1 H, C(4)H); 6.86 (s, 1 H, C(1)H); 7.24—7.47 (m, 5 H, Ph). ^{13}C NMR ($CDCl_3$), δ: 12.08 (C(18)); 23.19, 24.69, 24.75, 26.32, 26.93, 27.17, 27.24, 27.57, 28.57, 29.04, 31.73, 31.82, 34.59, 36.26, 36.91, 37.32, 38.42, 42.89, 44.04, 49.71, 56.23 (OMe); 71.02 ($O\underline{C}H_2Ph$); 76.69 ($C(2)_{Ad}$); 82.42 (C(17)); 109.72 (C(1)); 114.58 (C(4)); 127.21, 127.63, 128.40, 128.70, 132.72, 137.38, 146.28 (C(2)); 147.53 (C(3)); 172.92 (C(17)O(O)$\underline{C}$), 173.57 ($C(O)O_{Ad}$). Found (%): C, 77.51; H, 8.29. $C_{43}H_{56}O_6$. Calculated (%): C, 77.21; H, 8.44.

2-Adamantyl 3-benzyloxy-2-methoxyestra-1,3,5(10)-trien-17β-yl azelate (6b) was obtained from alcohol **5** (0.090 g, 0.23 mmol) and 9-(2-adamantyloxy)-9-oxononanoic acid (**2c**) (0.096 g, 0.30 mmol) in the presence of DCC (0.071 g, 0.34 mmol) and DMAP as described for compound **6a**. The yield of compound **6b** was 0.124 g (78%), clear oily liquid. ^1H NMR ($CDCl_3$), δ: 0.80 (s, 3 H, $C(18)H_3$); 1.26—1.59 (m, 16 H); 1.64—1.87 (m, 15 H); 1.96—2.06 (m, 4 H, Ad); 2.23 (m, 2 H); 2.31—2.37

(m, 4 H, $CH_2C(O)O$, J = 7.2 Hz, J = 7.3 Hz); 2.72—2.78 (m, 2 H, C(6)H_2); 3.87 (s, 3 H, OMe); 4.70 (dd, 1 H, C(17)H, J = 7.8 Hz, J = 8.7 Hz); 4.93 (m, 1 H, C(2)$_{Ad}$H); 5.11 (s, 2 H, OC$\underline{H}_2$Ph); 6.63 (s, 1 H, C(4)H); 6.85 (s, 1 H, C(1)H); 7.26—7.46 (m, 5 H, Ph). ^{13}C NMR ($CDCl_3$), δ: 12.11 (C(18)); 23.22, 25.00, 25.09, 26.35, 26.96, 27.20, 27.59, 28.95, 29.08, 31.76, 33.85, 34.81, 36.29, 36.92, 37.36, 38.45, 42.92, 44.06, 49.73, 56.27 (OMe); 71.04 (O$\underline{C}$H$_2$Ph); 76.68 (C(2)$_{Ad}$); 82.38 (C(17)); 109.74 (C(1)); 114.58 (C(4)); 127.24, 127.66, 128.43, 132.75, 137.40, 146.29 (C(2)); 147.53 (C(3)); 173.19 (C(17)O(O)$\underline{C}$); 173.83 (C(O)O$_{Ad}$). Found (%): C, 77.38; H, 8.79. $C_{45}H_{60}O_6$. Calculated (%): C, 77.55; H, 8.68.

2-Adamantyl 3-hydroxy-2-methoxyestra-1,3,5(10)-trien-17β-yl pimelate (7a). Hydrogen was bubbled through a mixture of benzyl ether **6a** (0.075 g, 0.11 mmol) and 5% Pd/C (0.100 g) in ethanol (5 mL) at 25 °C for 8 h. The precipitate that formed was filtered off. The filtrate was concentrated to give compound **7a** (0.051 g, 79%) as a yellow oily liquid. ^{1}H NMR ($CDCl_3$), δ: 0.84 (s, 3 H, C(18)H_3); 1.25—1.59 (m, 12 H); 1.65—1.89 (m, 15 H); 2.00—2.04 (m, 4 H, Ad); 2.22 (m, 2 H); 2.33—2.38 (m, 4 H, $CH_2C(O)O$, J = 7.3 Hz, J = 7.4 Hz); 2.76—2.80 (m, 2 H, C(6)H_2); 3.86 (s, 3 H, OMe); 4.72 (dd, 1 H, C(17)H, J = 8.0 Hz, J = 8.8 Hz); 4.93 (m, 1 H, C(2)$_{Ad}$H); 5.50 (br.s, 1 H, C(3)OH); 6.64 (s, 1 H, C(4)H); 6.79 (s, 1 H, C(1)H). ^{13}C NMR ($CDCl_3$), δ: 12.10 (C(18)); 23.21, 24.72, 24.78, 26.48, 26.96, 27.20, 27.25, 27.59, 28.60, 28.91, 31.76, 31.85, 34.34, 34.63, 36.29, 36.94, 37.35, 38.51, 42.92, 44.06, 49.76, 56.02 (OMe); 76.69 (C(2)$_{Ad}$); 82.47 (C(17)), 108.07 (C(1)); 114.58 (C(4)); 129.41, 131.55, 143.46 (C(3)OH); 144.57 (C(2)); 172.97 (C(17)O(O)$\underline{C}$); 173.63 (C(O)O$_{Ad}$). IR (KBr), ν/cm^{-1}: 985, 1025, 1101, 1118, 1211, 1241, 1265, 1357, 1454, 1511, 1590, 1617, 1729 (C=O); 2856—2923 (CH); 3463—3538 (OH). Found (%): C, 74.47; H, 8.82. $C_{36}H_{50}O_6$. Calculated (%): C, 74.71; H, 8.71. MS (MALDI-TOF), m/z: 578 [M]$^+$.

2-Adamantyl 3-hydroxy-2-methoxyestra-1,3,5(10)-trien-17β-yl azelate (7b) was obtained from benzyl ether **6b** (0.120 g, 0.17 mmol) and 5% Pd/C (0.1 g) in ethanol (5 mL) as described for compound **7a**. The yield of compound **7b** was 0.068 g (65%), yellow oily liquid. ^{1}H NMR ($CDCl_3$), δ: 0.80 (s, 3 H, C(18)H_3); 1.26—1.61 (m, 15 H); 1.66—1.85 (m, 16 H); 2.00—2.04 (m, 4 H, Ad); 2.24 (m, 2 H); 2.31—2.36 (m, 4 H, $CH_2C(O)O$, J = 7.0 Hz, J = 7.2 Hz); 2.76—2.80 (m, 2 H, C(6)H_2); 3.87 (s, 3 H, OMe); 4.72 (dd, 1 H, C(17)H, J = 9.0 Hz, J = 7.8 Hz); 4.93 (m, 1 H, C(2)$_{Ad}$H); 5.46 (br.s, 1 H, C(3)OH); 6.65 (s, 1 H, C(4)H); 6.79 (s, 1 H, C(1)H). ^{13}C NMR ($CDCl_3$), δ: 12.12 (C(18)); 23.22, 25.01, 25.10, 26.48, 26.95, 27.20, 27.25, 27.59, 28.93, 28.96, 31.76, 31.84, 34.54, 34.83, 36.92, 37.35, 38.50, 42.93, 44.06, 49.74, 56.02 (OMe); 76.68 (C(2)$_{Ad}$); 82.40 (C(17)); 108.02 (C(1)); 114.55 (C(4)); 131.58, 131.78, 143.41 (C(3)OH); 144.52 (C(2)); 173.24 (C(17)O(O)$\underline{C}$); 174.55 (C(O)O$_{Ad}$). Found (%): C, 75.91; H, 8.72. $C_{38}H_{54}O_6$. Calculated (%): C, 75.21; H, 8.97. MS (MALDI-TOF), m/z: 606 [M]$^+$.

MTT assay for cytotoxicity. A549 human lung carcinoma cells were cultured in 96-well plates (~3000 cells per well) at 37 °C. The culture medium (200 μL) consisted of DMEM and 10% FBS. The cells were kept for 24 h with solutions of test compounds (or colchicine as a positive control) in DMSO. The concentration of the test compounds was varied from 0.005 to 50 μmol L^{-1}; each concentration was present in eight wells. A phosphate-buffered solution of MTT (C = 5 mg mL^{-1}) was prepared and filtered through a filter with a pore diameter of 0.22 mm. Two hours before the exposure time of the test compounds elapsed, a sterile solution of MTT (20 μL) had been added to each well so that its final concentration was 0.45 mg mL^{-1}. The culture medium was removed, and a lysis buffer (100 μL) consisting of 10% sodium dodecyl sulfate and 0.6% acetic acid in DMSO was added to each well. The resulting formazan crystals were solubilized by thorough mixing on a plate shaker. Optical density was measured at 590 nm with a 690-nm reference filter on an EL808 Ultra Microplate Reader instrument (Bio-Tek Instruments, USA).

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