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# Synthesis and cytotoxic analysis of some disodium 3β,6β-dihydroxysterol disulfates

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ABSTRACT

Disodium 3β,6β-dihydroxy-5α-cholestane disulfate (**1**) was synthesized in 4 steps with a high overall yield from cholesterol. First, cholesterol (**4a**) was converted to cholest-4-en-3,6-dione (**5a**) via oxidation with pyridinium chlorochromate (PCC) and then **5a** was reduced by $NaBH_4$ in the presence of $NiCl_2$ to produce cholest-3β,6β-diol (**6a**). The reaction of **6a** with the triethylamine-sulfur trioxide complex generated diammonium 3β,6β-dihydroxy-5α-cholestane disulfate (**7a**) and the treatment of **7a** by cation exchange resin 732 (sodium form)($Na^+$) yielded the target steroid **1**. Disodium 24-ethyl-3β,6β-dihydroxycholest-22-ene disulfate (**2**) and disodium 24-ethyl-3β,6β-dihydroxycholestane disulfate (**3**) were synthesized using a similar method. The cytotoxicity of these compounds against Sk-Hep-1 (human liver carcinoma cell line), H-292 (human lung carcinoma cell line), PC-3 (human prostate carcinoma cell line) and Hey-1B (human ovarian carcinoma cell line) cells was investigated. Our results indicate that presence of a cholesterol-type side chain at position 17 is necessary for their biological activity.



## 1. Introduction

In recent years, a variety of steroids with unusual and interesting structures have been isolated from a wide range of marine organisms, particularly from sponges and echinoderms [1–3]. Among these steroidal compounds, many are sulfated polyhydroxysterols in sodium salt forms [4,5]. These compounds have exhibited a broad spectrum of biologic activities, such as suppression of HIV replication [6–9], inhibition of protein tyrosine kinases [10] and antitumor activities [11–13]. Therefore they greatly attract the attention of organic chemists and biomedical scientists [14–16]. Most of the sulfated polyhydroxysteroids isolated from these echinoderms or ophiuroids are found that the disulfation occurs on two hydroxyls located at C-2 and C-3 or C-3 and C-7, or C-3 and C-21 [17]. In this report, we synthesized three disulfated steroids with sulfate groups located at C-3 and C-6 on the ring A and B as shown in Fig. 1 and determined their cytotoxicity against cancer cells. Interestingly we found that the cytotoxicity of these compounds was differentially changed along with the structure of the R group. Our finding provides new evidence for the relationship between chemical structure and biofunctions.

## 2. Results and discussion

### 2.1. Chemistry

The synthesis of sulfated 3,6-dihydroxysterol was based on the methodology developed by Arnostova et al. [18]. Here we present a more efficient method for the synthesis of disodium 3β,6β-dihydroxysterol disulfates with a cholesterol-like or stigmasterol-like or sitosterol-like side chain at position 17 [19,20]. As shown in Scheme 1, cholesterol, stigmasterol, and β-sitosterol were used as raw materials.

Compounds (**4a**–**4c**) were converted to the corresponding 4-en-3, 6-dioxysteroids (**5a**–**5c**) via oxidation with PCC in $CH_2Cl_2$. The reduction of **5a**–**5c** by $NaBH_4$ in the presence of $NiCl_2$ gave 3β,6β-dihydroxysteroids (**6a**–**6c**) according to the synthetic method we previously developed [21]. The compounds **6a**–**6c** presented a typical difference in their spectrum from their parent compounds **5a**–**5c**. In the IR spectra the absorption at 1714 and 1686 $cm^{-1}$ for the original diketone carbonyl groups (CO) of **5b**, was replaced by a new broad absorption at 3427 $cm^{-1}$ for **6b**, indicating that the carbonyl groups in **5b** had been changed to the hydroxy groups in **6b**. In addition, the double bond of **5b** at 1609 $cm^{-1}$ had been eliminated in **6b**. In the $^1H$ NMR spectrum the chemical shifts at 3.650 ppm (1H, tt, $J$ = 11.0, 5.0, $C_3$-αH) and 3.803 ppm (1H, dd, $J$ = 5.5, 2.5, $C_6$-αH) for **6b** showed a α-configuration of 3-H and 6-H.

Based on the synthetic method developed by Comin et al. [14], the treatment of **6a**–**6c** with triethylammonium-sulfur tri-

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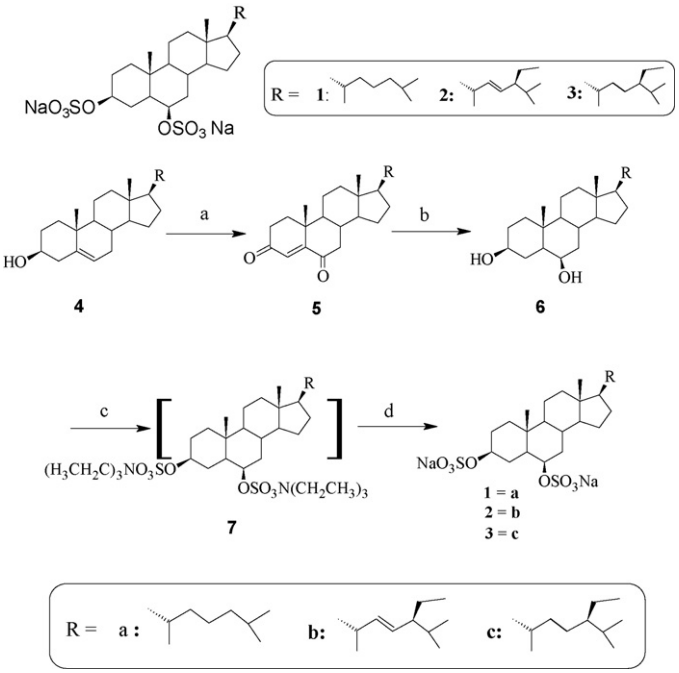


**Scheme 1.** Reagents and conditions: (a) $PCC/CH_2Cl_2$, (b) $NaBH_4/NiCl_2{\cdot}6H_2O$, (c) $Et_3N$-$SO_3$/DMF, and (d) cation exchange resin 732 (sodium form)($Na^+$)/MeOH.

oxide complex gave the ammonium sulfate of compounds **1**–**3**, which was converted to their disodium salt via ion exchange (Scheme 1). The presence of the sulfate group in **1**–**3** was confirmed by the downfield shift of 3-H and 6-H to 3.961–3.966 ppm and 4.140–4.152 ppm, and by comparing to the $^1$H NMR spectra of **6a** and **6c** (3.65–3.70 ppm and 3.80–3.82 ppm, respectively).

### 2.2. Antitumor activities

The antitumor activities of all these sulfated 3β,6β-dihydroxysterols were determined *in vitro* on Sk-Hep-1 (human liver carcinoma), H-292 (human lung carcinoma), PC-3 (human prostate carcinoma) and Hey-1B (human ovarian carcinoma) tumor cells. The results were summarized as $IC_{50}$ values in nmol/mL in Table 1.

Compound **1** displayed significantly higher cytotoxicity against these cancer cells when compared to compounds **2** and **3**. Interestingly, the cytotoxicity of the compounds against these cancer cells was increased along with the order of the side chain at 17-C: cholesterol-like side chain (**1**), stigmasterol-like side chain (**2**), and sitosterol-like side chain (**3**). Obviously the presence of a cholesterol-type side chain at 17-C is necessary for the best biological activity. The morphological change of the cancer cells induced by compound **1** was shown in Fig. 1A and B.

**Table 1**
*In vitro* antitumor activities ($IC_{50}$ in nmol/mL) of compounds **1**, **2** and **3**.[a]

| Compound | Sk-Hep-1 | H-292 | PC-3 | Hey-1B |
|---|---|---|---|---|
| **1** | 21 | 63 | 35 | 33 |
| **2** | 160 | 120 | 121 | 170 |
| **3** | >200 | >200 | >200 | >200 |

[a] The MTS method was used to analyze the antiproliferative activity.

## 3. Experiments

The sterol and $NaBH_4$ were purchased from Merck Co. All chemicals and solvents were analytical grade and solvents were purified by general methods before being used. Melting points were determined on an $X_4$ apparatus and were uncorrected. Infrared spectra were measured with a Nicolet FT-360 Spectrophotometer. The $^1$H and $^{13}$C NMR spectra were recorded in $CDCl_3$ or DMSO on a Bruker AV-500 spectrometer at working frequencies 500 and 125 MHz, respectively. Chemical shifts are expressed in ppm ($\delta$) values and coupling constants ($J$) in Hz. Mass spectra (ESI) were recorded on an LCMS-2010A instrument. The cell viability was determined by the MTS method using 96-well plates in a LD400 AD/LD analysis spectrometer (Beckman Coulter).

### 3.1. The synthesis of compounds *5a*–*5c*

*Cholest-4-en-3,6-dione* (***5a***): Pyridinium chlorochromate (PCC) (2.564 g, 11.87 mmol) was added to a solution of cholesterol (**4a**) (0.924 g, 2.2 mmol) in dried $CH_2Cl_2$ (40 mL) in one portion at room temperature. The reaction was completed in 28 h. To the mixture was then added 30 mL of $CH_2Cl_2$, and the suspension was poured over a silica gel column and eluted with $CH_2Cl_2$. The eluate was washed with cold water and saturated brines. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the crude product was purified by chromatography on silica gel using petroleum ether (60–90 °C)/EtOAc (5: 1) as the eluent to give 0.795 g (84%) of **5a** as pale yellow crystals, m.p. 90–91 °C; IR (KBr) $\nu$ 2953, 2865, 1693, 1600, 1486, 1249, 1221, 1117, 942 $cm^{-1}$; $^1$H NMR($CDCl_3$, 500 MHz): 0.746(3H, s, 18-$CH_3$), 0.886(3H, d, $J$ = 6.4, 26 or 27-$CH_3$), 0.899(3H, d, $J$ = 6.4, 26 or 27-$CH_3$), 0.952(3H, d, $J$ = 6.5, 21-$CH_3$), 1.172(3H, s, 19-$CH_3$), 2.546(1H, dd, $J$ = 14.6, 5.2, $C_2$-βH), 2.706(1H, dd, $J$ = 16.0, 4.0, $C_7$-αH), 6.196(1H, s, $C_4$-H).

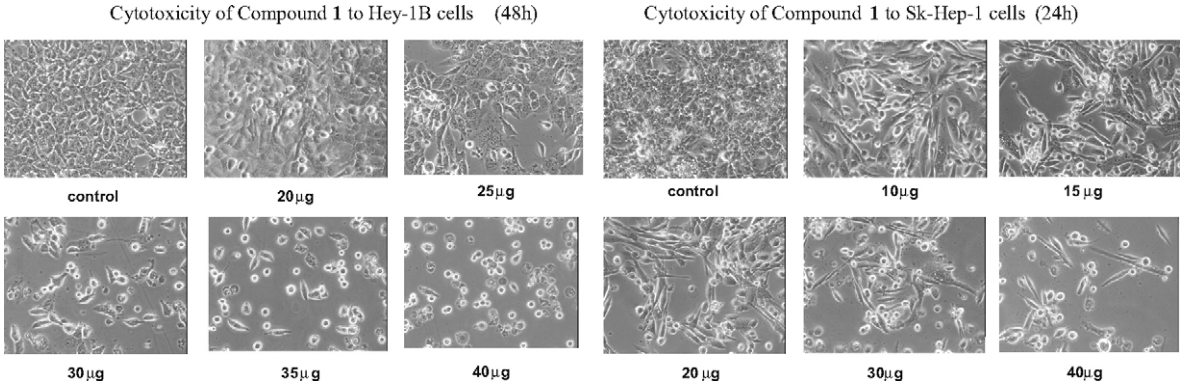


**Fig. 1.** The morphology of cancer cells. Hey 1B (A) and Sk-Hep-1 (B) cells were treated with compound **1** at various doses for 24 h and 48 h, respectively. The photos of cell morphology were taken under an Olympus CKX31 microscope at 100× magnification.

*24-Ethylcholest-4,22-dien-3,6-dione* (***5b***): Yield: 83%, m.p. 134–135 °C; IR (KBr) ν: 2959, 1714, 1686, 1609, 969, 864 $cm^{-1}$; $^1H$ NMR($CDCl_3$, 500 MHz): 0.743(3H, s, 18-$CH_3$), 0.805(3H, t, *J* = 7.0, 29-$CH_3$), 0.798(3H, d, *J* = 6.5, 26- or 27-$CH_3$), 0.849(3H, d, *J* = 6.5, 26- or 27-$CH_3$), 1.036(3H, d, *J* = 7.0, 21-$CH_3$), 1.169(3H, s, 19-$CH_3$), 5.040(1H, dd, *J* = 15.2, 9.0, $C_{22}$-H), 5.150(1H, dd, *J* = 15.2, 8.5, $C_{23}$-H), 6.171(1H, s, $C_4$-H).

*24-Ethylcholest-4-en-3,6-dione* (***5c***): Yield: 86%, m.p. 172–174 °C. IR (KBr) ν: 2959, 1683, 1601, 1581, 1461, 1377, 1246, 1124, 948, 871 $cm^{-1}$; $^1H$ NMR($CDCl_3$, 500 MHz): 0.724(3H, s, 18-$CH_3$), 0.816(3H, d, *J* = 7.0, 26- or 27-$CH_3$), 0.841(3H, d, *J* = 7.0, 26- or 27-$CH_3$), 0.848(3H, t, *J* = 8.0, 29-$CH_3$), 0.935(3H, d, *J* = 6.5, 21-$CH_3$), 1.167(3H, s, 19-$CH_3$), 2.13–2.17(1H, m, $C_2$-αH), 2.44–2.58 (2H, m, $C_7$-βH and $C_2$-βH), 2.682(1H, dd, *J* = 15.5, 4.5, $C_7$-αH), 6.170(1H, s, $C_4$-H).

### 3.2. The synthesis of compounds *6a–6c*

*Cholest-3β,6β-diol* (***6a***): $NaBH_4$ (90 mg, 2.38 mmol) was added to a solution of **5a** (200 mg, 0.50 mmol) and $NiCl_2.6H_2O$ (120 mg, 0.50 mmol) in $CH_3OH$ (20 mL) in 1 min at room temperature. After 20 min, the reaction was stopped. The solution was neutralized with 1 M HCl. After evaporating the majority of the MeOH under reduced pressure, ethyl acetate (40 mL) was added to the residue. The resulting solution was washed with cold water and saturated brines. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the crude product was then purified by flash chromatography on silica gel using petroleum ether /ethyl acetate (1:1) as the eluent, to give **6a** as a white solid (164 mg, 81%), m.p. 161–162 °C; IR(KBr) ν: 3399, 2925, 2868, 1470, 1360, 1319, 1172, 1078, 1041, 1021, 955, 767 $cm^{-1}$; $^1H$ NMR($CDCl_3$, 500 MHz): 0.709(3H, S, 18-$CH_3$), 0.878(3H, d, *J* = 2.2, 26 or 27-$CH_3$), 0.891(3H, d, *J* = 2.2, 26 or 27-$CH_3$), 0.927(3H, d, *J* = 6.4, 21-$CH_3$), 1.052(3H, s, 19-$CH_3$), 3.64–3.82(1H, m, $C_3$-αH), 3.825(1H, d, *J* = 2.4, $C_6$-αH).

*24-Ethylcholest-22-en-3β,6β-diol* (***6b***): Yield: 72%; m.p. 207–209 °C; IR(KBr) ν: 3427, 2929, 2864, 1581, 1462, 1380, 1037, 967 $cm^{-1}$; $^1H$ NMR($CDCl_3$, 500 MHz): 0.708(3H, s, 18-$CH_3$), 0.795(3H, d, *J* = 6.5, 26 or 27-$CH_3$), 0.845(3H, d, *J* = 6.5, 26 or 27-$CH_3$), 0.802(3H, t, *J* = 7.5, 29-$CH_3$), 1.013(3H, d, *J* = 7.0, 21-$CH_3$), 1.033(3H, s, 19-$CH_3$), 3.650(1H, tt, *J* = 11.0, 5.0, $C_3$-αH), 3.803(1H, dd, *J* = 5.5, 2.5, $C_6$-αH), 5.022(1H, dd, *J* = 15.0, 8.5, $C_{22}$-H), 5.140(1H, dd, *J* = 15.0, 8.5, $C_{23}$-H).

*24-Ethylcholest-3β,6β-diol* (***6c***): Yield: 72%; m.p. 172–173 °C; IR(KBr) ν: 3419, 3125, 2941, 2872, 1397, 1172, 1033 $cm^{-1}$; $^1H$ NMR($CDCl_3$, 500 MHz): 0.728(3H, s, 18-$CH_3$), 0.831(6H, d, *J* = 7.6 Hz, 26 and 27-$CH_3$), 0.866(3H, d, *J* = 6.2 Hz, 29-$CH_3$), 0.896(3H, s, 21-$CH_3$), 1.027(3H, s, 19-$CH_3$), 3.667(1H, t, *J* = 5.5, $C_3$-H), 3.821(1H, s, $C_6$-H).

### 3.3. General procedure for the synthesis of compounds *1–3*

*Disodium 3β,6β-dihydroxy-5α-cholestane disulfate* (***1***): The triethylamine-sulfur trioxide complex (173 mg, 0.96 mmol) was added to a solution of cholest-3β,6β-diol (**6a**) (50 mg, 0.12 mmol) in DMF (0.6 mL) under an argon atmosphere, and the mixture was stirred at 95 °C for 3 h. Then the reaction mixture was quenched with water (0.2 mL). The solution was poured over a silica gel column to remove excess $SO_3{\cdot}NEt_3$. The product was eluted by using petroleum ether /$CH_2Cl_2$ (1:1) as the eluent, and then continued to be eluted with MeOH/$CHCl_3$(1:12), and followed by evaporation of the solvent to yield a white solid (diammonium 3β,6β-dihydroxy-5α-cholestane disulfate). To the solution of the solid in methanol (15 mL) was added Cation exchange resin 732 (sodium form) ($Na^+$) (10 g), and stirred for 5 h at room temperature. The resin was removed by filtration, and the filtrate was concentrated. This process was repeated one more time with 15 g of the resin. Finally compound **1** was obtained as a colorless solid (45 mg), yield: 60%, m.p. 173–174 °C; IR(KBr) ν: 3464, 2937, 2868, 1470, 1383, 1226, 1066, 980, 947, 853, 820, 624 $cm^{-1}$; $^1H$ NMR(DMSO, 500 MHz): 0.657(3H, s, 18-$CH_3$), 0.851(3H, d, *J* = 6.5, 26- or 27-$CH_3$), 0.855(3H, d, *J* = 6.5, 26- or 27-$CH_3$), 0.896(3H, d, *J* = 6.5, 21-$CH_3$), 0.932(3H, s, 19-$CH_3$), 1.935(1H, brd, *J* = 12.5, $C_2$-βH), 3.966(1H, m, $C_3$-αH), 4.140(1H, s, $C_6$-αH); $^{13}C$ NMR(DMSO, 125 MHz): 36.1(1-C), 28.2(2-C), 70.1(3-C), 33.4(4-C), 35.4(5-C), 76.1(6-C), 42.8(7-C), 30.5(8-C), 47.7(9-C), 38.6(10-C), 21.1(11-C), 39.4(12-C), 40.6(13-C), 56.2(14-C), 24.4(15-C), 27.8(16-C), 54.2(17-C), 12.4(18-C), 15.8(19-C), 35.6(20-C), 19.0(21-C), 36.1(22-C), 23.7(23-C), 40.5(24-C), 29.2(25-C), 22.8(26-C), 23.1(27-C); (-)LRESIMS *m/z*: 585$[M-Na]^{-1}$, 563$[M-2Na+H]^{-1}$.

*Disodium 24-ethyl-3β,6β-dihydroxycholest-22-ene disulfate* (***2***): Yield: 66%; m.p. 174–175 °C; IR(KBr) v: 2937, 2864, 1646, 1466, 1368, 1209, 1066, 967, 853, 812 $cm^{-1}$; $^1H$ NMR(DMSO, 500 Hz): 0.679(3H, s, 18-$CH_3$), 0.778 (3H, t, *J* = 4.4, 29-$CH_3$), 0.795(3H, d, *J* = 6.1, 26- or 27-$CH_3$), 0.835(3H, d, *J* = 6.1, 26- or 27-$CH_3$), 0.935(3H, s, 19-$CH_3$), 0.998(3H, d, *J* = 6.5, 21-$CH_3$), 1.917(1H, d, *J* = 12.5, $C_2$-βH), 2.031(1H, m, $C_{20}$-H), 3.961(1H, m, $C_3$-αH), 4.152(1H, d, *J* = 3.4, $C_6$-αH), 5.031(1H, dd, *J* = 15.1, 8.6, $C_{22}$-H), 5.161(1H, dd, *J* = 15.1, 8.8, $C_{23}$-H); $^{13}C$ NMR(DMSO, 125 MHz): 35.5(1-C), 27.4(2-C), 70.1(3-C), 33.4(4-C), 31.8(5-C), 76.1(6-C), 45.7(7-C), 29.2(8-C), 54.2(9-C), 38.6(10-C), 21.4(11-C), 40.6(12-C), 44.2(13-C), 56.4(14-C), 25.3(15-C), 29.0(16-C), 56.0(17-C), 12.7(18-C), 15.9(19-C), 42.6(20-C), 21.6(21-C), 138.6(22-C), 129.3(23-C), 47.8(24-C), 30.5(25-C), 21.1(26-C), 19.4(27-C), 24.4(28-C), 12.6(29-C); (-)LRESIMS *m/z*: 611$[M-Na]^{-1}$, 588$[M-2Na]^{-2}$.

*Disodium 24-ethyl-3β,6β-dihydroxycholestane disulfate* (***3***): Yield: 65%; m.p. 242–249 °C; IR(KBr) v: 3427, 2925, 1634, 1401, 1209, 815 $cm^{-1}$; $^1H$ NMR(DMSO, 500 MHz): 0.650(3H, s, 18-$CH_3$), 0.798(3H, d, *J* = 7.0, 26- or 27-$CH_3$), 0.818(3H, d, *J* = 7.0, 26- or 27-$CH_3$), 0.825(3H, t, *J* = 6.5, 29-$CH_3$), 0.896(3H, d, *J* = 6.3, 21-$CH_3$), 0.923(3H, s, 19-$CH_3$), 1.924(1H, brad, *J* = 12.5, $C_2$-βH), 3.961(1H, m, $C_3$-H), 4.181(1H, d, *J* = 3.5, $C_6$-H); (-)LRESIMS *m/z*: 613$[M-Na]^{-1}$, 590$[M-2Na]^{-2}$.

### 3.4. Assay for cell viability

#### 3.4.1. Materials and methods

Stock solutions of compounds **1**, **2** and **3**, were prepared in sterile dimethyl sulfoxide (DMSO) (Sigma) at a concentration of 10 mg/mL, and diluted to proper concentrations according to the experimental design with the cell culture medium.

#### 3.4.2. Cell culture

Sk-Hep-1, H-292, PC-3 (ATCC) and Hey-1B (a gift from Dr. Yan Xu, University of Indiana) cells were cultured in a proper medium supplemented with 10% fetal bovine serum and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate in a humidified atmosphere of 5% $CO_2$ at 37 °C.

#### 3.4.3. Treatment of cancer cells

Cancer cells ($4 \times 10^4$ cells/mL, 200 μL) were seeded into each well of a 96-well microtiter plate. After incubation for 24 h, the compounds with a series of concentrations (range 5–100 μg/mL) were added to the cells. An equal amount of DMSO was added to the cells as negative controls. All were treated in triplicate.

#### 3.4.4. Determination of cell viability

MT Stetrazolium salt ((3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (CellTiter 96 $AQ_{ueous}$ Non-Radioactive Cell Proliferation Assay, Cat #G5421, Promega Corporation) dye reduction assay was used to determine the cell viability. The assay is dependent on the

reduction of MTS by mitochondrial dehydrogenases of viable cells to a blue water soluble formazan product, which can be measured spectrophotometrically. The absorbance of the formazan product at 490 nm is in linear proportion to cell numbers. Briefly, after treatment (see 3.4.3) with the compounds for 48 h, the medium was removed and the cells were incubated with 100 μL of fresh medium plus 20 μL of MTS solution for additional 4 h according to the instruction provided by the manufacturer. The absorbance (A) at 490 nm was measured using a LD400 AD/LD analysis spectrometer (Beckman coulter). The $IC_{50}$ value was calculated as the concentration of drug yielding 50% cell survival.

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