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

JURI TAKAHASHI[1], TAKASHI SEKINE[1], MASAYUKI NISHISHIRO[1], ATSUHIRO ARAI[1], HIDETSUGU WAKABAYASHI[1], TERUO KURIHARA[1], KEN HASHIMOTO[2], KAZUE SATOH[3], NOBORU MOTOHASHI[4] and HIROSHI SAKAGAMI[2]

[1]*Faculty of Science, Josai University, Sakado, Saitama;*
[2]*Division of Pharmacology, Meikai University School of Dentistry, Sakado, Saitama;*
[3]*Department of Anatomy, School of Medicine, Showa Unversity, Tokyo;*
[4]*Meiji Pharmaceutical University, Kiyose, Tokyo, Japan*

Abstract. *The effect of 20 trihaloacetylazulene derivatives with one halogen atom, on nitric oxide (NO) production by mouse macrophage-like cells Raw 264.7 was investigated. 2-Methoxyazulenes and 2-ethoxyazulenes exhibited comparable cytotoxicity. Trichloroacetylazulenes generally exhibited higher cytotoxicity, as compared with the corresponding trifluoroacetylazulenes. Substitution of chloride, bromide or iodine at the C-3 position further enhanced their cytotoxicity. All of these compounds failed to stimulate the Raw 264.7 cells to produce detectable amounts of NO, but did inhibit NO production by LPS-activated Raw 264.7 cells to different extents. 1-Trichloroacetyl-2-methoxyazulene and 1-trichloroacetyl-2-ethoxyazulene, with less cytotoxic activity, inhibited NO production to the greatest extent, producing the highest selectivity index (SI) of >24.7 and >28.7, respectively. This was accompanied by the efficient inhibition of inducible NO synthase (iNOS) mRNA expression, but not by iNOS protein abundance. Electron spin resonance (ESR) spectroscopy showed that neither of these compounds produced radicals, nor scavenged NO, superoxide anion or diphenyl-2-picrylhydrazyl radicals. The present study suggests that the inhibitory effects of trifluoroacetylazulenes and trichloroacetylazulenes on NO production by activated macrophages might be derived from the perturbation of NO anabolism (inhibition of iNOS mRNA expression and possibly the inactivation of iNOS protein) rather than NO catabolism (NO scavenging).*

Correspondence to: Hidetsugu Wakabayashi, Faculty of Science, Josai University, Sakado, Saitama 350-0295, Japan. Tel: +81 049 2717959, Fax: +81 049 2717985, e-mail: hwaka@josai.ac.jp

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

Azulene (1-4), an isomer of naphthalene, has a dipole moment and a resonance energy having intermediate values between that of benzene and naphthalene, and is considerably more reactive, when compared with the two arenes. Azulene derivatives have been investigated for their synthesis and chemical reactions (5-7). They have been shown to have several biological activities, including antibacterial activity (8), anti-ulcer activity (9), relaxant activity (10), inhibition of thromboxane A_2-induced vasoconstriction and thrombosis (11), acute toxicity and local anesthetic activity (12), and chemotherapeutic activity against mucous membrane diseases (13, 14). However, the effects of azulene derivatives on cellular functions have not yet been investigated in detail.

We initiated the structure–activity study of azulene, tropolone and azulenequinone-related compounds (15-23). Among 107 compounds so far analyzed, 2-acetylamino-azulene, diethyl 2-chloroazulene-1,3-dicarboxylate, methyl 7-isopropyl-2-methoxyazulene-1-carboxylate (16), tropolone derivatives with a phenolic OH group, hinokithiol and its tosylate and methyl ethers (18), 3-(3-guaiazulenyl)-1,5-azulenequinone, 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone (20), 2,3-dimethyl-1-trichloroacetylazulene and 1,3-ditrichloroacetyl-4,6,8-trimethylazulene (22) showed higher tumor-specific cytotoxic activity than other compounds.

1,3-Difluoroazulene (15), 2,4-dibromo-7-methoxytropone (17), 3-morpholino-1,5-azulenequinone, 3,7-dibromo-1,5-azulenequinone (19), 3-trifluoroacetylguaiazulene, 1-trifluoroacetyl-4,6,8-trimethylazulene, 3-methyl-1-trichloroacetylazulene and 3-ethyl-1-trichloroacetylazulene (21) inhibited nitric oxide (NO) production by lipopolysaccharide (LPS)-activated mouse macrophages, with (17, 19, 21) or without (15) the inhibition of inducible NO synthase (iNOS) mRNA and protein expression. However, the extent of inhibition of iNOS expression varied considerably from compound to compound (17, 19, 21).

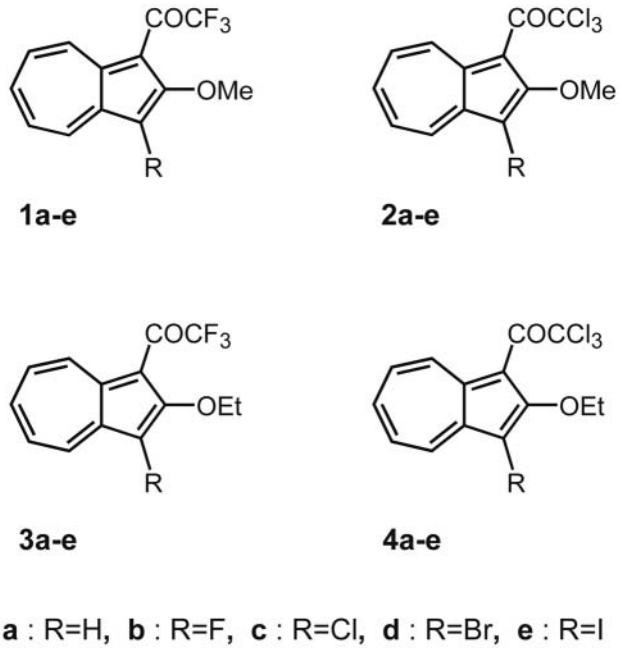

Figure 1. *Structure of ten trifluoroacetylazulenes (**1a-e**, **3a-e**) and ten trichloroacetylazulenes (**2a-e**, **4a-e**).*

We recently reported that 1-trichloroacetyl-3-bromo-2-methoxyazulene and 1-trichloroacetyl-3-chloro-2-ethoxyazulene (**2d** and **4c** respectively) among twenty trihaloacetylazulene derivatives with either one atom of fluorine, chlorine, bromine or iodine (Figure 1) showed the highest tumor specificity (23). These compounds induced apoptotic cell death in HL-60 cells, whereas they induced autophagic cell death characterized by autophagosome formation in human squamous cell carcinoma HSC-4 cells, suggesting the diversity of the type of cell death induced in human tumor cell lines by trihaloacetylazulene derivatives (23). Here, we investigated whether these twenty trihaloacetylazulene derivatives inhibit NO production by unstimulated- and LPS-stimulated mouse macrophage-like Raw 264.7 cells, and if so, whether they affect the NO anabolism (iNOS protein and mRNA expressions, assessed by western blotting and RT-PCR analyses) and NO catabolism (NO scavenging activity, assessed by electron spin resonance (ESR) spectroscopy).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the companies indicated: Dulbecco's modified Eagle's medium (DMEM), phenol red-free DMEM (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); LPS from *Escherichia coli* (Serotype 0111:B4), hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriaminepentaacetic acid (DETAPAC), diphenyl-2-picrylhydrazyl (DPPH), phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem. Co., St. Louis, MO, USA); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-1-3-oxide (carboxy-PTIO; a spin trap agent), 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7; a NO generator) (Dojin, Kumamoto, Japan).

Synthesis of trihaloacetylazulenes. Trihaloacetylazulene derivatives were synthesized, according to methods previously published: 1-trifluoroacetyl-2-methoxyazulene [**1a**] (24-27), 1-trifluoroacetyl-3-fluoro-2-methoxyazulene [**1b**] (25, 28-30), 1-trifluoroacetyl-3-chloro-2-methoxyazulene [**1c**] (25, 31-34), 1-trifluoroacetyl-3-bromo-2-methoxyazulene [**1d**] (25, 31-34), 1-trifluoroacetyl-3-iodo-2-methoxyazulene [**1e**] (25, 31, 32), 1-trichloroacetyl-2-methoxyazulene [**2a**] (24-27), 1-trichloroacetyl-3-fluoro-2-methoxyazulene [**2b**] (25, 28-30), 1-trichloroacetyl-3-chloro-2-methoxyazulene [**2c**] (25, 31-34), 1-trichloroacetyl-3-bromo-2-methoxyazulene [**2d**] (25, 31-34), 1-trichloroacetyl-3-iodo-2-methoxyazulene [**2e**] (25, 31, 32), 1-trifluoroacetyl-2-ethoxyazulene [**3a**] (24-27, 34), 1-trifluoroacetyl-2-ethoxy-3-fluoroazulene [**3b**] (25, 28-30), 1-trifluoroacetyl-3-chloro-2-ethoxyazulene [**3c**] (25, 31-34), 1-trifluoroacetyl-3-bromo-2-ethoxyazulene [**3d**] (25, 31-34), 1-trifluoroacetyl-2-ethoxy-3-iodoazulene [**3e**] (25, 31, 32), 1-trichloroacetyl-2-ethoxyazulene [**4a**] (24-27, 34), 1-trichloroacetyl-2-ethoxy-3-fluoroazulene [**4b**] (25, 28-30), 1-trichloroacetyl-3-chloro-2-ethoxyazulene [**4c**] (25, 31-34), 1-trichloroacetyl-3-bromo-2-ethoxyazulene [**4d**] (25, 31-34), 1-trichloroacetyl-2-ethoxy-3-iodoazulene [**4e**] (25, 31, 32) (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 (15, 17, 19, 21).

Assay for cytotoxic activity. Cytotoxic activity of azulenes was determined by the 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 (15, 17, 19, 21).

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 in the presence or absence of 100 ng/mL LPS, and the NO production by Raw 264.7 cells was quantified by Greiss reagent, using a standard curve of NO_2^-. To eliminate the interaction between sample and Greiss reagent, the NO concentration in the culture medium without cells was measured and was subtracted from the value obtained using the cells. The concentration that inhibited the LPS-stimulated NO production by 50% (50% inhibitory concentration: IC_{50}) was determined from the dose-response curve (15, 17, 19, 21). The efficacy of inhibition of NO production was evaluated by the selectivity index (SI), which was calculated using the following equation: $SI = CC_{50} / IC_{50}$.

Western blotting. The pelleted cells 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 iced 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 insoluble materials and the supernatant was collected. The protein concentrations of supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). An equal amount of the protein from cell lysates (10 μg) was mixed with 2x 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-mercaptoethanol), boiled for 10 minutes, applied to the SDS-8% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skimmed milk in phosphate-buffered saline (PBS(-)) plus 0.05% Tween 20 for 90 minutes and incubated for 90 minutes at room temperature with anti-iNOS antibody (1:1,000, Santa Cruz Biotechnology, Delaware, CA, USA), or anti-actin antibody (1:2000-4000, Sigma), and then incubated with horseradish peroxidase-conjugated anti-mouse or goat IgG for 90 minutes at room temperature. Immunoblots were developed with a Western Lightning™ Chemiluminescence Reagent *Plus* (Perkin Elmer Life Sciences, Boston, MA, USA) and analyzed on a Macintosh (Power Macintosh 7600/120) computer using the public domain NIH Image program (National Technical Information Service, Springfield, Virginia, USA, part number PB95-500195GEI).

Assay for mRNA expression. Total RNA was isolated by PURESCRIPT RNA Isolation kit (Gentra systems, Minneapolis, MN, USA) protocol. Raw 264.7 cells were lysed in 300 μL cell lysis solution, then 100 μL Protein-DNA precipitation solution were added. Cell lysates were centrifuged at 15,000 xg for 3 minutes. To the supernatant, 300 μL isopropanol was added. After centrifugation at 15,000 xg for 3 minutes, the pellet was washed in 300 mL 75% ethanol. After centrifugation at 15,000 xg 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, Japan) with oligo $(dT)_{20}$ primer (15, 17, 19, 21). Single-strand cDNA obtained by the RT reaction was amplified using the KOD plus (Toyobo Co.) with iNOS specific primers (5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3') and β-actin specific primers (5'-GAGGCCCAGAGCAAGAGAGG-3' and 5'-TACAT GGCTGGGGTGTTGAA-3'), according to the manufacturer's protocol. The RT-PCR products were applied to 2% agarose gel and the ethidium bromide-stained gel was then photographed under UV light and analyzed as described above.

Radical scavenging activity. The radical intensity of trihaloacetylazulenes was determined at 25˚C, using ESR spectroscopy (JEOL JES REIX Tokyo, Japan, X-band, 100 kHz modulation frequency) (15, 17, 19, 21). Samples (24 mM dissolved in DMSO) were mixed with an equal volume of 0.2 M KOH (pH 13.5). The radical intensity was measured 40 seconds thereafter (center field, 336.0±5.0 mT; microwave power, 16 mW; modulation amplitude, 0.1 mT; gain, 630 mW; time constant, 0.03 s; scanning time, 2 minutes).

The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 60 μM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO (microwave power and gain were changed to 5 mW and 400 mW, 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. Samples were added 3 minutes after mixing. The NO radical intensity was defined as the ratio of signal intensity of the first peak of carboxy-PTI to that of MnO (15, 17, 19, 21) and expressed as the ratio to the height of MnO, an external marker.

For determination of superoxide anion (O_2^-) produced by HX and XOD reactions (total volume: 200 μL) [50 μL of 2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB)], 20 μL of 0.5 mM DETAPAC, 30 μL of 8% DMPO, 40 μL of sample in DMSO, 30 μL of H_2O or SOD, 30 μL of XOD (0.5 U/mL in PB) were used and the gain was changed to 400. The O_2^- scavenging activity was expressed as the ratio of the intensity of the first peak of DMPO-OOH to that of MnO.

For determination of DPPH radical scavenging activity, samples were mixed with the reaction mixture (total volume: 120 μL) (60 μL of 100 μM DPPH, 60 μL of sample in DMSO), and 1 minute thereafter subjected to ESR spectroscopy (35). Microwave power and gain were changed to 8 mW and 500 mW, respectively.

Statistics. Student's *t*-test was used to assess the statistical significance between the two groups.

Table I. *Cytotoxicity of trihaloacetylazulenes and their effect on NO production by LPS-stimulated Raw 264.7 cells.*

Compound	MW.	CC_{50} (μM)		IC_{50} (μM)	SI
		(–)LPS	(+)LPS	(+)LPS	
1a	254.2	>500.0	>500.0	219.1	>2.6
1b	272.2	>500.0	>500.0	238.1	>2.4
1c	288.7	>117.0	>109.9	32.3	>9.7
1d	333.1	178.3	124.0	52.9	2.4
1e	380.1	174.2	86.5	28.2	8.4
2a	303.6	>393.6	>400.6	22.7	>24.7
2b	321.6	248.7	157.4	36.8	6.4
2c	338.0	112.7	96.5	30.9	5.7
2d	382.5	46.5	74.2	20.8	5.4
2e	429.5	130.9	124.7	17.6	8.6
3a	268.3	>500.0	>500.0	247.7	>2.5
3b	286.2	>500.0	>341.6	199.2	>1.4
3c	302.7	169.7	119.5	61.6	1.9
3d	347.1	>166.7	>152.8	43.0	>4.2
3e	394.1	>166.7	>125.0	74.3	>1.7
4a	317.6	358.8	>500.0	21.2	>28.7
4b	335.6	>365.6	>258.0	20.6	>12.5
4c	352.0	106.7	66.1	16.5	4.9
4d	396.5	66.1	63.8	13.3	4.7
4e	443.5	>98.1	77.7	10.6	7.6

SI: Selectivity index (CC_{50}/IC_{50}). Each value represents the mean from 3 ~ 6 independent experiments. Raw 264.7 cells were incubated for 24 hours with various concentrations of each compound (2-500 μM) in the presence (+) or absence (–) of 100 ng/mL LSP. The CC_{50} and IC_{50} values were determined by the dose–response curve.

Results

Structure and activity relationship. There was no apparent difference in the cytotoxic activity between 2-methoxyazulenes (**1a-e**, **2a-e**) and 2-ethoxyazulenes (**3a-e**, **4a-e**) (Table I).

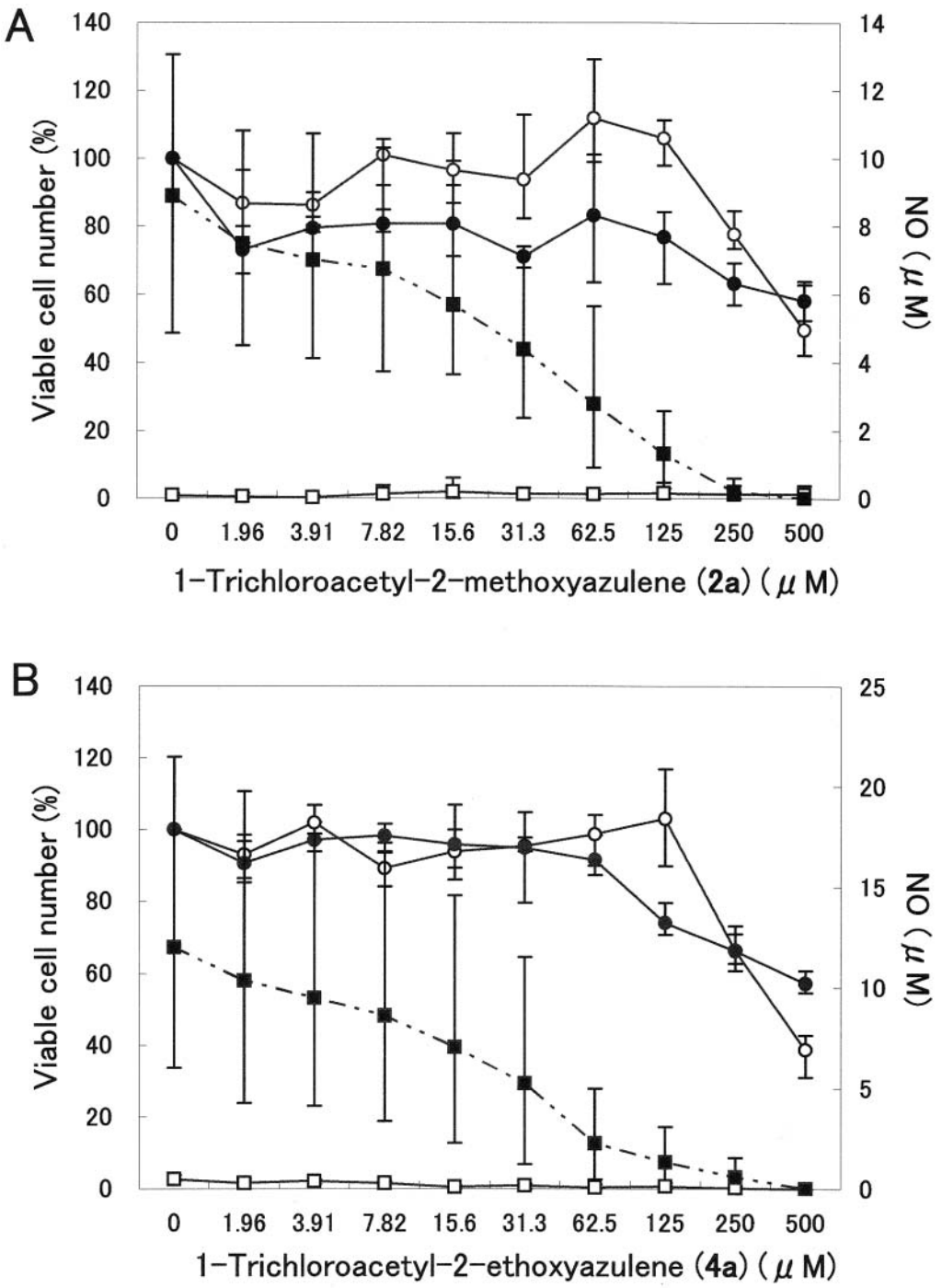

Figure 2. *Effect of trihaloacetylazulenes on the NO production by LPS-stimulated Raw 264.7 cells. Near-confluent Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of (A) 1-trichloroacetyl-2-methoxyazulene (**2a**) or (B) 1-trichloroacetyl-2-ethoxyazulene (**4a**) in the absence (open symbols) or presence (closed symbols) of 100 ng/mL LPS in phenol red-free DMEM supplemented with 10% FBS. The viable cell number (expressed as % of control) (○, ●) and extracellular concentration of NO (□, ■) were determined by MTT assay and Griess reagent, respectively. Each value represents the mean from 3 independent experiments.*

Trichloroacetylazulenes (**2a-e**, **4a-e**) generally exhibited higher cytotoxicity as compared with their corresponding trifluoroacetylazulenes (**1a-e**, **3a-e**). In particular, trifluoroacetylazulenes (**1a**, **1b**, **3a**, **3b**) showed low cytotoxic activity whereas 1-trichloroacetyl-3-bromo-2-methoxyazulene (**2d**) and 1-trichloroacetyl-3-bromo-2-ethoxyazulene (**4d**) showed the

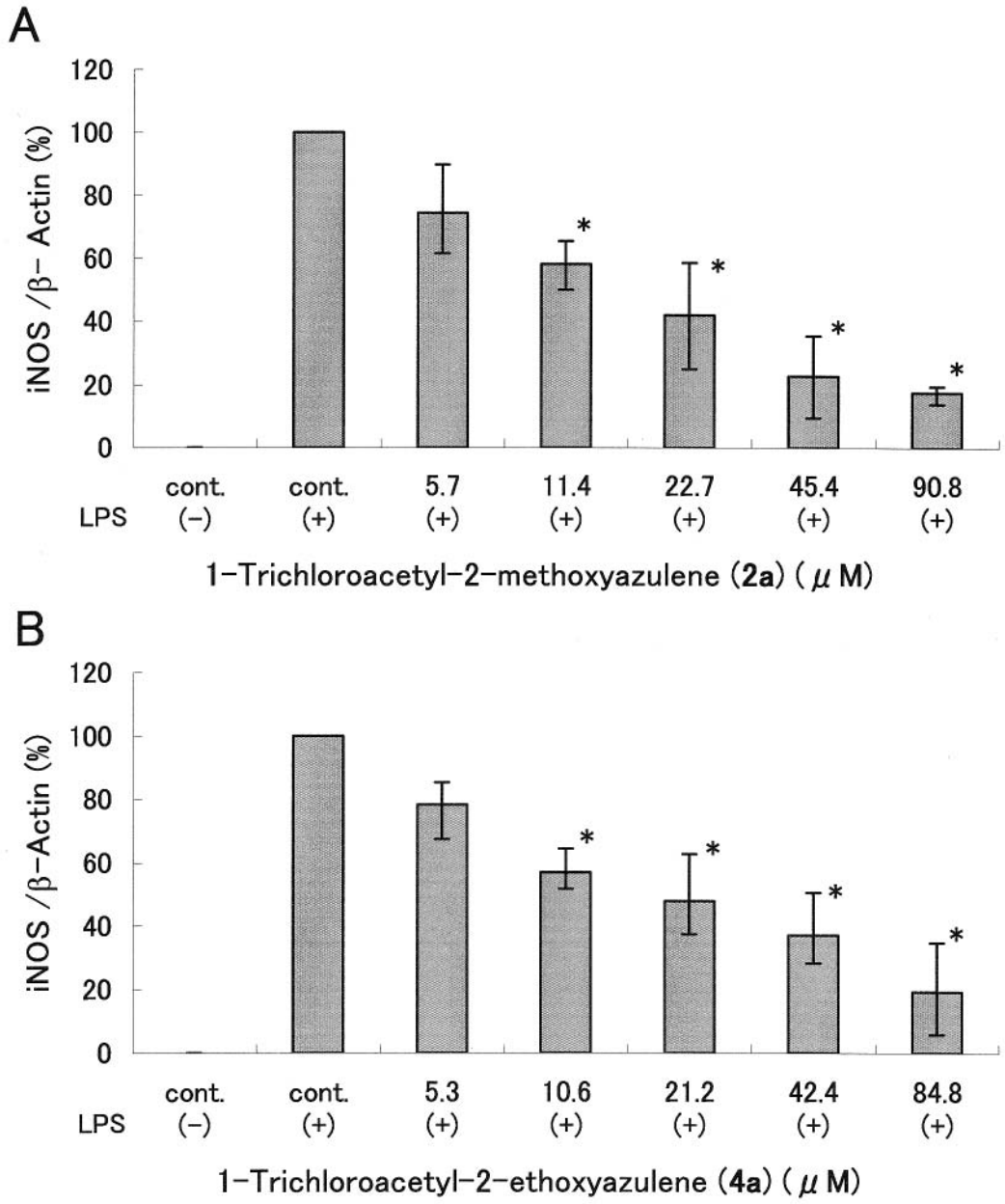

Figure 3. *Effect of trihaloacetylazulenes on LPS-stimulated iNOS mRNA expression. Raw 264.7 cells were incubated for 24 hours with (A) 1-trichloroacetyl-2-methoxyazulene (**2a**) or (B) 1-trichloroacetyl-2-ethoxyazulene (**4a**) in the presence of 100 ng/mL LPS. RNA was then isolated, and the RT-PCR product was applied to agarose gel electrophoresis and quantified by NIH image. The expression of iNOS mRNA was expressed as its ratio to that of β-actin mRNA. Each value represents the mean from 3 independent experiments; (+) and (–) represent the presence or absence of 100 ng/mL LPS. *p<0.05.*

highest cytotoxicity (Table I). Substitution of chloride (**1c**, **2c**, **3c**, **4c**), bromide (**1d**, **2d**, **3d**, **4d**) or iodine (**1e**, **2e**, **3e**, **4e**) at the C-3 position further enhanced cytotoxicity (Table I).

All of these compounds failed to stimulate Raw 264.7 cells to produce detectable amounts of NO, but did inhibit NO production by LPS-activated Raw 264.7 cells to various extents (Table I). 1-Trichloroacetyl-2-methoxyazulene (**2a**) and 1-trichloroacetyl-2-ethoxyazulene (**4a**), with low cytotoxic activity (CC_{50} >400 and >500, respectively), inhibited NO production to the greatest extent (IC_{50}=22.7 and 21.2 μM, respectively) (Figure 2), producing the highest selectivity index (SI) of >24.7 and >28.7, respectively (Table I).

Effect on iNOS expression. We next investigated whether the inhibition of NO production by (**2a**) and (**4a**) was due to the reduced expression of iNOS mRNA expression. RT-PCR

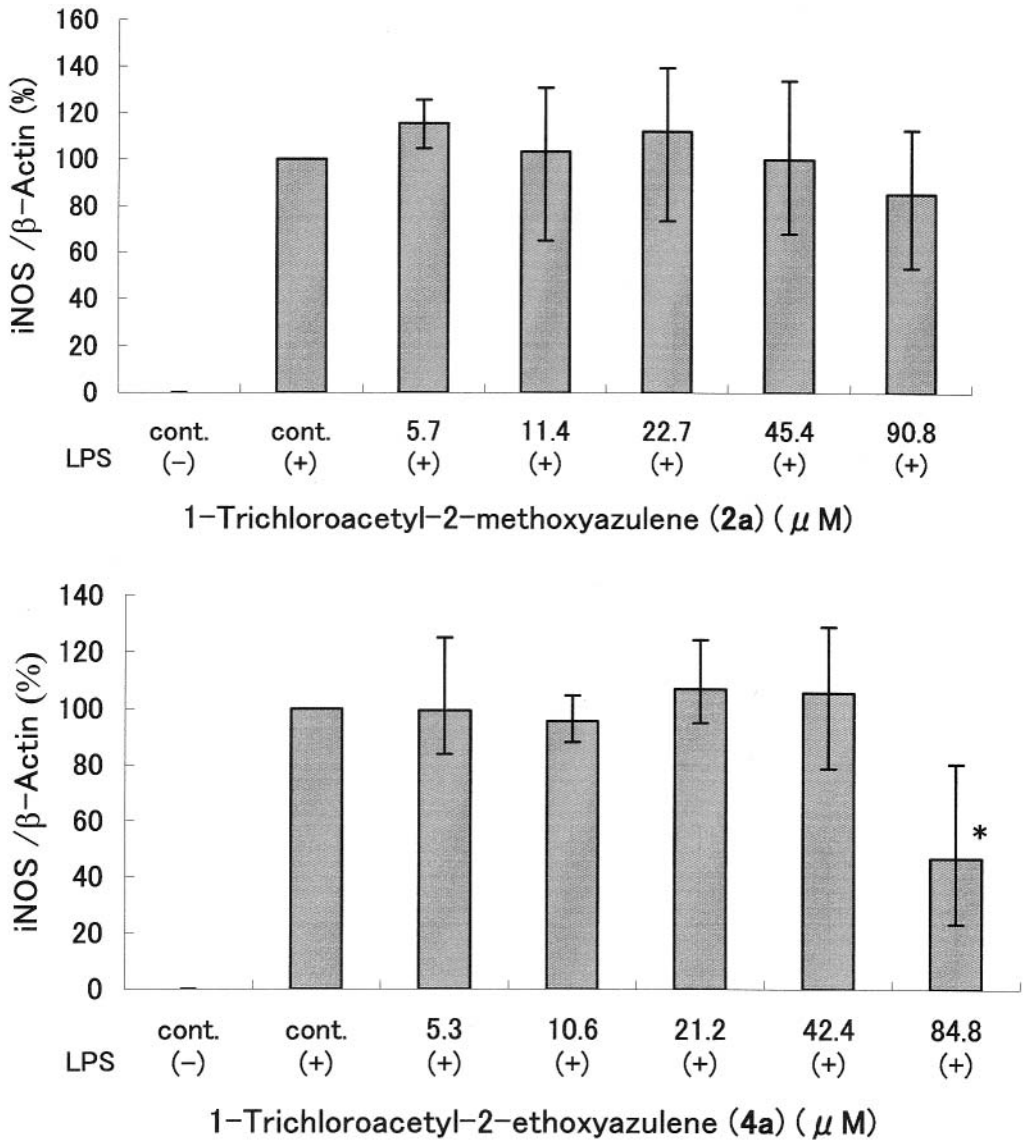

Figure 4. *Effect of trihaloacetylazulenes on the intracellular concentration of iNOS proteins in Raw 264.7 cells. Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of 1-trichloroacetyl-2-methoxyazulene (**2a**) or 1-trichloroacetyl-2-ethoxyazulene (**4a**) in the presence of 100 ng/mL LPS. Cell lysate was applied to SDS-PAGE and iNOS protein was quantified by western blot analysis, followed by NIH imaging. The intracellular iNOS protein was expressed as its ratio to that of actin protein. The iNOS protein concentration without LPS was below the detection limit. Each value represents the mean from 3 independent experiments; (+) and (–) represent the presence or absence of 100 ng/mL LPS. *p<0.05.*

analysis showed that this was in fact the case. Compounds **2a** and **4a** inhibited iNOS mRNA expression (IC_{50}=17.0 and 18.8 μM, respectively), as efficiently as they did NO production (Figure 3). However, western blot analysis showed that these compounds reduced the intracellular concentration of iNOS protein only at higher concentrations (IC_{50}≥90.8 and 82.4 μM, respectively) (Figure 4).

Radical production and scavenging activity. ESR spectroscopy showed that compounds (**2a** and **4a**) did not produce any detectable amounts of radical at pH 13.5 (Table II). These compounds slightly scavenge O_2^- at higher concentration (>1.2 mM), whereas they did not affect the radical intensity of NO or DPPH at a wide range of concentrations (0.2-12 mM) (Table II).

Discussion

The present study demonstrates for the first time that ten trichloroacetylazulenes (**2a-e**, **4a-e**) generally showed higher

Table II. *Radical production and scavenging activity of trihaloacetylazulenes.*

Compound	mM	Radical intensity at pH 13.5	Radical scavenging activity against		
			NO	O_2^-	DPPH
Control	0	<0.08	1.76 (100)	1.58 (100)	1.17 (100)
2a	0.2		2.17 (123)		
	0.4		1.69 (96)		
	0.6				1.80 (154)
	1.2			1.24 (78)	1.42 (121)
	2		2.15 (122)		
	2.4			1.11 (70)	
	4		1.84 (105)		
	6				1.72 (147)
	12	<0.08			1.21 (103)
4a	0.2		1.89 (107)		
	0.4		1.92 (109)		
	0.6				1.76 (150)
	1.2			1.03 (65)	1.83 (156)
	2		1.76 (100)		
	2.4			0.73 (46)	
	4		1.68 (95)		
	6				1.49 (127)
	12	<0.08			1.03 (88)

The number in parentheses represents the value obtained as a percentage of that of the control.

cytotoxicity against Raw264.7 cells as compared with their corresponding trifluoroacetylazulenes (**1a-e**, **3a-e**), confirming the result from our previous study (21). We recently reported that the former compounds showed higher cytotoxicity against human tumor cell lines than did the latter (23). Among these twenty compounds, compounds **2a** and **4a** most efficiently inhibited NO production by LPS-activated Raw 264.7 cells, with the highest selectivity index (Table I). The NO concentration in the medium is influenced by both the intracellular concentration and activity of iNOS protein and the quenching of NO radicals. NO radicals interact with superoxide anions (O_2^-) (36). We found that compounds **2a** and **4a** did not scavenge the NO radical (produced from NOC-7, an NO donor), even at 4 mM, a two hundred-fold higher concentration that induced 50% reduction in the NO production by LPS-stimulated Raw 264.7 cells. Furthermore, none of these compounds increased O_2^- but rather scavenged it, which is known to interfere with the NO molecule. These data suggest that inhibition of NO production by compounds **2a** and **4a** is not due to the NO scavenging activity. We also found that compounds **2a** and **4a** inhibited iNOS mRNA expression at the same concentration at which it inhibited NO production. However, these compounds reduced the intracellular concentration of iNOS protein to a lesser extent. These data suggest that these compounds may inactivate the iNOS protein.

Our recent studies demonstrated that trihaloacetylazulene derivatives show diverse biological activity. Although 1-trichloroacetyl-3-bromo-2-methoxyazulene (**2d**) and 1-trichloroacetyl-3-chloro-2-ethoxyazulene (**4c**) showed the highest cytotoxicity and tumor specificity (TS of >3.5 and >2.5, respectively) (23), they inhibited NO production by activated macrophages very weakly (SI=5.4 and 4.9, respectively) (Table I). Although compounds (**2a** and **4a**) inhibited NO production by activated macrophages very potently (SI of >24.7 and >28.7, respectively) (Table I), they showed very weak tumor-specific cytotoxicity (TS of >1.7 and >2.0, respectively) (23). These data indicate that these biological activities of trihaloacetylazulenes do not overlap with each other. Further studies are required to test the possible anti-inflammatory action of trihaloacetylazulenes.

Acknowledgements

We thank Prof. Dr. Masafumi Yasunami (Faculty of Engineering, Nihon University) for his very generous gift of a large amount of cyclohepta[*b*]furan-2-ones.

References

1 Nozoe T and Asao T: *In*: Azulenes and heptafulvenes. Dai-Yuki Kagaku, Kotake M (ed.). Tokyo, Asakura-Shoten, Vol. 13, pp. 439-534, 1960 [in Japanese].
2 Nozoe T, Breslow R, Hafner K, Ito S and Murata I: Topics in nonbenzenoid aromatic chemistry. Tokyo, Hirokawa Publ. Co., Vol I, pp. 1-295, 1973.
3 Nozoe T, Breslow R, Hafner K, Ito S and Murata I: Topics in nonbenzenoid aromatic chemistry. Tokyo, Hirokawa Publ. Co., Vol II, pp. 1-379, 1977.
4 Zeller KP: Methoden der organischen chemie. New York, Houben-Weyl, Band V/2c, pp. 127-418, 1985.
5 Ziegler K and Hafner K: Eine rationelle synthese des azulens. Angew Chem *67*: 301, 1955.
6 Hafner K: Zur kenntnis der azulene I Eine neue azulen-synthese. Liebigs Ann Chem *606*: 79-87, 1957.
7 Hafner K: Uber substituierte azulene. Angew Chem *69*: 393, 1957.
8 Frigola J, Pares J, Corbera J, Vano D, Merce R, Torrens A, Mas J and Valenti E: 7-Azetidinylquinolones as antibacterial agents. Synthesis and structure-activity relationship. J Med Chem *36*: 801-810, 1993.
9 Wakabayashi S, Sekiguchi H, Kosakai K, Mochizuki S, Kashima M and Tomiyama A: General pharmacological properties of an anti-ulcer drug, azuletil sodium (KT1-21). Folia Pharmacol Japon *96*: 185-204, 1990 [in Japanese].
10 Tanaka M, Yamaki F, Saitoh M, Nakazawa T, Tanaka H, Noguchi K, Hashimoto K and Shigenobu K: Relaxant action of azulene-1-carboxamidine derivative N^1,N^1-dimethyl-N^2-(2-pyridylmethyl)-5-isopropyl-3,8-dimethylazulene-1-carboxamidine (HNS-32) in pig coronary artery. Pharm Pharmacol Commun *6*: 397-404, 2002.

11 Tomiyama T, Yokota M, Wakabayashi S, Kosakai K and Yanagisawa T: Design, synthesis, and pharmacology of 3-substituted sodium azulene-1-sulfontes and related compounds: non-prostanoid thromboxane A2 receptor antagonists. J Med Chem *36*: 791-800, 1993.
12 Doukas PH, Speaker TJ and Thompson RS: Azulene analogs of pharmacological agents III. Acute toxicity and local anesthetic activity of azulylamides and azulenecarboxamides. J Pharm Sci *64*: 158-161, 1975.
13 Hamajima R, Fujimoto T and Okuda H: Studies on azulene derivatives (II). Absorption, distribution, metabolism and excretion of sodium guaiazulene-3-sulfonate. Applied Pharmacol *12*: 501-510, 1976 [in Japanese].
14 Fujimoto T, Yoshimura A, Nishimura H and Okuda H: Studies on azulene derivatives (III). Pharmacokinetic studies of sodium guaiazulene-3-sulfonate (35S-GAS) through the rat mucosa. Applied Pharmacol *12*: 511-520, 1976 [in Japanese].
15 Hashiba K, Yokoyama K, Wakabayashi H, Hashimoto K, Satoh K, Kurihara T, Motohashi N and Sakagami H: Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by azulenes. Anticancer Res *24*: 3939-3944, 2004.
16 Wakabayashi H, Hashiba K, Yokoyama K, Hashimoto K, Kikuchi H, Nishikawa H, Kurihara T, Satoh K, Shioda S, Saito S, Kusano S, Nakashima H, Motohashi N and Sakagami H: Cytotoxic activity of azulenes against human oral tumor cell lines. Anticancer Res *23*: 4747-4756, 2003.
17 Yokoyama K, Hashiba K, Wakabayashi H, Hashimoto K, Satoh K, Kurihara T, Motohashi N and Sakagami H: Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by tropolones. Anticancer Res *24*: 3917-3922, 2004.
18 Wakabayashi H, Yokoyama K, Hashiba K, Hashimoto K, Kikuchi H, Nishikawa H, Kurihara T, Satoh K, Shioda S, Saito S, Kusano S, Nakashima H, Motohashi N and Sakagami H: Cytotoxic activity of tropolones against human oral tumor cell lines. Anticancer Res *23*: 4757-4764, 2003.
19 Nishishiro M, Arikawa S, Wakabayashi H, Hashimoto K, Satoh K, Yokoyama K, Unten S, Kakuta H, Kurihara T, Motohashi N and Sakagami H: Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by azulenequinones. Anticancer Res *25*: 4157-4164, 2005.
20 Wakabayashi H, Nishishiro M, Arikawa S, Hashimoto K, Kikuchi H, Nishikawa H, Kurihara T, Terakubo S, Shoji Y, Nakashima H, Motohashi N and Sakagami H: Cytotoxic activity of azulenequinones against human oral tumor cell lines. Anticancer Res *25*: 350-312, 2005.
21 Ohshima N, Akatsu Y, Yamagishi Y, Nishishiro M, Wakabayashi H, Kurihara T, Satoh K, Motohashi N and Sakagami H: Inhibition of NO production in LPS-stimulated mouse macrophage-like cells by trihaloacetylazulenes. Anticancer Res *26*: 2921-2928, 2006.
22 Akatsu Y, Ohshima N, Yamagishi Y, Nishishiro M, Wakabayashi H, Kurihara T, Kikuchi H, Katayama T, Motohashi N, Shoji Y, Nakashima H and Sakagami H: Apoptosis-inducing activity of trihaloacetylazulenes against human oral tumor cell lines. Anticancer Res *26*: 1917-1924, 2006.
23 Sekine T, Takahashi J, Nishishiro M, Arai A, Wakabayashi H, Kurihara T, Kobayashi M, Hashimoto K, Kikuchi H, Katayama T, Kanda Y, Kunii S, Motohashi N and Sakagami H: Tumor-specificity and type of cell death induced by trihaloacetylazulenes in human tumor cell lines. Anticancer Res *27*: 133-144, 2007.
24 Nozoe T, Wakabayashi H, Shindo K, Ishikawa S, Wu C-P and Yang P-W: A convenient, one-pot azulene synthesis from cyclohepta[*b*]furan-2-ones and vinyl ether and its analogues. (III) Orthoesters as reagent. Heterocycles *32*: 213-220, 1991.
25 Anderson AG, Anderson AR and Replogle LL: Reaction of azulenes with trifluoroacetic anhydride. Proc Chem Soc, pp. 72, 1960.
26 Anderson AG and Anderson AR: The reaction of azulenes with trifluoro- and trichloroacetic anhydride. J Org Chem *27*: 3578-3581, 1962.
27 McDonald RN, Reitz RR and Richmond JM: Nonbenzenoid aromatic systems. XIII. Certain substituent group effects on the pKa of 1-azulenecarboxylic acid. J Org Chem *41*: 1822-1828, 1976.
28 Ueno T, Toda H, Yasunami M and Yoshifuji M: Synthesis of 1-fluoro- and 1,3-difluoroazulenes. Chem Lett pp. 169-170, 1995.
29 Ueno T, Toda H, Yasunami M and Yoshifuji M: Synthesis and properties of fluoroazulenes. II. Electrophilic fluorination of azulenes with *N*-fluoro reagents. Bull Chem Soc Jpn *69*: 1645-1656, 1996.
30 Rajeev MS and Robert LS: Synthesis of fluorinated azulenes. J Fluorine Chem *89*: 173-175, 1998.
31 Hafner K, Patzelt H and Kaiser H: Azulenes. XI. Nucleophilic substitution of halogenated azulenes. Ann *656*: 24-33, 1962.
32 Anderson AG, Nelson JA and Tazuma JJ: Azulene. III. Electrophilic substitution. J Am Chem Soc *75*: 4980-4989, 1953.
33 Nozoe T, Shindo K, Wakabayashi H, Kurihara T, Ishikawa S. Unusual reactions of guaiazulene with *N*-bromosuccinimide and synthesis of variously functionalized Azulenes using these reactions. Collect Czech Chem Commun *56*: 991-1010, 1991.
34 Dehmlow EV and Balschukat D: Azulenes by vacuum pyrolysis of halotricyclo[7.1.0.0^{4,6}]decadienes. Chem Ber *118*: 3805-3816, 1985.
35 Satoh K, Ida Y, Tomioka M, Shiohara K, Sakagami H and Fujisawa S: Interaction between antioxidants and hydroquinone/bisphenol. Anticancer Res *19*: 5457-5462, 1999.
36 Sawa T, Akuta T, Arimoto H and Akaike T: Unique signal pathway *via* nitration reaction induced by NO. "Saibo-Kogaku" *25*: 132-136, 2006 [in Japanese].

Received March 23, 2007
Revised June 8, 2007
Accepted June 20, 2007