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

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Abstract. *We investigated the effect of twenty-seven azulenes on nitric oxide (NO) production by mouse macrophage-like Raw 264.7 cells. No azulene derivative alone induced NO production by the Raw 264.7 cells, but inhibited lipopolysaccharide (LPS)-stimulated NO production to various extents. The ability of azulenes to inhibit NO generation by activated macrophages was generally increased when their cytotoxic activity declined. Western blot and RT-PCR analyses demonstrated that the most potent compound, 1,3-difluoroazulene **[11]**, slightly inhibited the expression of inducible NO synthase (iNOS), but only at extremely high concentrations. ESR spectroscopy showed that **[11]** did not produce radical under alkaline condition, nor scavenged O_2^- (generated by HX-XOD reaction) or NO (generated by NOC-7). These data suggest that the inhibitory effect of **[11]** may be produced via a mechanism other than iNOS induction and a radical-mediated mechanism.*

Azulene **[1]** (Figure 1), an isomer of naphthalene, has a dipole moment and a resonance energy between that of benzene and naphthalene, and is considerably more reactive than the two arenes (1-4). Azulene derivatives have shown various biological activities, including antibacterial (5), anti-ulcer (6), relaxant (7), inhibition of thromboxane A_2-induced vasoconstriction and thrombosis (8), acute toxicity and local anesthetic (9), and chemotherapeutic activity against mucous membrane diseases (10, 11). Recently, we found that 2-acetylaminoazulene **[4]**, diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** and methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]** showed higher tumor- specific cytotoxicity than the parent compounds such as azulene **[1]** and guaiazulene **[2]**. Four 1- and 3-halogenated derivatives showed a lower tumor specificity. The tumor-specific cytotoxic activity seemed not to be related to the position of functional groups. Compound **[24]** induced apoptotic cell death (characterized by internucleosomal DNA fragmentation and caspase 3 activation) in HL-60 cells, without involvement with radical-mediated oxidation mechanism (12).

We investigated whether a total of 27 azulene derivatives (Figure 1) can modify the nitric oxide (NO) production by unstimulated- and lipolysaccharide (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 quenching of NO by radical scavengers, we also investigated whether azulene 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: 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 (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 azulene derivatives. Azulene **[1]**, guaiazulene **[2]**, 2-aminoazulene **[3]**, 2-acetylaminoazulene **[4]**, 2-methoxyazulene **[5]**, 2-chloroazulene **[6]**, diethyl 2-aminoazulene-1,3-dicarboxylate

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

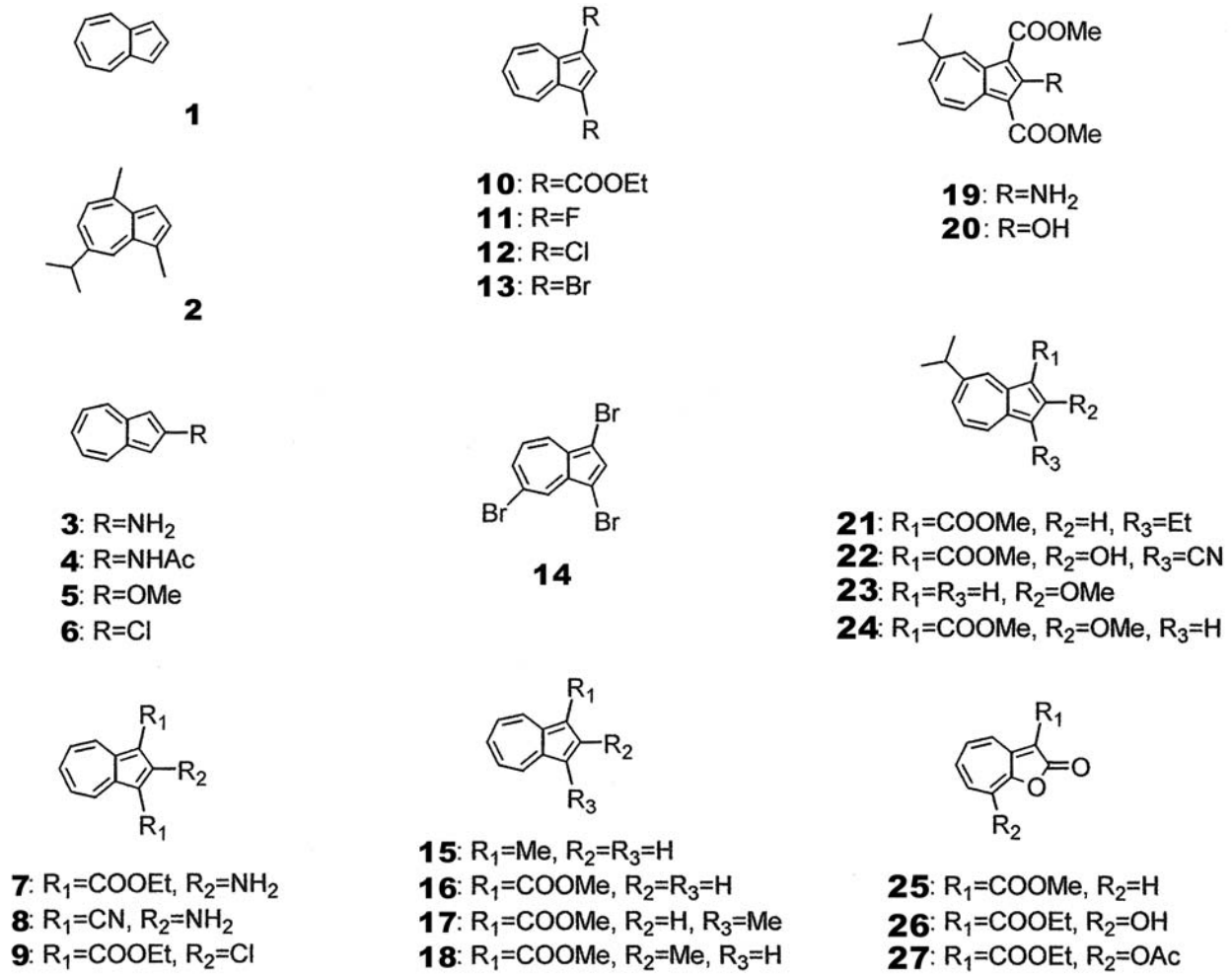

Figure 1. *Structure of azulene derivatives used in this study.*

[7], 2-amino-1,3-dicyanoazulene **[8]**, diethyl 2-chloroazulene-1,3-dicarboxylate **[9]**, diethyl azulene-1,3-dicarboxylate **[10]**, 1,3-difluoroazulene **[11]**, 1,3-dichloroazulene **[12]**, 1,3-dibromoazulene **[13]**, 1,3,5-tribromoazulene **[14]**, 1-methylazulene **[15]**, methyl azulene-1-carboxylate **[16]**, methyl 3-methylazulene-1-carboxylate **[17]**, methyl 2-methylazulene-1-carboxylate **[18]**, dimethyl 2-amino-5-isopropylazulene-1,3-dicarboxylate **[19]**, dimethyl 2-hydroxyazulene-5-isopropyl-1,3-dicarboxylate **[20]**, methyl 3-ethyl-7-isopropylazulene-1-carboxylate **[21]**, methyl 3-cyano-2-hydroxy-7-isopropylazulene-1-carboxylate **[22]**, 5-isopropyl-2-methoxyazulene **[23]**, methyl 7-isopropyl-2-methoxyazulene-1-carboxylate **[24]**, methyl 2*H*-cyclohepta[*b*]furan-2-one-3-carboxylate **[25]**, ethyl 8-hydroxy-2*H*-cyclohepta[*b*]furan-2-one-3-carboxylate **[26]** and ethyl 8-acetoxy-2*H*-cyclohepta[*b*]furan-2-one-3-carboxylate **[27]** were prepared, according to the references cited in a previous paper (12) (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 (13, 14).

Assay for cytotoxic activity. The cytotoxic activity of azulenes was determined by MTT method, and expressed as absorbance at 540 nm of the MTT-stained cells. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (13, 14).

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, using the standard curve of NO_2^-. To eliminate the interaction between the sample and Greiss reagent, we also measured the NO concentration in the culture medium without the cells, and subtracted this value from that obtained 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 (13, 14). 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

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

Compd.	MW	Cytotoxic activity CC_{50} (μM) LPS(+)	Cytotoxic activity CC_{50} (μM) LPS(-)	Inhibition of NO production EC_{50} (μM) LPS(+)	SI
[1]	128.17	>624	>624	535	>1.2
[2]	198.31	29.8	30.8	10.1	3.0
[3]	143.19	>559	>559	60.1	>9.3
[4]	201.23	74.5	89.4	4.97	15.0
[5]	158.20	242	506	31.6	7.7
[6]	162.62	>492	410	365	>1.3
[7]	287.32	15.0	63.7	12.9	1.2
[8]	193.21	133	90.6	21.2	6.3
[9]	306.76	>261	>261	17.0	>15.4
[10]	272.30	>294	>294	>294	><1.0
[11]	164.16	368	353	12.2	30.2
[12]	197.06	152	406	>406	<0.4
[13]	285.97	>280	>280	>280	><1.0
[14]	364.86	122	164	196	0.6
[15]	142.20	>563	>563	>563	><1.0
[16]	186.20	61.2	79.5	7.52	8.1
[17]	200.24	36.0	37.5	19.5	1.8
[18]	200.24	49.9	57.4	51.4	1.0
[19]	301.34	14.9	12.6	16.6	0.9
[20]	302.33	3.64	23.2	4.3	0.8
[21]	256.35	65.1	41.7	22.6	2.9
[22]	269.30	41.2	39.0	28.6	1.4
[23]	200.14	56.5	63.0	23.0	2.5
[24]	258.32	92.9	34.8	12.0	7.7
[25]	204.19	>392	>392	>392	><1.0
[26]	234.21	>342	>342	>342	><1.0
[27]	276.25	>290	>290	14.5	>20

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 the 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, and 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 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. 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 (14). Single strand cDNA obtained by RT reaction was amplified, using the KOD plus (Toyobo Co., Ltd.), using the iNOS- specific primer of (5'-CCCTTCCGAAGTTTC TGGCA GCAGC-3' and 5'-GGCTGTCAGAGCCTCGTGGCTT TGG-3'), β-actin specific primers (5'- GAGGCCCAGAGC AAGAGAGG -3', 5'-TACATGGCTGGGGTGTTGAA -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 azulenes 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) (12). 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. The 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 (14).

Results

Relationship between cytotoxicity and inhibition of LPS-stimulated NO production. We first investigated the cytotoxic activity of 27 azulene- related compounds against Raw 264.7 cells, determined without LPS (Table I). Compound **[19]** (CC_{50}=12.6 μM) showed the highest cytotoxicity, followed by **[20]** (CC_{50}=23.2 μM) > **[2]** (CC_{50}= 30.8 μM) > **[24]** (CC_{50}=34.8 μM) > **[17]** (CC_{50}=37.5 μM) > **[22]** (CC_{50}= 39.0 μM) > **[21]** (CC_{50}=41.7 μM) > **[18]** (CC_{50}= 57.4 μM) > **[23]** (CC_{50}=63.0 μM) > **[7]** (CC_{50}= 63.7 μM). These compounds inhibited NO production by LPS-activated Raw 264.7 cells less efficiently (SI=0.9, 0.8, 3.0, 7.7, 1.8, 1.4, 2.9, 1.0, 2.5 and 1.2, respectively).

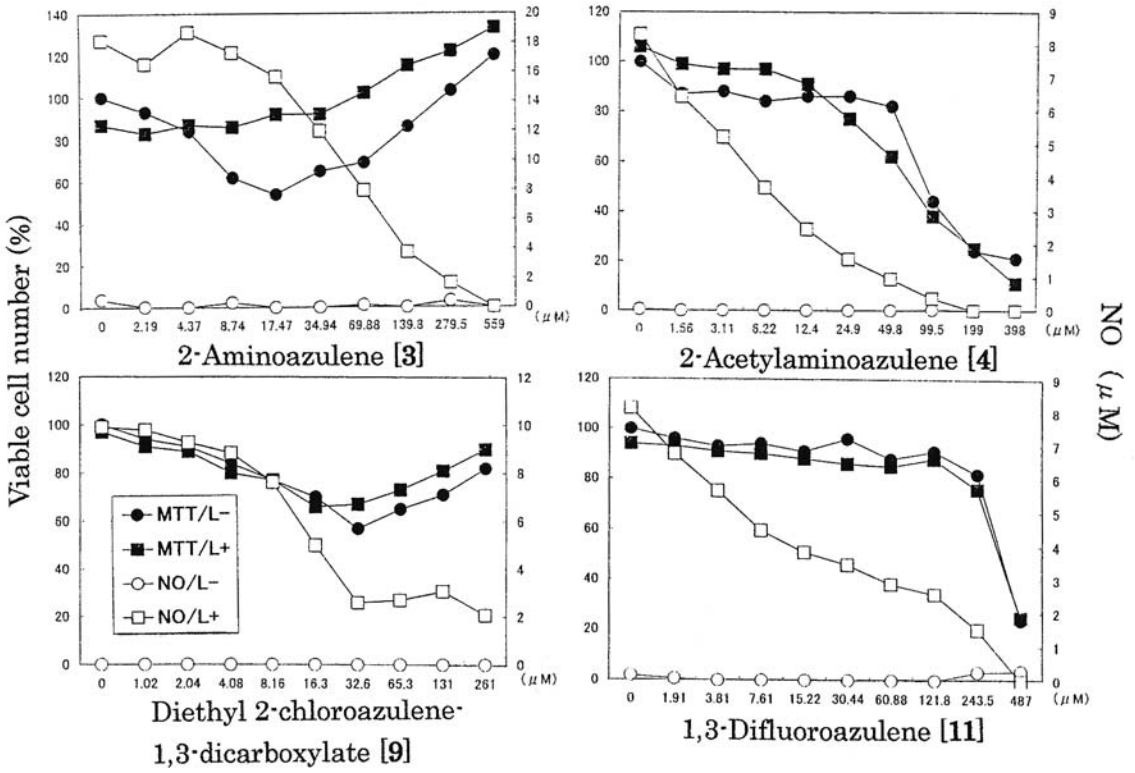

Figure 2. *Azulenes inhibited the NO production by LPS-stimulated Raw 264.7 cells. Near-confluent Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of 2-aminoazulene **[3]**, 2-acetylaminoazulene **[4]**, diethyl 2-chloroazulene-1,3-dicarboxylate **[9]** or 1,3-difluoroazulene **[11]**, 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 assay and Griess reagent, respectively. Each value represents mean from 4 assays.*

The ability of azulenes to inhibit NO generation by activated macrophages was generally increased when their cytotoxic activity declined. Compounds **[4** (Figure 2), **8, 16]** showed slightly lower cytotoxic activity (CC_{50}= 89.4, 90.6 and 79.5 μM, respectively) and more efficiently inhibited NO production (SI=15.0, 6.3 and 8.1, respectively).

Compounds **[3, 9, 11, 27]** were much less cytotoxic (CC_{50}= >559, >261, 353, >290 μM, respectively) and most efficiently inhibited NO production by activated Raw 264.7 cells (SI=>9.3, >15.4, 30.2 and >20, respectively) (Figure 2).

All azulene compounds including compounds **[3, 9, 11** (indicated by open circles in Figure 2)**, 27]** failed to stimulate the Raw 264.7 cells to produce any detectable amount of NO (data not shown).

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

Among five haloazulenes, compounds **[6, 12, 13, 14]** poorly inhibited the NO production by LPS-activated Raw 264.7 cells, as reflected by the lower SI values (>1.3, <0.4, ><1.0, 0.6), whereas 1,3-difluoroazulene **[11]** potently inhibited NO production, as reflected by the much higher value (SI=30.2). All three 2-aminoazulenes **[3, 4, 8]** efficiently inhibited the NO production (SI=>9.3, 15.0 and 6.3, respectively). All five methyl 7-isopropylazulene-1-carboxylate derivatives **[19, 20, 21, 22, 24]** were highly cytotoxic (CC_{50}=12.6, 23.2, 41.7, 39.0 and 34.8 μM, respectively), with lower SI values (0.9, 0.8, 2.9, 1.4 and 7.7, respectively). 2-Methoxyazulenes **[23, 24]** and 2-hydroxyazulenes **[20, 22]** were also highly cytotoxic. Among three cyclohepta[*b*]furan-2-ones **[25-27]**, only acetylated compound **[27]** potently inhibited the NO production (SI= >20). Guaiazulene **[2]** and compounds **[7, 19, 20]** were highly cytotoxic. The cytoxic activity of **[7, 19]** may be due to their stereoprotective effect on the amino group in azulene. Among compounds having 1,3-carboxylic esters **[7, 9, 10, 19, 20]**, only the 2-chloroazulene derivative **[9]** showed higher SI value.

iNOS expression. Since 1,3-difluoroazulene **[11]** most effectively inhibited NO production by LPS-activated Raw 264.7 cells, we next investigated whether this compound would reduce the intracellular concentration of iNOS, using Western blot analysis. Unstimulated Raw 264.7 cells expressed only a background level of iNOS protein, but, upon stimulation with LPS, they began to express detectable

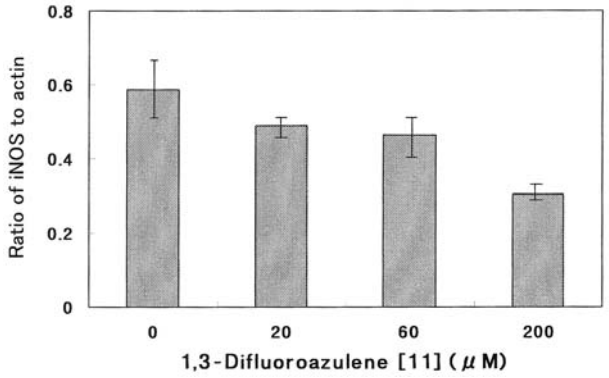

Figure 3. *Effect of 1,3-difluoroazulene **[11]** on the intracellular concentration of iNOS protein in Raw 264.7 cells. Raw 264.7 cells were incubated for 24 hours in the absence or presence of 100 ng/mL LPS with the indicated concentrations of 1,3-difluoroazulene **[11]**. Cell lysate was applied to 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 actin protein. The iNOS protein concentration without LPS was below the detection limit and therefore omitted. Each value represents mean±S.D. from three independent experiments.*

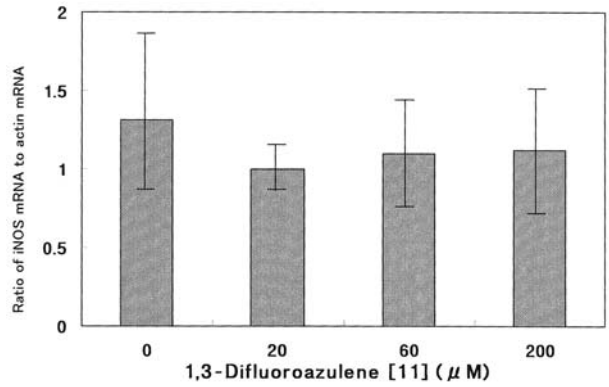

Figure 4. *Effect of 1,3-difluoroazulene **[11]** on LPS-stimulated iNOS mRNA expression. Raw 264.7 cells were incubated for 24 hours in the absence or presence of 100 ng/mL LPS with the indicated concentrations of 1,3-difluoroazulene **[11]**. RNA was then isolated and the RT-PCR product was applied 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.*

amounts of iNOS protein. This LPS-induced iNOS expression was dose-dependently inhibited by simultaneous addition of 1,3-difluoroazulene **[11]** (Figure 3). However, significantly higher amounts (200 μM) of **[11]** were required to achieve the 50% reduction of LPS-stimulated iNOS protein expression (Figure 3), as compared to that required to inhibit the NO production by 50% (12 μM) (Figure 2).

RT-PCR analysis demonstrated that 1,3-difluoroazulene **[11]** did not significantly affect the iNOS mRNA expression (Figure 4).

Table II. *Radical scavenging activity of azulenes.*

Addition of azulene (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]	0.4	1.76	(87)		
	4	1.17	(58)		
	40	0.34	(17)	1.00	(77)
		(108 SOD unit/mg)			
[4]	0.4	1.83	(90)		
	4	1.19	(59)		
	40	0.34	(17)	0.45	(35)
	400	0.10	(5)	(IC_{50}=27 μg/mL)	
		(103 SOD unit/mg)			
[9]	40			1.10	(85)
	400	1.33	(66)		
[11]	40			1.50	(115)
	400	1.90	(94)		

[1)] % of control

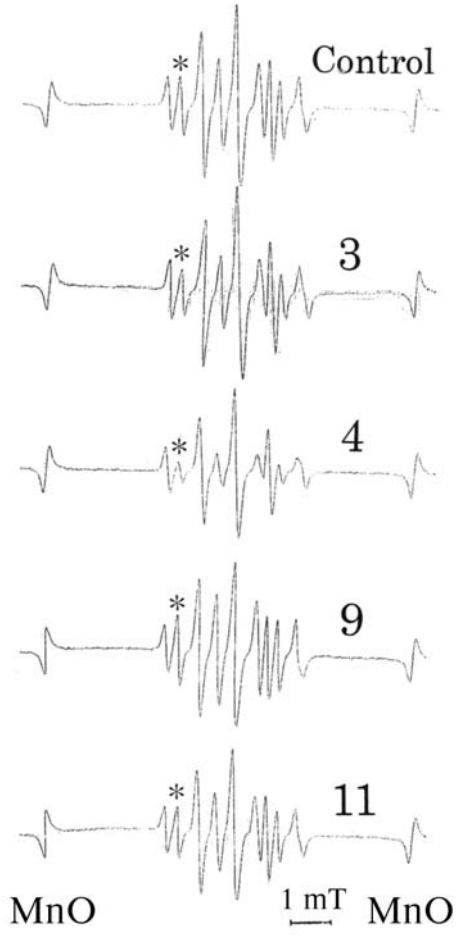

*Figure 5. ESR spectra of radical species generated by NOC-7 and carboxy-PTIO in the absence (control) or presence of 40 μg/mL of **[3]**, **[4]**, **[9]** or **[11]**. *, the first peak of carboxy-PTI; mT, milli-tesla.*

Radical scavenging activity. ESR spectroscopy shows that **[3]**, but not **[4, 9, 11]**, produced radical under alkaline condition (data not shown). Compound **[3]** most potently scavenged O_2^- (generated by HX-XOD reaction), followed by **[4]** and **[9]**, whereas **[11]** was inactive (Table II). However, **[4]** most efficiently scavenged NO (generated from NOC-7 in the presence of c-PTIO, indicated by the symbol in Figure 5), followed by **[3]** and **[9]**, whereas **[11]** was inactive (Table II).

Discussion

The present study demonstrated that 2-aminoazulene **[3]**, diethyl 2-chloroazulene-1,3-dicarboxylate **[9]**, 1,3-difluoroazulene **[11]** and ethyl 8-acetoxy-2*H*-cyclohepta[*b*] furan-2-one-3-carboxylate **[27]** potently inhibited NO production by LPS-activated macrophages, even at their non-cytotoxic concentrations. Among these four compounds, **[11]** was the most potent. The inhibitory activity of **[11]** cannot simply be explained by the changes in iNOS metabolism, since this compound did not significantly affect the iNOS expression (at both protein and mRNA levels), and had no apparent NO scavenging activity. As far as we know, this is the first report of the effect of azulene-related compounds on the macrophage functions. The property of **[11]** is quite different from tropolones, which inhibited the NO production by inhibiting the iNOS expression (15). Furthermore, azulenes were generally less cytotoxic than tropolones (15). It remains to be investigated whether **[11]** directly inactivates the iNOS enzyme, or turns off the signal transduction mechanism in which iNOS is not involved.

We have previously reported that compounds **[4, 9]** induced tumor-specific cytotoxic activity (12). The present study showed that compounds **[4, 9]** efficiently inhibited NO production by activated macrophages. The relationship between the tumor-specificity and inhibition of NO production by these azulenes should be further clarified, since they obviously influence the function of macrophages.

Acknowledgements

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