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Inhibition of LPS-stimulated NO Production in Mouse Macrophage-like Cells by Tropolones

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Abstract. *We investigated the effect of 27 tropolones on nitric oxide (NO) production by mouse macrophage-like Raw 264.7 cells. All of these compounds failed to stimulate the Raw 264.7 cells to produce detectable amounts of NO, but inhibited NO production by lipopolysaccharide (LPS)-activated Raw 264.7 cells to various extents. Generally, the ability of tropolones to inhibit LPS-stimulated NO production was inversely related to their cytotoxic activity. Western blot and RT-PCR analyses demonstrated that the most active compound, 2,4-dibromo-7-methoxytropone* ***[21]****, significantly reduced both the intracellular concentration of iNOS protein and the expression of iNOS mRNA. ESR spectroscopy showed that* ***[21]*** *did not produce radicals under alkaline condition, nor scavenged NO, produced by NOC-7. These data suggested that the inhibitory effect of* ***[21]*** *on NO production might be generated via the inhibition of iNOS expression, rather than a radical-mediated mechanism.*

Hinokitiol (compound **[1]** in Figure 1), and related compounds with a tropolone skeleton (1-3), have shown a broad spectrum of biological activities such as antimicrobial (4), antifungal (5), phytogrowth-inhibitory (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 (9) or antimicrobial activity, nor metalloprotease inhibition (4), suggesting that these 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, no detailed study of cytotoxicity induction by tropolone-related compounds has been performed to date. We recently found that tropolone derivatives with the phenolic OH group, hinokitiol, its tosylate and methyl ethers showed a tumor-specific cytotoxic activity. 5-Aminotropolone **[6]** showed the highest tumor-specificity, whereas 2-aminotropone **[25]** and its derivatives showed little or no specificity. 5-Aminotropolone **[6]** induced apoptotic cell death characterized by internucleosomal DNA fragmentation and caspase 3 activation in the human promyelocytic leukemic HL-60 cell line, possibly by radical-mediated redox reaction (11).

We investigated here whether a total of 27 tropolone derivatives (Figure 1) modify the nitric oxide (NO) production by unstimulated- and lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells. Since the NO concentration is determined by many factors, including the intracellular concentration of inducible NO synthase (iNOS), the enzyme activity of iNOS and the quenching of NO radical by radical scavengers present in the assay systems, we also investigated whether tropolone-related compounds affect the expression of iNOS protein and mRNA (by Western blot and RT-PCR analyses) and scavenge NO, generated from 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7, a NO generator) in the presence of 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxy1-3-oxide (carboxy-PTIO, a spin trap agent) (by ESR spectroscopy).

Materials and Methods

Materials. 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); Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriaminepentaacetic acid

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Key Words: Tropolones, Raw 264.7 cells, NO, iNOS, ESR, radical.

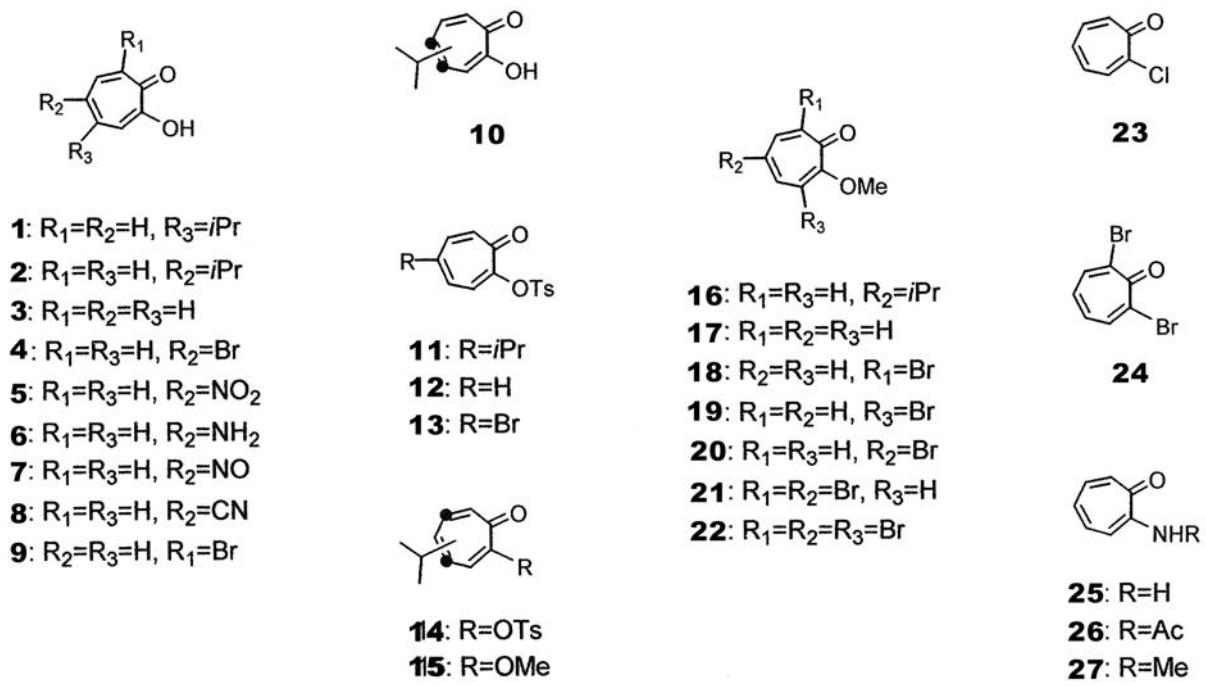

Figure 1. *Structures of tropolone derivatives used in this study.*

(DETAPAC), phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem. Co., St. Louis, MO, USA); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), carboxy-PTIO, NOC-7, superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan).

Synthesis of tropolones. Hinokitiol (β-thujaplicin) **[1]**, γ-thujaplicin **[2]**, tropolone **[3]**, 5-bromotropolone **[4]**, 5-nitrotropolone **[5]**, 5-aminotropolone **[6]**, 5-nitrosotropolone **[7]**, 5-cyanotropolone **[8]**, 3-bromotropolone **[9]**, hinokitiol mixture **[10]**, γ-thujaplicin tosylate **[11]**, tropolone tosylate **[12]**, 5-bromotropolone tosylate **[13]**, hinokitiol tosylate mixture **[14]**, hinokitiol methyl ether mixture **[15]**, γ-thujaplicin methyl ether **[16]**, 2-methoxytropone **[17]**, 2-bromo-7-methoxytropone **[18]**, 3-bromo-2-methoxytropone **[19]**, 5-bromo-2-methoxytropone **[20]**, 2,4-dibromo-7-methoxytropone **[21]**, 2,4,6-tribromo-7-methoxytropone **[22]**, 2-chlorotropone **[23]**, 2,7-dibromotropone **[24]**, 2-aminotropone **[25]**, *N*-acetyl-2-aminotropone **[26]** and *N*-methyl-2-aminotropone **[27]** were prepared, according to the references cited in a previous report (11) (Figure 1).

Cell culture. Mouse macrophage-like Raw 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere (12, 13).

Assay for cytotoxic activity. The cytotoxic activity of tropolones was determined by MTT method and expressed as absorbance at 540nm of the MTT-stained cells. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (12, 13).

Assay for NO concentration. Near confluent Raw 264.7 cells were incubated for 24 hours with each test sample in phenol red-free DMEM supplemented with 10% FBS and the NO production by Raw 264.7 cells was quantified by Greiss reagent (Molecular Probes Inc, Netherlands), using the standard curve of NO_2^-. To eliminate the interaction between sample and Greiss reagent, we also measured the NO concentration in the culture medium without the cells, and subtracted it from that with the cells. The concentration which inhibited the LPS-stimulated NO production by 50% (50% effective concentration: EC_{50}) was determined from the dose-response curve (12, 13). The efficacy of inhibition of NO production was estimated by the selectivity index SI, which was calculated by the following equation:

$$SI = \frac{CC_{50}}{EC_{50}}$$

Western blotting. The cell pellets were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 minutes in ice water, and then incubated for 50 minutes at 4˚C with RT-5 ROTATOR (Titec, Saitama, Japan). The cell lysates were centrifuged at 16,000 xg for 20 minutes at 4˚C to remove the insoluble materials and the supernatant was collected. The protein concentrations of supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). Equal amounts of the protein from cell lysates (10 μg) were mixed with 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-β mercaptoethanol), boiled for 10 minutes, applied to the SDS-7% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in Tris-HCl-buffered saline plus 0.05% Tween 20 for 90 minutes and incubated with anti-iNOS antibody (1:1,000, Santa Cruz Biotechnology, Delaware, CA, USA) for 90

Table I. *Inhibition of NO production by LPS-stimulated Raw 264.7 cells by tropolones.*

Compd.	MW	Cytotoxic activity CC_{50} (μM) LPS(+)	Cytotoxic activity CC_{50} (μM) LPS(-)	Inhibition of NO production EC_{50} (μM) LPS(+)	SI
[1]	164.20	30.5	13.4	2.44	12.5
[2]	164.20	27.4	12.8	<1.95	>14.1
[3]	122.12	353	145	11.5	30.8
[4]	201.02	93.5	93.5	7.96	11.8
[5]	167.12	97.5	160	25.1	3.9
[6]	137.14	10.9	8.75	10.2	1.1
[7]	151.12	1.99	2.65	<2.12	>0.9
[8]	147.13	510	181	46.9	10.9
[9]	201.02	321	233	10.4	30.7
[10]	164.20	25.6	14.0	135	0.2
[11]	318.39	22.3	22.3	1.57	14.2
[12]	276.31	18.1	18.1	<1.16	>15.6
[13]	355.21	1.13	2.53	<0.90	>1.3
[14]	318.39	9.42	15.7	<1.01	>9.4
[15]	178.23	233	277	17.4	13.4
[16]	178.23	389	393	65.6	5.9
[17]	136.15	542	588	109	5.0
[18]	215.05	>372	>372	41.4	>9.0
[19]	215.05	14.4	16.3	2.33	6.2
[20]	215.05	366	288	23.3	15.8
[21]	293.94	88.1	102	<1.09	>80.9
[22]	372.84	<0.86	1.07	<0.86	><1.0
[23]	140.57	45.5	53.4	<2.28	>20
[24]	263.92	<1.21	<1.21	<1.21	><1.0
[25]	121.14	>660	>660	58.6	>11.3
[26]	163.17	409	490	58.8	6.9
[27]	135.16	131	262	22.2	5.9

minutes at room temperature or overnight at 4˚C, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 hour at room temperature. Immunoblots were detected by Western Lightning™ Chemiluminescence Reagent *Plus* (Perkin Elmer Life Sciences, Boston, MA, USA).

Assay for mRNA expression. Total RNA was isolated by the PURESCRIPT RNA Isolation kit (Gentra systems) protocol. Raw 264.7 cells were lysed in 300 μL cell lysis solution, then 100 μL Protein-DNA precipitation solution was added. The cell lysates were centrifuged at 15,000 x g for 3 minutes. To the supernatant, 300 μL isopropanol was added. After centrifugation at 15,000 x g for 3 minutes, the pellet was washed in 300 μL 75% ethanol. After centrifugation at 15,000 x g for 1 minute, the pellet was air-dried for 15 minutes and dissolved in DEPC-treated H_2O. A reverse transcriptase reaction (RT) was performed with 1.0 μg of total RNA, using the Rever Tra Ace (Toyobo Co., Ltd.), using oligo $(dT)_{20}$ primer (13). Single strand cDNA obtained by RT reaction was amplified, using the KOD plus (Toyobo Co., Ltd.), using iNOS specific primer of (5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and 5'-GGCTGTCA GAGCCTCGTGGCTTTGG-3') and β-actin specific primers (5'-GAGGCCCAGAGCAAGAGAGG-3' and 5'-TACATGGCTGGGG TGTTGAA -3'), according to the protocol. The RT-PCR products were applied to 2 % agarose gel, and the ethidium bromide-stained gel was then photographed under UV light.

Radical scavenging activity. The radical intensity of tropolones was determined at 25˚C in 0.1 M Tris-HCl (pH 7.4, 8.0), 0.1 M Na_2CO_3/$NaHCO_3$ (pH 9.0, 10.5) or 0.1 M KOH (pH 12.5), using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (3, 4). Instrument settings; center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT: gain, 500, time constant, 0.1 second; scanning time, 2 minutes.

For determination of O_2^- produced by HX and XOD reactions (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB) 50 μL, 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 time constant and scanning time was changed to 0.03 second and 2 minutes, respectively. The O_2^- scavenging activity was expressed as SOD units/mg sample, by calibration with the standard curve of erythrocyte SOD.

The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 50 μM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO. The microwave power and gain were changed to 5 mW and 250, respectively. When NOC-7 (NO generator) and carboxy-PTIO (spin trapping agent) were mixed, NO was oxidized to NO_2, and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. NO radical intensity was defined as the ratio of signal intensity of the first peak of carboxy-PTI (indicated by symbols in Figure 5) to that of MnO (12, 13).

Results

Relationship between cytotoxicity and inhibition of LPS-stimulated NO production. We first investigated the cytotoxic activity of 27 tropolone compounds against Raw 264.7 cells, determined without LPS (Table I). Generally, the ability of tropolones to inhibit LPS-stimulated NO production was inversely related to their cytotoxic activity. Compound **[22]** (CC_{50}= 1.07 μM) showed the highest cytotoxicity, followed by **[24]** (CC_{50}= <1.21 μM) > **[13]** (CC_{50}= 2.53 μM) > **[7]** (CC_{50}= 2.65 μM) > **[6]** (CC_{50}= 8.75 μM). These compounds inhibited NO production by LPS-activated Raw 264.7 cells less efficiently (SI=><1.0, ><1.0, >1.3, >0.9 and 1.1, respectively).

Compounds **[1, 2, 11, 12, 14, 19]** showed slightly lower cytotoxic activity (CC_{50}= 13.4, 12.8, 22.3, 18.1, 15.7 and 16.3 μM, respectively), and more efficiently inhibited NO production (SI=12.5, >14.1, 14.2, >15.6, >9.4 and 6.2, respectively).

Compounds **[3, 9, 21, 23]** were much less cytotoxic (CC_{50}= 145, 233, 102 and 53.4 μM, respectively) and most efficiently inhibited NO production by activated Raw 264.7 cells (SI=30.8, 30.7, >80.9 and >20, respectively) (Figure 2).

All the tropolones, including compounds **[3, 9, 21, 23]** (indicated by open circles in Figure 2), failed to stimulate the Raw 264.7 cells to produce any detectable amount of NO (data not shown).

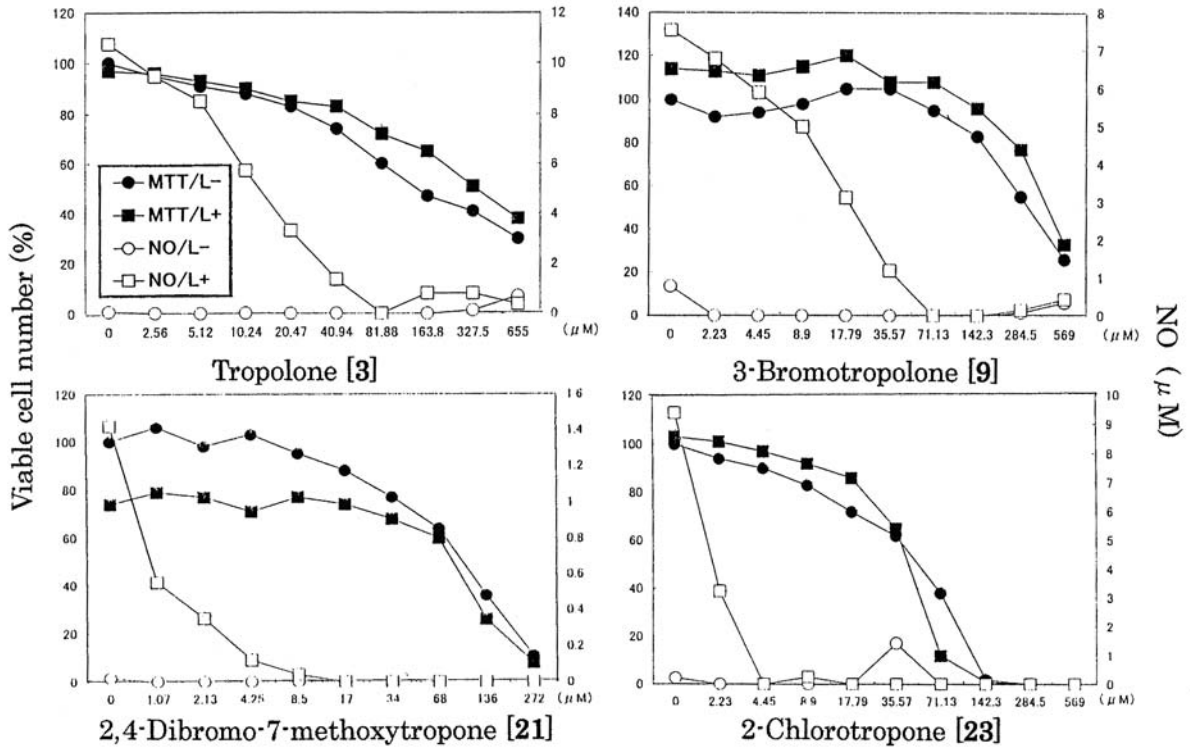

Figure 2. *Tropolones inhibited NO production by LPS-stimulated Raw 264.7 cells. Near-confluent Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of tropolone **[3]**, 3-bromotropolone **[9]**, 2,4-dibromo-7-methoxytropone **[21]** or 2-chlorotropone **[23]**, in the absence (●, ○) or presence (■, □) of 100 ng/mL LPS in phenol red-free DMEM supplemented with 10% FBS, and then the viable cell number (●, ■) and extracellular concentration of NO (○, □) were determined by MTT method and Griess reagent, respectively. Each value represents the mean from 4 assays.*

Structure and activity relationship. There was no clear-cut relationship between the positions of functional groups of tropolones and their ability to inhibit the NO production by activated Raw 264.7 cells (Figure 1, Table I).

Tropolone **[3]** and its alkylating derivatives **[1, 2]** efficiently inhibited NO production by activated Raw 264.7 cells, showing relatively high SI values (SI=30.8, 12.5 and >14.1, respectively). Tosylates of tropolone and its alkylating compound **[11, 12]** had higher SI values (SI=14.2 and >14.7, respectively), whereas the SI values of their methyl esters **[16, 17]** were much lower (SI=5.9 and 5.0, respectively). 3-Bromo-2-methoxytropone **[19]**, 2,4-dibromo-7-methoxytropone **[21]** and 2-chlorotropone **[23]** showed relatively high SI values (SI=6.2, >80.9 and >20, respectively), whereas monobromo- **[18, 20]** and tribromo-compounds **[22]** showed lower SI values (SI=>9.0, 15.8 and ><1.0, respectively). Compounds **[6, 7, 13, 22, 24]** were highly cytotoxic (CC_{50}=8.75, 2.65, 2.53, 1.07 and <1.21 μM, respectively), thereby giving lower SI values (SI= 1.1, >0.9, >1.3, ><1.0 and ><1.0, respectively). Hinokitiols, such as β-thujaplicin **[1]** and γ-thujaplicin **[2]**, showed lower EC_{50} value (EC_{50}=2.44 and <1.95 μM, respectively) than the hinokitiol mixture **[10]** (EC_{50}=135 μM).

iNOS expression. Based on our finding that 2,4-dibromo-7-methoxytropone **[21]** most effectively reduced the extracellular NO production by LPS-activated Raw 264.7 cells, we next investigated whether it could reduce the intracellular concentration of iNOS. Western blot analysis showed that unstimulated Raw 264.7 cells expressed only background levels of iNOS protein but, upon stimulation with LPS, they began to express detectable amounts of iNOS protein. This LPS-induced iNOS expression was dose-dependently inhibited by **[21]** (Figure 3). The inhibitory effect of 2,4-dibromo-7-methoxytropone **[21]** was detected above 2 μM. At 20 μM of **[21]**, the intracellular concentration of iNOS declined nearly to the baseline level (Figure 3).

RT-PCR analysis demonstrated that treatment with 2 μM 2,4-dibromo-7-methoxytropone **[21]** resulted in a 34% decline in iNOS mRNA expression (Figure 4). At 20 μM of **[21]**, iNOS mRNA expression declined to nearly half of the control level.

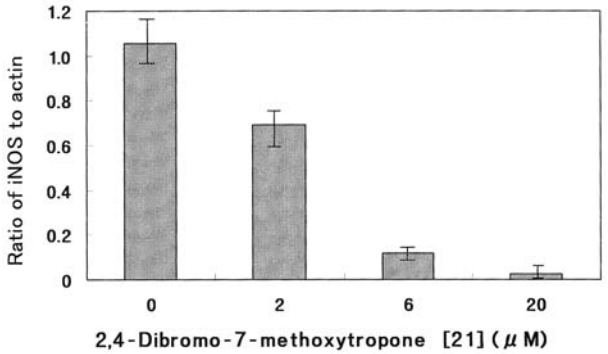

Figure 3. *Effect of 2,4-dibromo-7-methoxytropone **[21]** on the intracellular concentration of iNOS protein in Raw 264.7 cells. Raw 264.7 cells were incubated for 24 hours in the absence or presence of 100 ng/mL LPS with the indicated concentrations of 2,4-dibromo-7-methoxytropone **[21]**. The cell lysate was applied to SDS-PAGE and iNOS protein was quantified by Western blot analysis, followed by densitometry. The intracellular iNOS protein was expressed as the ratio to that of β-actin. The iNOS protein concentration without LPS was below the detection limit and therefore omitted. Each value represents mean±S.D. from three independent experiments.*

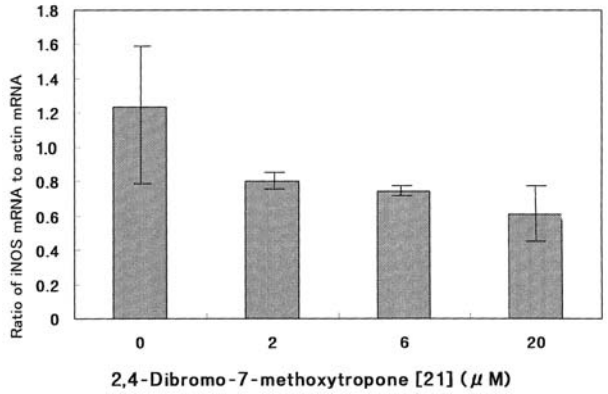

Figure 4. *Effect of 2,4-dibromo-7-methoxytropone **[21]** on LPS-stimulated iNOS mRNA expression. Raw 264.7 cells were incubated for 24 hours in the absence or presence of 100 ng/mL LPS with the indicated concentrations of 2,4-dibromo-7-methoxytropone **[21]**. RNA was then isolated and the PT-PCR product was applied to agarose gel electrophoresis and quantified by densitometry. The expression of iNOS mRNA was expressed as the ratio to that of β-actin mRNA. The iNOS mRNA expression without LPS was below the detection limit and therefore omitted. Each value represents mean±S.D. from three independent experiments.*

Radical scavenging activity. ESR spectroscopy showed that all four compounds **[3, 9, 21, 23]** failed to produce radical under alkaline condition (data not shown) and scavenge O_2^- (generated by HX-XOD reaction) (Table II). However, compounds **[3]** and **[9]** scavenged NO (generated from NOC-7), while **[21]** and **[23]** were inactive (Figure 5, Table II).

Table II. *Radical scavenging activity of tropolones.*

Addition of tropolone (μg/mL)		Intensity of DMPO-OOH (used to measure O_2^- scavenging activity)		Intensity of NO radical (used to measure NO scavenging activity)	
None	0 (control)	2.03	(100)[1)]	1.30	(100)[1)]
[3]	40	2.29	(113)	0.68	(52)
	400	2.23	(110)		
[9]	40	2.16	(106)	1.0	(77)
	400	2.20	(108)		
[21]	40	1.88	(93)	1.45	(112)
[23]	40	1.93	(95)	1.15	(88)

1) % of control

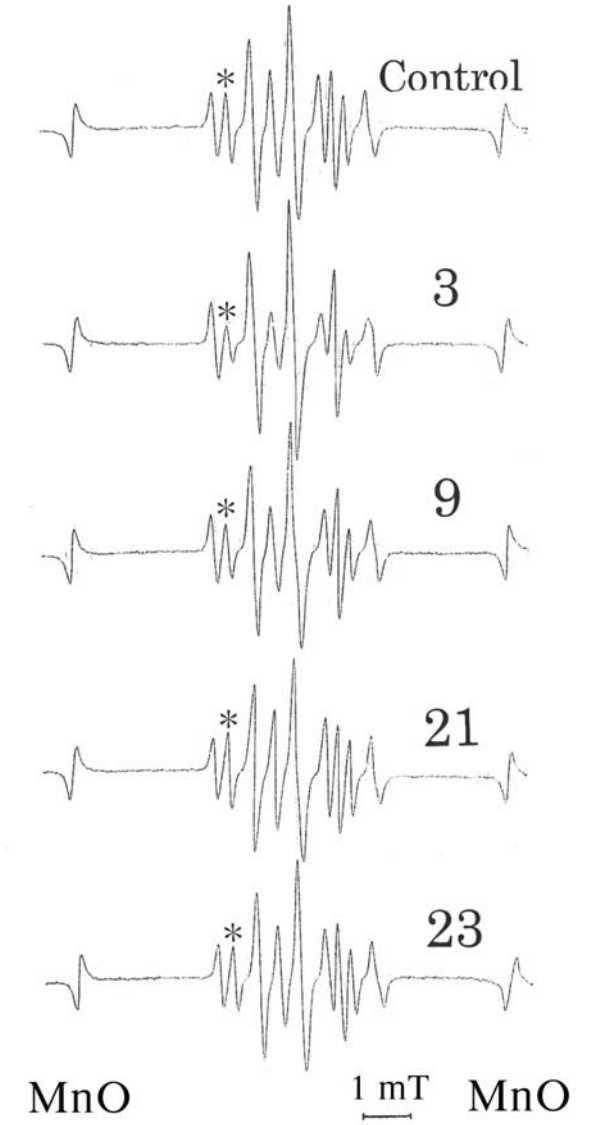

Figure 5. *ESR spectra of radical species produced by NOC-7 and carboxy-PTIO in the absence (control) or presence of 40 μg/mL of **[3]**, **[9]**, **[21]** or **[23]**. *, the first peak of carboxy-PTI; mT, milli-tesla.*

Discussion

The present study demonstrated that tropolone **[3]**, 3-bromotropolone **[9]**, 2,4-dibromo-7-methoxytropone **[21]** and 2-chlorotropone **[23]** inhibited NO production by LPS-activated macrophages at their non-cytotoxic concentrations. Among these four compounds, **[21]** was the most potent. The inhibitory activity of **[21]** was tightly associated with the decline of iNOS protein expression, rather than that of iNOS mRNA expression, without involvement of a radical-mediated mechanism (such as radical generation and scavenging). These data suggested that **[21]** might interfere with the activation pathway triggered by LPS in Raw 264.7 cells. It is possible that **[21]** may inhibit the LPS-stimulated translation of iNOS protein more efficiently than transcription of iNOS mRNA. Further investigation is necessary to determine the action point of **[21]**. We have recently found that azulenes also inhibited NO production by LPS-stimulated Raw 264.7 cells. However, azulenes generally showed much lower cytotoxic activity (14). Furthermore, the inhibitory effect of azulenes seems not to be linked to iNOS expression, in contrast to tropolones (14).

As far as we know, this is the first report of the effect of tropolones and related compounds on macrophage functions. The inhibitory effect of tropolones on NO production by macrophages suggests that they may play a role in inflammation.

Acknowledgements

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