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Cytotoxic Activity of Tropolones Against Human Oral Tumor Cell Lines

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Abstract. *Twenty-seven tropolone derivatives were investigated for their tumor-specific cytotoxicity, using 3 normal human cells and 3 human oral tumor cell lines. Tropolone derivatives with phenolic OH group, hinokithiol, its tosylate and methyl ethers have relatively higher tumor specificity. 5-Aminotropolone showed the highest specificity, whereas 2-aminotropone and its derivatives showed little or no specificity. 5-Aminotropolone induced apoptotic cell death characterized by internucleosomal DNA fragmentation and caspase 3 activation in the human promyelocytic leukemic HL-60 cell line. ESR spectroscopy showed that 5-aminotropolone produced radical under alkaline condition, and efficiently scavenged O_2^- and NO produced by HX-XOD reaction and NOC-7, respectively. These data suggest that 5-aminotropolone may induce cytotoxicity by radical-mediated redox reaction.*

Hinokitiol (compound **[1]** in Figure 1) and related compounds with a tropolone skeleton (1-3), have shown a broad spectrum of biological activities, including antimicrobial activity (4), antifungal activity (5), phytogrowth-inhibitory activity (6, 7), cytotoxic effect on mammalian tumor cells (8, 9) and inhibitory activity on catechol-*O*-methyltransferase (10) and metalloproteases (4). Hinokitiol acetate did not show the cytotoxic activity (9), antimicrobial activity or metalloprotease inhibition (4), suggesting that the biological effects of hinokitiol-related compounds may result from the metal chelation between the carbonyl group at C-1 and the hydroxyl group at C-2 in the tropolone skeleton. However, a detailed study of cytotoxicity induction by tropolone-related compounds has not been performed so far. We investigated here whether a total of 27 compounds, which have a tropolone skeleton, display tumor-specific cytotoxic activity, using three normal human cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)] and three human oral tumor cell lines [submandibular gland carcinoma (HSG) and oral squamous cell carcinoma (HSC-2, HSC-3)] and, and if so, whether they induce apoptosis-associated characteristics (such as DNA fragmentation and caspase activation) in human tumor cells. We also investigated whether a radical-mediated mechanism is involved in the tropolone-induced cytotoxicity, using ESR spectroscopy.

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Key Words: Tropolones, cytotoxic activity, apoptosis, caspase, DNA fragmentation.

Materials and Methods

Methods. The following chemicals and reagents were obtained from the indicated companies: Hinokitiol **[1]**, tropolone **[3]**, tropolone tosylate **[12]** (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan); Dulbeccos's modified Eagle medium (DMEM), RPMI 1640 (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA); dimethyl sulfoxide (DMSO), diethylenetriaminepentaacetic acid (DETAPAC) (Wako Pure Chem, Ind, Ltd, Osaka, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD), 3'-azido-2', 3'-dideoxythymidine (AZT), dideoxycytidine (ddC)(Sigma Chem Co., St. Louis, MO, USA); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), superoxide dismutase (SOD) from bovine erythrocytes, 1-hydroxyl-2-oxo-3-*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (Dojin, Kumamoto, Japan).

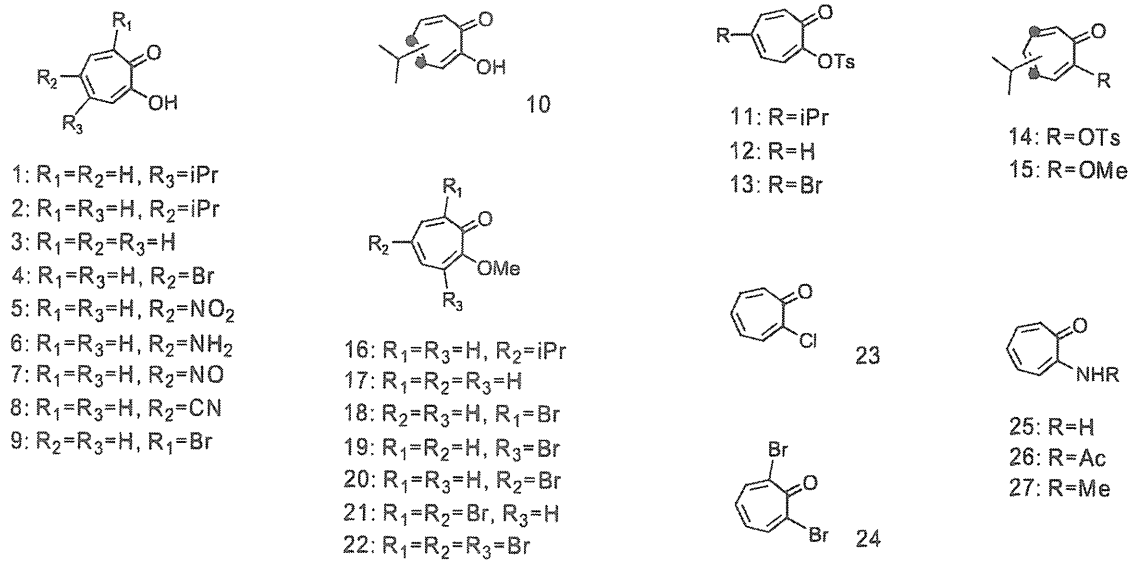

Figure 1. *Structure of tropolone-related compounds.*

Synthesis of tropolones. Tropolones were synthesized, according to the published reports: Hinokitiol (β-thujaplicin) **[1]** (11, 12), γ-thujaplicin **[2]** (12-14), tropolone **[3]** (15, 16), 5-bromotropolone **[4]** (17), 5-nitrotropolone **[5]** (18), 5-aminotropolone **[6]** (19, 20), 5-nitrosotropolone **[7]** (21, 22), 5-cyanotropolone **[8]** (23), 3-bromotropolone **[9]** (24, 25), hinokitiol mixture **[10]** (12, 14), γ-thujaplicin tosylate **[11]** (26), tropolone tosylate **[12]** (27), 5-bromotropolone tosylate **[13]** (28), hinokitiol tosylate mixture **[14]** (26), hinokitiol methyl ether mixture **[15]** (29), γ-thujaplicin methyl ether **[16]** (29), 2-methoxytropone **[17]** (30), 2-bromo-7-methoxytropone **[18]** (25), 3-bromo-2-methoxytropone **[19]** (25), 5-bromo-2-methoxytropone **[20]** (30), 2,4-dibromo-7-methoxytropone **[21]** (30), 2,4,6-tribromo-7-methoxytropone **[22]** (30), 2-chlorotropone **[23]** (31), 2,7-dibromotropone **[24]** (31), 2-aminotropone **[25]** (16, 32), *N*-acetyl-2-aminotropone **[26]** (16), *N*-methyl-2-aminotropone **[27]** (16).

Cell culture. Three human oral tumor cell lines (HSG, HSC-2, HSC-3) and three human normal cells [HGF (5-8 population doubling level (PDL)), HPC (5-8PDL), HPLF (5-8PDL)] were cultured in DMEM supplemented with 10% heat-inactivated FBS. Human promyelocytic leukemic HL-60 cells were cultured in RPMI 1640 supplemented with 10% FBS. Normal cells were prepared from periodontal tissues, according to the guidelines of Meikai University Ethics Committee, after obtaining informed consent from the patients.

Assay for cytotoxic activity. Cells (other than HL-60 cells) were inoculated at 12 x 10^3 cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 24 hours, the medium was removed by suction with an aspirator and replaced with 0.1 mL of fresh medium containing various concentrations of test compounds. Cells were incubated for another 24 hours and the relative viable cell number was then determined by MTT method. In brief, cells were replaced with fresh culture medium containing 0.2 mg/mL MTT and incubated for another 4 hours. The cells were lysed with 0.1 mL of DMSO, and the absorbance at 540 nm of the cell lysate was determined, using a microplate reader (Biochromatic Labsystem, Helsinki, Finland) (33). A_{540} of control cells were usually in the range of 0.40 to 0.90. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. Tumor specificity (TS) was determined by the following equation.

$$TS = \frac{[CC_{50}(HGF)] + [CC_{50}(HPC)] + [CC_{50}(HPLF)]}{[CC_{50}(HSG)] + [CC_{50}(HSC\text{-}2)] + [CC_{50}(HSC\text{-}3)]}$$

The viability of HL-60 cells was determined by trypan blue exclusion cell. HL-60 cells were inoculated at 5 x 10^4/0.1 mL in 96-microwell and various concentrations of test compounds were added. After incubation for 24 hours, the viable cell number was determined as described previously. The cell density of control cells at cell harvest was in the range of 8-9 x 10^5/mL.

Assay for DNA fragmentation. Cells were lysed with 50 μL lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate solution]. The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1-2 hours at 50°C. After incubation, the lysate was mixed with 50 μL of NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0]. Two hundred and fifty μL of ethanol was added and centrifuged for 20 minutes at 20,000 xg. The precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). A sample (10-20 μL) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA molecular marker (Takara) and DNA from apoptotic HL-60 cells induced by UV irradiation were used for calibration (34). The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Table I. *Cytotoxic activity of tropolones.*

Compd.	Molecular Weight	Cytotoxic activity (CC_{50}: mM)						
		Normal human cells			Human tumor cell lines			
		HGF	HPC	HPLF	HSG	HSC-2	HSC-3	TS
1	164.20	1.93	1.72	1.73	1.52	0.17	0.17	2.9
2	164.20	1.79	1.55	1.55	1.22	0.13	0.13	3.3
3	122.12	2.10	1.12	1.23	0.81	0.14	0.14	4.1
4	201.02	1.40	1.37	1.49	0.96	0.39	0.39	2.5
5	167.12	2.05	1.52	2.27	1.93	0.53	0.53	2.0
6	137.14	0.63	1.04	0.85	0.14	0.058	0.058	9.9
7	151.12	0.13	0.066	0.12	0.02	0.046	0.16	1.4
8	147.13	>2.72	>2.72	>2.72	>2.72	0.082	0.082	><2.8
9	201.02	1.91	1.60	1.59	1.30	0.17	0.17	3.1
10	164.20	1.86	1.63	1.36	1.29	0.10	0.10	3.4
11	318.39	0.11	0.17	0.12	0.022	0.041	0.041	3.8
12	276.31	0.076	0.069	0.033	0.025	0.022	0.022	2.6
13	355.21	0.0084	0.014	0.014	0.0056	0.014	0.014	1.1
14	318.39	0.14	0.10	0.094	0.019	0.031	0.031	4.1
15	178.23	1.64	1.58	1.48	0.84	0.51	0.51	2.5
16	178.23	>2.24	2.11	1.68	1.01	1.39	1.39	>1.6
17	136.15	>2.94	>2.94	>2.94	2.24	2.59	2.59	>1.2
18	215.05	>1.86	1.76	>1.86	1.30	0.38	0.38	>2.7
19	215.05	0.11	0.16	0.14	0.074	0.074	0.084	1.8
20	215.05	>1.86	>1.86	>1.86	2.20	1.71	1.71	>1.0
21	293.94	0.22	0.34	0.16	0.011	0.27	0.27	>1.3
22	372.84	0.027	0.075	0.048	0.013	0.008	0.011	4.7
23	140.57	0.23	0.18	0.085	0.05	0.19	0.19	1.1
24	263.92	0.0076	0.049	>0.034	0.045	0.027	0.015	1.0
25	121.14	3.12	2.91	2.97	2.94	2.42	2.42	1.2
26	163.17	>2.45	>2.45	>2.45	2.26	>2.45	>2.45	><1.0
27	135.16	0.62	1.27	2.23	0.34	1.40	1.40	1.3

Assay for caspase activation. Cells were washed with PBS and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 10 minutes on ice and centrifugation for 5 minutes at 10,000 xg, the supernatant was collected. The lysate (50 μL, equivalent to 200 μg protein) was mixed with 50μL 2x reaction buffer (MBL) containing substrates for caspase 3 (DEVD-*p*NA (*p*-nitroanilide)), caspase 8 (IETD-*p*NA) or caspase 9 (LEHD-*p*NA). After incubation for 3 hours at 37°C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by plate reader.

Assay for radical intensity. The radical intensity of the test sample was determined at 25°C in 0.1 M Tris-HCl buffer (pH 7.4), 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9, 10) or in 0.1 M KOH (pH 12.5), using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.03 seconds; scanning time, 2 minutes. The radical intensity was defined as the ratio of peak height of these radicals to that of MnO (35).

To determine O_2^-, produced by HX-XOD reaction (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB) 50μL, 0.5 mM DETAPAC 20 μL, 8 % DMPO 30 μL, sample (in DMSO) 40 μL, H_2O or SOD 30 μL, XOD (0.5 U/mL in PB) 30 μL], the gain, time constant and scanning time were changed to 500, 0.1 seconds and 1 minute, respectively. The radical intensity was determined 1 minute after mixing. The O_2^- scavenging activity was expressed as SOD unit/mg sample, by calibration with the standard curve of SOD (9). The concentration required to reduce the radical intensity of DMPO-OOH by 50% (IC_{50}) was determined from the dose-response curve.

For the determination of NO radical, sample was added to the reaction mixture of 20 μL C-PTIO and 50 μL NOC-7 in 0.06 M phosphate buffer, pH 7.4. The gain and scanning time were changed to 250 and 2 minutes, respectively. The NO radical intensity was defined as the ratio of peak height of the 1st peak of carboxy-PTI, which was produced by the reaction of NO (derived from NOC-7) and carboxy-PTIO to that of MnO (35).

Assay for anti-human immunodeficiency virus (HIV) activity. MT-4 cells were infected with $HIV\text{-}1_{IIIB}$ at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock- infected (control) MT-4 cells (1.5 x 10^5/mL, 200μL/well) were placed into 96-well microtiter plates and incubated in the presence of various concentrations of test samples. After incubation for 5 days at 37°C in a 5% CO_2 incubator, cell viability was quantified by a colorimetric assay (at 540 nm and 690 nm), monitoring the ability of viable cells to reduce MTT to a blue formazan product. The CC_{50} and 50% effective concentration (EC_{50}) were determined from the dose-response curve with mock-

Table II. *Anti-HIV activity of tropolones.*

Compd.	CC_{50} (mM)	EC_{50} (mM)	SI
1	0.00783	>0.00974	< 1
2	0.00156	>0.00195	< 1
3	0.0103	>0.0131	< 1
4	0.0200	>0.0398	< 1
5	0.136	>0.239	< 1
6	0.0363	>0.0583	< 1
7	0.00102	>0.00212	< 1
8	0.204	>0.272	< 1
9	0.0224	>0.0398	< 1
10	0.00273	>0.00974	< 1
11	0.0441	>0.126	< 1
12	0.0266	>0.0290	< 1
13	0.00583	>0.0225	< 1
14	0.00946	>0.0251	< 1
15	0.594	>1.12	< 1
16	0.336	>1.12	< 1
17	0.551	>1.47	< 1
18	0.272	>0.930	< 1
19	0.0115	>0.0372	< 1
20	0.483	>0.930	< 1
21	0.0638	>0.136	< 1
22	0.00156	>0.00429	< 1
23	0.0301	>0.0569	< 1
24	0.00268	>0.00606	< 1
25	0.807	>1.65	< 1
26	0.722	>1.23	< 1
27	0.551	>1.27	< 1
AZT	0.213	0.000021	9973
ddC	>5.000	0.00309	>1616

infected or HIV-infected cells, respectively (36). All data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by selectivity index (SI), which was calculated by the following equation:

$$SI=CC_{50}/EC_{50}$$

Results

Structure and activity relationship. We first investigated 27 tropolones for their relative cytotoxicity against three human normal cells (HGF, HPC, HPLF) and three human oral tumor cell lines (HSG, HSC-2, HSC-3) (Table I). Tropolone **[3]** showed higher cytotoxicity against tumor cell lines [CC_{50} (HSG) = 0.81 mM; CC_{50} (HSC-2) = 0.14 mM; CC_{50} (HSC-3) = 0.14 mM] than against normal cells [CC_{50} (HGF) = 2.10 mM; CC_{50} (HPC) = 1.12 mM; CC_{50} (HPLF) = 1.23 mM], yielding the tumor-specific cytotoxicity index (TS) of 4.1. In general, tropolone **[3]** and its derivatives with phenolic OH group **[1-2, 4-10]** showed relatively higher TS values. Among them, 5-aminotropolone **[6]** showed the highest tumor specificity (TS=9.9). However, 2-aminotropone **[25]** and its derivatives **[26, 27]** showed little or no tumor specificity [TS=1.0-1.3]. Hinokithiol **[1]** and its tosylate **[11, 14]** and methyl ethers **[15, 16]** showed slightly lower tumor specificity (TS=2.9, 3.8, 4.1, 2.5, 1.6) than tropolone **[3]**. Compounds **[7, 13, 19, 23, 24]** were highly cytotoxic, but showed essentially no tumor specificity (TS=1.4, 1.1, 1.8, 1.1, 1.0).

All compounds showed no anti-HIV activity (selectivity index (SI)>1), whereas two positive controls, such as AZT and ddC, showed potent anti-HIV activity (SI=5617 and 2002, respectively) (Table II).

Apoptosis induction. 5-Aminotropolone **[6]** induced internucleosomal DNA fragmentation, a biochemical hallmark, in HL-60 cells (Figure 2). The optimal concentration was 0.029-0.058 mM and higher concentration (0.117 mM) was much less active. 5-Aminotropolone **[6]** activated the caspase 3 to a comparable extent as that attained by actinomycin D, without affecting caspase 8 and 9 activity (Figure 3).

Radical generation. 5-Aminotropolone **[6]** produced radical under alkaline condition. At an optimal condition (pH 9.0), multiple peaks of radical were produced (Figure 4). 5-Aminotropolone **[6]** efficiently scavenged O_2^- (generated by HX-XOD reaction) (IC_{50}=0.029 mM) (Figure 5) and NO (generated from NOC-7) (IC_{50}=0.017 mM) (Figure 6).

Discussion

We found that tropolone **[3]** showed higher cytotoxic activity and tumor-specific cytotoxicity (TS=4.1) than hinokitiol **[1]** (TS=2.9). Since 2-methoxytropone **[17]** showed much reduced cytotoxicity compared to tropolone, metal chelation between the carbonyl group at C-1 and the hydroxyl group at C-2 might be responsible for the cytotoxicity induction, as has been reported in hinokitiol-related compounds (4, 9). It was unexpected that 2-chlorotropone **[23]**, which has replaced the hydroxyl group at C-2 by Cl, showed comparable cytotoxicity against both normal and tumor cells, yielding essentially no tumor specificity (TS=1.1).

The present study demonstrated, for the first time, that introduction of the amino group at C-5 in tropolone **[6]** significantly enhanced the tumor-specific cytotoxicity (TS=9.9), and that this compound induced apoptotic cell death characterized by caspase 3 activation and DNA fragmentation. At present, the mechanism by which 5-aminotropolone **[6]** activated caspase 3 is unclear, since this compound did not significantly activate caspase 8 (mitochondria-independent extrinsic pathway) and caspase 9 (mitochondria-dependent intrinsic pathway) (37).

We found that 5-aminotropolone **[6]** was more cytotoxic, produced higher amounts of radical and scavenged O_2^- and NO radicals more efficiently than azulenes (38). This suggests that 5-aminotropolone **[6]** may induce cytotoxicity by radical-mediated redox reaction. Higher dipole moment of 5-

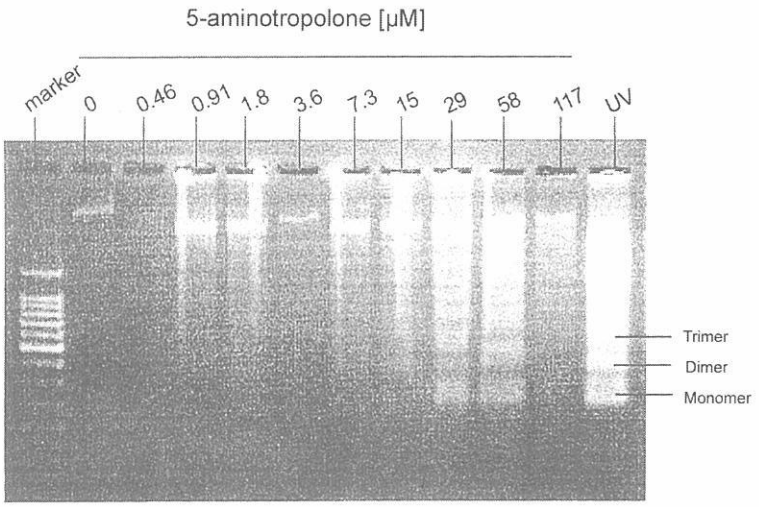

Figure 2. *Induction of DNA fragmentation by 5-aminotropolone* ***[6]*** *in HL-60 cells. HL-60 cells were inoculated at 5 x* 10^5 *cells/1 mL in 24-well plate, in fresh culture medium (RPMI1640 +10% FBS) with the indicated concentrations of 5-aminotropolone* ***[6]****. After incubation for 6 hours, DNA was extracted and applied to agarose gel electrophoresis. Marker, DNA molecular marker; UV, DNA from apoptotic HL-60 cells induced by UV irradiation.*

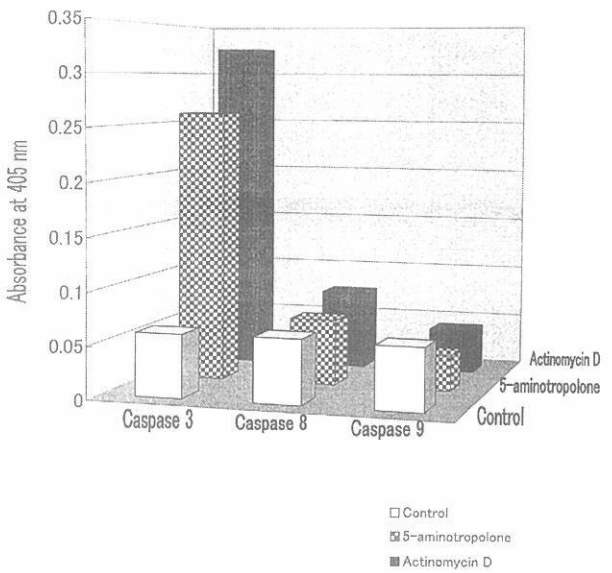

Figure 3. *Activation of caspase 3 by 5-aminotropolone* ***[6]*** *in HL-60 cells. HL-60 cells were incubated for 4 hours without (control) or with 44 μM 5 aminotropolone* ***[6]*** *or 1 μg/mL actinomycin D (positive control).*

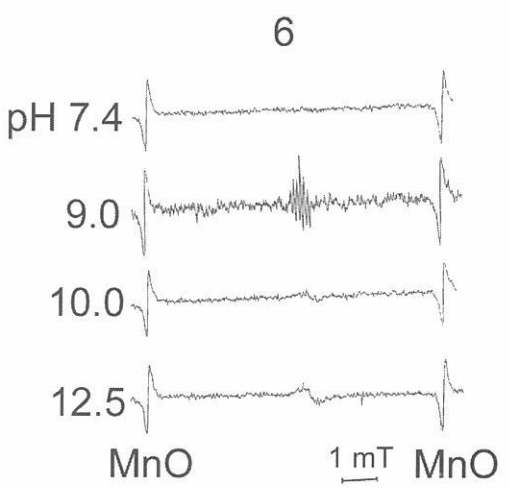

Figure 4. *ESR spectra of 5-aminotropolone* ***[6]*** *(1 mg/mL) at increasing pH.*

aminotropolone **[6]** may produce higher SI value. Since 2-aminotropolone **[25]** had a much lower SI value, the effect of the functional group may be small. Further studies are underway to test this possibility.

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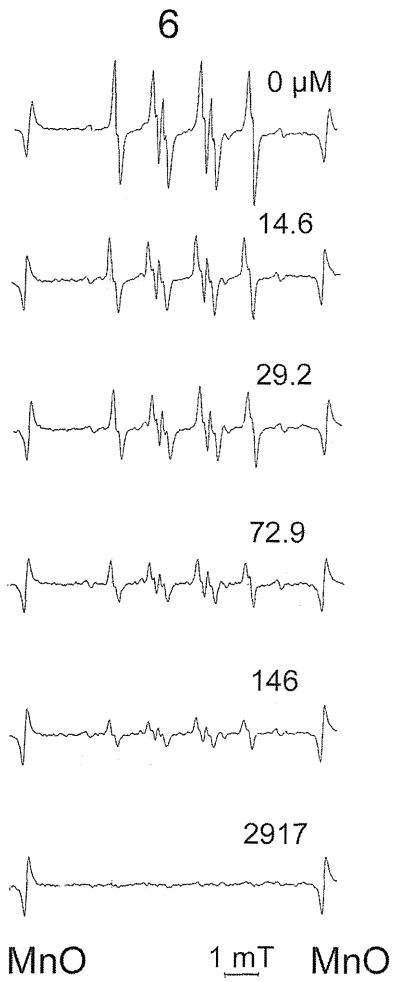

Figure 5. *ESR spectra of DMPO-OOH adduct produced by HX-XOD reaction mixture in the presence of the indicated concentrations of 5-aminotropolone [6].*

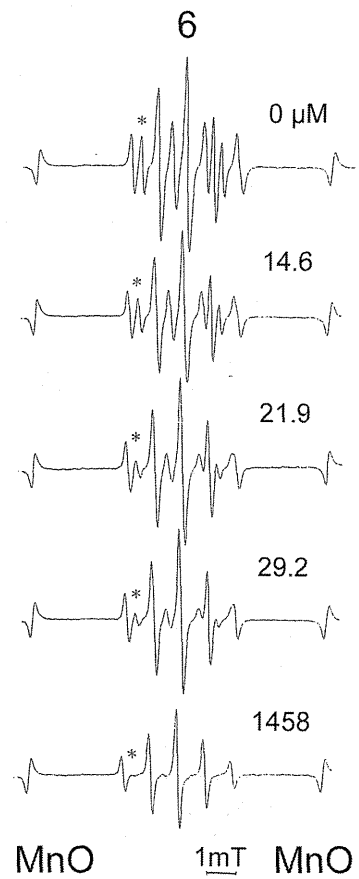

Figure 6. *ESR spectra of carboxy-PTIO + NOC-7 in the presence of the indicated concentrations of 5-aminotropolone [6]. Measured at 3 minutes. The second peak (indicated by asterisks) (derived from carboxy-PTI) was used for the calculation of NO radical intensity.*

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