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

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Abstract. *The effects of 26 trihaloacetylazulene derivatives on nitric oxide (NO) production by the mouse macrophage-like Raw 264.7 cells was investigated. The trichloroacetylazulenes* ***[1b-13b]*** *generally showed higher cytotoxicity as compared with the corresponding trifluoroacetylazulenes* ***[1a-13a]****. All the compounds inhibited NO production by lipopolysaccharide (LPS)-activated Raw 264.7 cells to various extents. 3-Trifluoroacetylguaiazulene* ***[8a]****, 1-trifluoroacetyl-4,6,8-trimethylazulene* ***[10a]****, 3-methyl-1-trichloroacetylazulene* ***[2b]*** *and 3-ethyl-1-trichloroacetylazulene* ***[3b]*** *showed lower cytotoxic activity and most effectively inhibited NO production. Western blot analysis revealed that compounds* ***[8a, 10a]*** *dose-dependently reduced the intracellular concentration of inducible NO synthase (iNOS), whereas compounds* ***[2b, 3b]*** *only marginally affected the iNOS protein expression. RT-PCR analysis showed that compounds* ***[8a, 2b]*** *reduced the iNOS mRNA expression by approximately 50%. These compounds affected cyclooxygenase-2 protein and mRNA expression, depending on the concentrations. ESR spectroscopy revealed that compounds* ***[8a, 10a, 2b, 3b]*** *neither produced radical, nor scavenged NO, superoxide anion or diphenyl-2-picrylhydrazyl radicals. The present study showed the inhibitory effects of trifluoroacetylazulenes and trichloroacetylazulenes on NO production by activated macrophages.*

Azulene (1-4), an isomer of naphthalene, has a dipole moment and a resonance energy with intermediate values between those of benzene and naphthalene, and is considerably more reactive when compared with two arenes. Azulene derivatives have been investigated for their synthesis and chemical reactions (5-7). These derivatives have shown 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). Since the effects of azulene derivatives on cellular function have not yet been investigated in detail, the structure-activity study of azulene, tropolone and azulenequinone-related compounds was initiated (15-20). Among 81 compounds analyzed to date, 2-acetylaminoazulene, diethyl 2-chloroazulene-1,3-dicarboxylate, methyl 7-isopropyl-2-methoxyazulene-1-carboxylate (16), tropolone derivatives with the phenolic OH group, hinokithiol, its tosylate and methyl ethers (18), 3-(3-guaiazulenyl)-1,5-azulenequinone and 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone (20) showed relatively higher tumor-specific cytotoxic activity. 1,3-Difluoroazulene (15), 2,4-dibromo-7-methoxytropone (17), 3-morpholino-1,5-azulenequinone and 3,7-dibromo-1,5-azulenequinone (19) inhibited nitric oxide (NO) production by lipopolysaccharide (LPS)-activated mouse macrophages, without (15) or with (17, 19) the inhibition of inducible NO synthase (iNOS) mRNA and protein expression.

We recently found that trichloroacetylazulenes **[1b-13b]** (Figure 1) generally showed higher cytotoxicity and higher tumor-specific cytotoxicity as compared with the corresponding trifluoroacetylazulenes **[1a-13a]** (Figure 1). Especially, 2,3-dimethyl-1-trichloroacetylazulene **[5b]** and 1,3-ditrichloroacetyl-4,6,8-trimethylazulene **[11b]** showed the highest cytotoxicity and tumor-specificity (TS >35.6 and >44.1, respectively) (21). Whether these 26 trihaloacetylazulene derivatives, like azulenes (15), tropolones (17) and azulenequinones (19), inhibit NO production by unstimulated- and LPS-stimulated mouse macrophage-like Raw 264.7 cells

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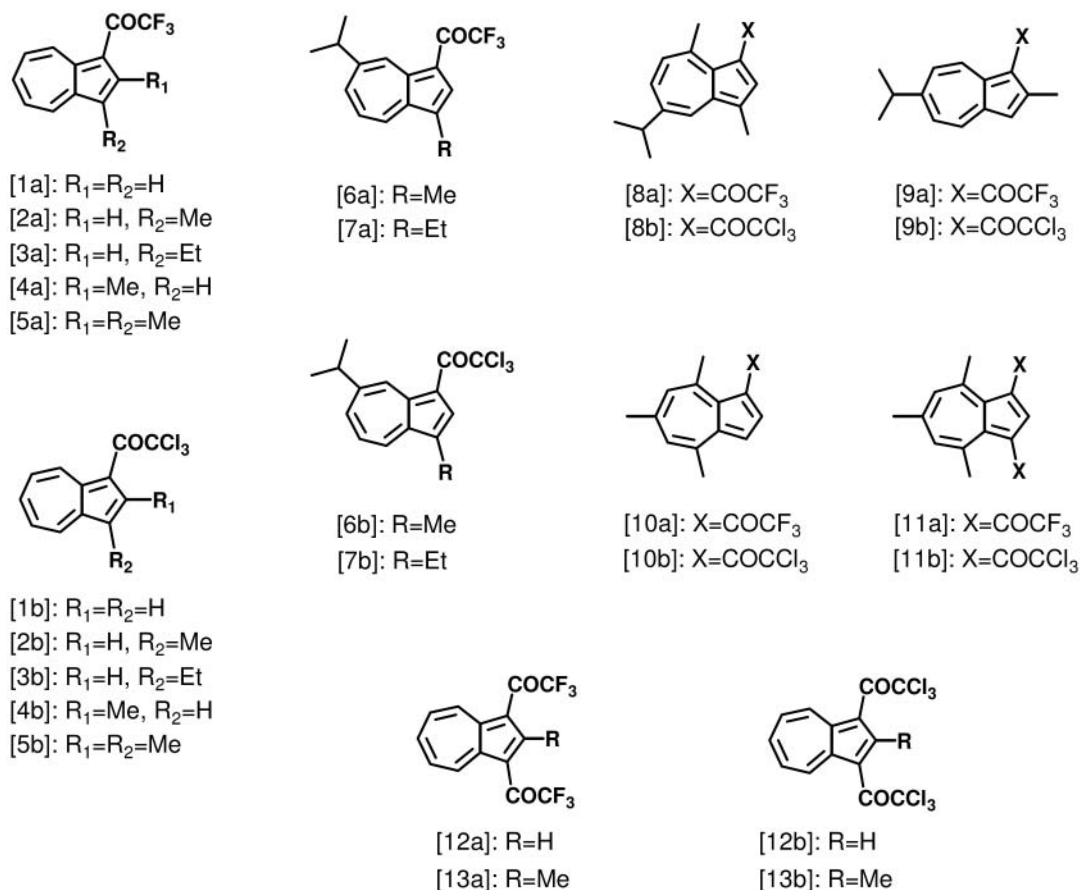

Figure 1. *Chemical structures of the trifluoroacetylazulenes **[1a-13a]** and trichloroacetylazulenes **[1b-13b]**.*

was investigated and their effects on NO anabolism (iNOS protein and mRNA expressions, assessed by Western blotting and RT-PCR analyses) and catabolism (NO scavenging activity, assessed by ESR spectroscopy) were also examined. To investigate the possible anti-inflammatory activity of the trihaloacetylazulenes, their effect on the expression of cyclooxygenase-2 (COX-2), which is induced by inflammation and is invoved in the production of PGE_2 (22), was also studied.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle 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-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); LPS from *Escherichia coli*. (Serotype 0111:B4), hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriamine-pentaacetic 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-tetramethylimi-dazoline-1-oxy1-3-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. The trihaloacetylazulene derivatives were synthesized, according to the published methods for the following compounds: 1-trifluoroacetylazulene **[1a]** (23, 24), 3-methyl-1-trifluoroacetylazulene **[2a]** (25), 3-ethyl-1-trifluoroacetyl-azulene **[3a]** (24-26), 2-methyl-1-trifluoroacetylazulene **[4a]** (25), 2,3-dimethyl-1-trifluoroacetylazulene **[5a]** (24-26), 7-isopropyl-3-methyl-1-trifluoro-acetylazulene **[6a]** (24-26), 3-ethyl-7-isopropyl-1-trifluoroacetylazulene **[7a]** (24-26), 3-trifluoroacetylguaiazulene **[8a]** (26, 27), 6-isopropyl-2-methyl-1-trifluoroacetylazulene **[9a]** (24-26), 1-trifluoroacetyl-4,6,8-trimethylazulene **[10a]** (26, 27), 1,3-ditrifluoroacetyl-4,6,8-trimethyl-azulene **[11a]** (28-30), 1,3-ditrifluoro-acetylazulene **[12a]** (28-30), 2-methyl-1,3-ditrifluoroacetylazulene **[13a]** (28-30), 1-trichloroacetyl-azulene **[1b]** (24), 3-methyl-1-trichloroacetylazulene **[2b]** (24-26), 3-ethyl-1-trichloroacetylazulene **[3b]** (24-26), 2-methyl-1-trichloro-acetylazulene **[4b]** (24-26), 2,3-dimethyl-1-trichloroacetylazulene **[5b]** (24-26), 7-isopropyl-3-methyl-1-trichloroacetylazulene **[6b]** (24-26), 3-ethyl-7-isopropyl-1-trichloroacetylazulene **[7b]** (24-26), 3-trichloro-acetylguaiazulene **[8b]** (26), 6-isopropyl-2-methyl-1-trichloroacetyl-azulene **[9b]** (24-26), 1-trichloroacetyl-4,6,8-trimethylazulene **[10b]** (26), 1,3-ditrichloro-acetyl-4,6,8-trimethylazulene **[11b]** (28-30), 1,3-ditrichloroacetylazulene **[12b]** (28-30), 2-methyl-1,3-ditrichloro-acetylazulene **[13b]** (28-30).

Cell culture. The 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).

Assay for cytotoxic activity. The cytotoxic activity of the azulenes was determined by the MTT method and was expressed as the 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).

Assay for NO concentration. Near confluent Raw 264.7 cells were incubated for 24 h 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 the Greiss reagent, the NO concentration in the culture medium without the cells was also measured, and this value was subtracted from that obtained with 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). The efficacy of inhibition of NO production was evaluated by the selectivity index (SI), which was calculated by the following equation:

$$SI=CC_{50} / IC_{50}.$$

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 min in ice water and were then incubated for 50 min at 4˚C with RT-5 ROTATOR (Titec, Saitama, Japan). The cell lysates were centrifuged at 16,000 xg for 20 min at 4˚C to remove the insoluble material and the supernatant was collected. The protein concentrations of the supernatant was measured using the Protein Assay Kit (Bio Rad, Hercules, CA, USA). An equal amount of the protein from the cell lysates (10 μg) was 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-mercaptoethanol), boiled for 10 min, applied to SDS-8% polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in phosphate-buffered saline [PBS(–)] plus 0.05% Tween 20 for 90 min and were incubated with anti-iNOS antibody (1:1,000, Santa Cruz Biotechnology, Delaware, CA, USA), anti-COX-2 antibody (1:1,000, Santa Cruz), or anti-actin antibody (1:2000-4000, Sigma) for 90 min at room temperature, followed by incubation with horseradish peroxidase-conjugated anti-mouse or goat IgG for 90 min at room temperature. The immunoblots were developed with Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA) and were analyzed on a Macintosh (Power Macintosh 7600/120) computer using the public domain NIH Image program (National Technical Information Service, Springfield, VA, USA, part number PB95-500195GEI).

Assay for mRNA expression. Total RNA was isolated using the PURESCRIPT RNA Isolation kit (Gentra systems) protocol. Raw 264.7 cells were lysed in 300 μL cell lysis solution, followed by the addition of 100 μL Protein-DNA precipitation solution. The cell lysates were centrifuged at 15,000 xg for 3 min. To the supernatant, 300 μL isopropanol were added. After centrifuging at 15,000 xg for 3 min, the pellet was washed in 300 μL 75% ethanol. Following further centrifugation at 15,000 xg for 1 min, the pellet was air dried for 15 min 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). Single-strand cDNA obtained by the RT reaction was amplified, using the KOD plus (Toyobo Co.), iNOS specific primers (5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and 5'-GGCT GTCAGAGCCTCGTGGCTTTGG-3'), COX-2 specific primers (5'-TTTGTTGAGTCATTCACCAGACAG-3' and 5'-CAGTATT GAGGAGAACAGATGGGATT-3') and β-actin specific primers (5'-GAGGCCCAGAGCAAGAGAGG-3', 5'-TACATGGCTGGGG TGTTGAA-3'), according to the protocol. The RT-PCR products were applied to 2% agarose gel, the ethidium bromide-stained gel was then photographed under UV light and analyzed as described above.

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

Compound	MW.	CC_{50} (μM)		IC_{50} (μM)	SI
		(–)LPS	(+)LPS	(+)LPS	
[1a]	224	323.2	321.6	21.5	15.0±2.8
[2a]	238	>500.0	>500.0	30.1	>16.6±6.1
[3a]	252	>500.0	>500.0	52.9	>9.4±6.6
[4a]	238	281.4	>340.8	109.6	>3.1±3.6
[5a]	252	>500.0	>500.0	107.4	>4.7±1.7
[6a]	280	>411.8	>375.6	55.9	>6.7±3.5
[7a]	294	239.8	164.9	29.3	5.6±2.0
[8a]	294	>408.0	>400.5	16.6	>24.2±5.1
[9a]	280	412.4	365.0	101.2	3.6±2.0
[10a]	266	>500.0	>500.0	22.1	>22.7±10.3
[11a]	362	>500.0	>500.0	34.1	>14.7±2.6
[12a]	320	>500.0	>500.0	86.5	>5.8±2.1
[13a]	334	>344.3	>195.9	39.7	>4.9±4.3
[1b]	273.5	51.1	53.9	8.7	6.2±0.9
[2b]	287.5	>494.6	>500.0	13.3	>37.7±6.6
[3b]	301.5	>500.0	>500.0	13.9	>36.1±2.7
[4b]	287.5	82.3	67.3	22.0	3.1±3.1
[5b]	301.5	19.7	12.3	9.2	1.3±1.2
[6b]	329.5	>218.7	92.2	13.3	6.9±3.5
[7b]	343.5	120.7	101.0	9.5	10.7±3.4
[8b]	343.5	17.0	19.5	4.7	4.2±2.8
[9b]	329.5	67.7	60.2	14.3	4.2±1.5
[10b]	315.5	9.9	10.0	<2.0	>5.1±0.9
[11b]	461	3.0	<2.0	<2.0	><1.0±0.0
[12b]	419	136.0	>232.3	24.0	>9.7±3.4
[13b]	433	>217.3	68.7	11.1	6.2±3.5

Each value represents the mean±S.D. from three to six independent experiments.

Radical scavenging activity. The radical intensity of the trihalo-acetylazulenes was determined at 25˚C, using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (15, 17, 19). The compounds (8 mM dissolved in DMSO) were mixed with an equal volume of 0.2 M sodium phosphate buffer (PB) (pH. 4.0), 0.2 M Tris-HCl (pH 7.4), 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 10.0) or 0.2 M KOH (pH 13.5). The radical intensity was measured 40 sec thereafter (center field, 336.0±5.0 mT; microwave power, 12 mW; modulation amplitude, 0.1 mT: gain, 500, time constant, 0.1 sec; scanning time, 4 min).

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

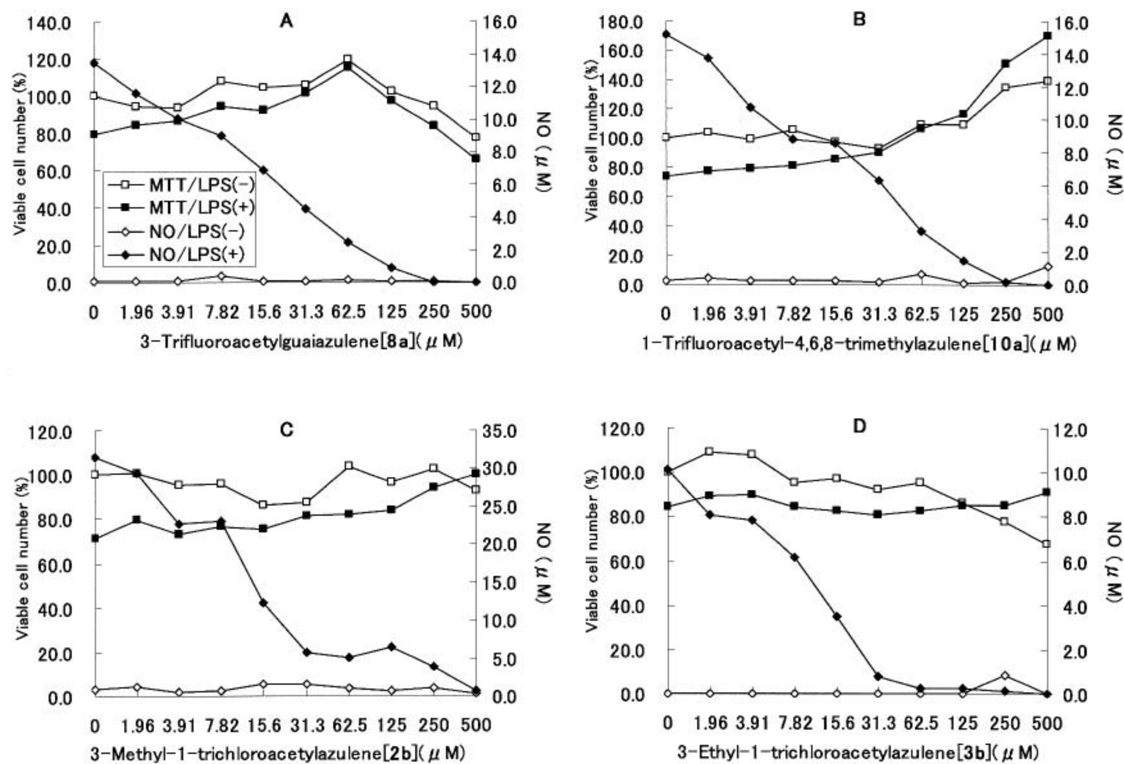

Figure 2. *Effect of trihaloacetylazulenes on the NO production by LPS-stimulated Raw 264.7 cells. Near-confluent Raw 264.7 cells were incubated for 24 h with the indicated concentrations of 3-trifluoroacetylguaiazulene **[8a]** (A), 1-trifluoroacetyl-4,6,8-trimethylazulene **[10a]** (B), 3-methyl-1-trichloroacetylazulene **[2b]** (C) or 3-ethyl-1-trichloroacetylazulene **[3b]** (D) 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 (expressed as % of control) (□, ■) and extracellular concentration of NO (◇, ◆) were determined by MTT assay and Griess reagent, respectively. Each value represents the mean from three independent experiments.*

PB, pH 7.4, in the presence of 30% DMSO. 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 produce seven-line signals. 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) and was expressed as the ratio to the height of MnO, an external marker.

For determination of the superoxide anion (O_2^-) produced by HX and XOD reactions (total volume: 200 μL) [2 mM HX in 0.1 M PB (pH 7.4) 50 μL, 0.5 mM DETAPAC 20 μL, 8% DMPO 30 μL, sample compound (7.5 mM in DMSO) 40 μL, H_2O or SOD 30 μL, XOD (0.5 U/mL in PB) 30 μL], 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 the determination of the DPPH radical scavenging activity, the compound was mixed with the reaction mixture (total volume: 200 μL) [100 μM DPPH 140 μL, H_2O 20 μL, sample in DMSO 40 μL] and, after 1 min, was subjected to ESR spectroscopy (31).

Results

Structure and activity relationship. The 13 trichloroacetylazulenes **[1b-13b]** generally showed higher cytotoxicity against the Raw 264.7 cells, compared to the corresponding 13 trifluoroacetylazulenes **[1a-13a]** (Table I). All of these compounds failed to stimulate the Raw 264.7 cells to produce detectable amounts of NO, but inhibited the NO production by LPS-activated Raw 264.7 cells to various extents (Table I). Compounds **[8a, 10a, 2b, 3b]**, with lesser cytotoxic activity (CC_{50}=>400, >500, >500 and >500 μM, respectively), inhibited NO production to the greatest extent (IC_{50}=16.6, 22.1, 13.3 and 13.9 μM, respectively) (Figure 2), producing the highest selectivity index (SI)= >24.2, >22.7, >37.7 and >36.1, respectively (Table I).

Effect on iNOS expression. Western blot analysis showed that compounds **[8a, 10a]** dose-dependently reduced the intracellular concentration of inducible NO synthase (Figure 3A, 3B), overlapping the dose-response curve of inhibition of NO production (Figure 2A, 2B). On the other hand, compounds **[2b, 3b]** slightly increased the iNOS protein expression at lower concentrations (3-13 μM), but were weakly inhibitory at higher concentrations (27-56 μM) (Figure 3C, 3D). RT-PCR analysis showed that the inhibition of iNOS mRNA expression by compounds **[8a,**

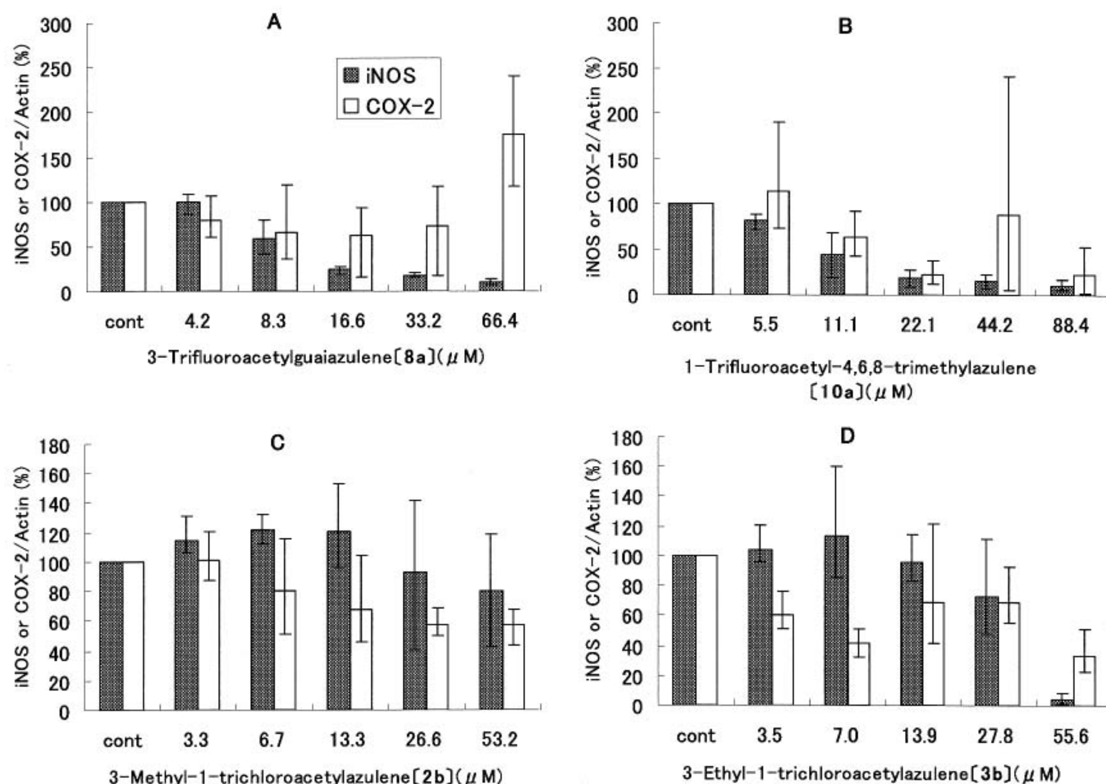

Figure 3. *Effect of trihaloacetylazulenes on the intracellular concentration of iNOS and COX-2 proteins in Raw 264.7 cells. Raw 264.7 cells were incubated for 24 h with the indicated concentrations of 3-trifluoroacetylguaiazulene **[8a]** (A), 1-trifluoroacetyl-4,6,8-trimethylazulene **[10a]** (B), 3-methyl-1-trichloroacetylazulene **[2b]** (C) or 3-ethyl-1-trichloroacetylazulene **[3b]** (D) in the presence of 100 ng/mL LPS. The cell lysate was applied to SDS-PAGE and iNOS (grey bars) or COX-2 (white bars) proteins were quantified by Western blot analysis, followed by densitometry. The intracellular iNOS or COX-2 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 the mean from three independent experiments.*

2b] was incomplete, with only a 50% reduction of iNOS mRNA expression (Figure 4).

Radical production and scavenging activity. ESR spectroscopy revealed that compounds **[8a, 10a, 2b, 3b]** did not produce any detectable amounts of radical at wide ranges of pH (pH 4.0-13.5) (Table II). Solutions of compounds **[2b, 3b]** at pH 10 became turbid, but upon elevation of the pH to 13.5, the solutions became clear again but the color changed to blue.

Compounds **[8a, 10a, 2b, 3b]** (1.5 mM) only slightly reduced the NO radical intensity (at most 10-17%), but did not scavenge the O_2^- radical at all. All these compounds (4 mM) only slightly reduced the DPPH radical (at most 5-33%) (Table II).

Relation between iNOS and COX-2 expression. The effects of compounds **[8a, 10a, 2b, 3b]** on COX-2 protein expression were more complicated and roughly biphasic: inhibition at lower concentrations and stimulation at higher concentrations (Figure 3). Compounds **[8a, 2b]** showed a similar biphasic effect on COX-2 mRNA expression (Figure 4).

Discussion

The present study demonstrated for the first time, that 13 trichloroacetylazulenes **[1b-13b]** generally showed higher cytotoxicity against Raw 264.7 cells than the corresponding 13 trifluoroacetylazulenes **[1a-13a]**, in agreement with the higher cytotoxicity of the trichloroacetylazulenes against human tumor cell lines (21). Among a total of 26 compounds, **[8a, 10a, 2b, 3b]** most efficiently inhibited the NO production in LPS-activated Raw 264.7 cells, with the highest selectivity index (SI=>23->38). The NO concentration is influenced by both the intracellular concentration and activity of iNOS protein and quenching of the NO radical. The NO radical interacts with superoxide anion (O_2^-) (32). We found that compounds **[8a, 10a, 2b, 3b]** only marginally scavenged the NO radical (produced from NOC-7, a NO donor), even at 1.5 mM, a 100-fold higher concentration that induced a 50% reduction in the NO production by LPS-stimulated Raw 264.7 cells. Furthermore, none of these compounds increased O_2^-, known to interfere with NO. These data reduce the possibility

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

Compd.	Radical intensity at pH[a]				Radical scavenging activity against		
	4.0	7.4	10.0	13.5	NO[b]	O_2^{-c}	DPPH[d]
Control	–	–	–	–	2.31 (100)	2.60 (100)	1.60 (100)
[8a]	<0.08	<0.08	<0.08	<0.08	1.91 (83)	2.67 (103)	1.07 (67)
[10a]	<0.08	<0.08	<0.08	<0.08	1.96 (85)	2.75 (106)	1.51 (95)
[2b]	<0.08	<0.08	<0.08	<0.08	1.92 (83)	2.59 (100)	1.32 (83)
[3b]	<0.08	<0.08	<0.08	<0.08	2.08 (90)	2.67 (103)	1.40 (88)

[a]Determined 40 sec after dissolving the compound (final: 4 mM) in 0.1 M buffer adjusted to the indicated pH; [b]Determined 3 min after dissolving the compound (final 1.5 mM) in the assay buffer from the ratio of the intensity of the first peak of carboxy-PTI to that of MnO, an external marker; [c]Determined 1 min after dissolving the compound (final 1.5 mM) in the assay buffer from the ratio of the intensity of the first peak of DMPO-OOH to that of MnO; [d]Determined at 1 min after dissolving the compound (final 4 mM) in the assay buffer from the ratio of the intensity of the third peak of DPPH to that of MnO. The number in parenthesis represents percent of the control.

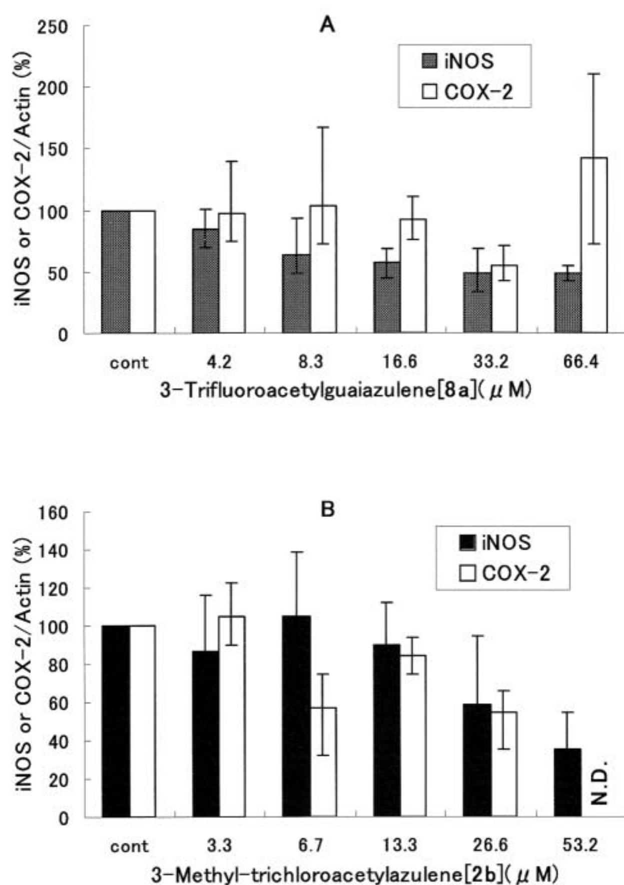

Figure 4. *Effect of trihaloacetylazulenes on LPS-stimulated iNOS and COX-2 mRNA expressions. Raw 264.7 cells were incubated for 24 h with the indicated concentrations of 3-trifluoroacetylguaiazulene* ***[8a]*** *(A) or 3-methyl-1-trichloroacetylazulene* ***[2b]*** *(B) in the presence of 100 ng/mL LPS. The RNA was then isolated and the RT-PCR product was applied to agarose gel electrophoresis and quantified by densitometry. The expression of iNOS (grey bars) or COX-2 (white bars) mRNA was expressed as the ratio to that of β-actin mRNA. Each value represents the mean from three independent experiments. N.D., not determined.*

that inhibition of NO production by compounds **[8a, 10a, 2b, 3b]** is due to NO scavenging activity. Alternatively, we found that compounds **[8a, 10a]** inhibited iNOS production at the same concentration that inhibited NO production, indicating that compounds **[8a, 10a]** inhibited NO production possibly by inhibiting iNOS protein expression. Since compounds **[8a, 10a]** did not completely inhibit the iNOS mRNA expression, the involvement of a post-transcriptional regulation mechanism was suggested. On the other hand, compounds **[2b, 3b]** did not affect the iNOS protein expression, indicating the possibility that these compounds inactivate the iNOS protein.

Compounds **[8a, 10a]** did not completely inhibit COX-2 protein expression, but rather increased it at higher concentrations, suggesting that iNOS and COX-2 expressions are not correlated with each other. This may be partly explained by the difference in the basal level and stimulation-fold between iNOS and COX-2 expressions after LPS stimulation. It was recently reported that COX-2 is activated after nitrosylation by binding with iNOS at a specific binding site (33). This suggests that the reduction of iNOS protein expression in Raw 264.7 cells on treatment with compounds **[8a, 10a]** might activate COX-2 less efficiently.

Our recent studies demonstrated that trihaloacetylazulene derivatives show diverse biological activity. Although 2,3-dimethyl-1-trichloroacetylazulene **[5b]** and 1,3-ditrichloro-acetyl-4,6,8-trimethylazulene **[11b]** showed the highest cytotoxicity and tumor specificity (TS >35.6 and >44.1, respectively) (21), they inhibited NO production by activated macrophages very weakly (SI=1.3, ><1.0, respectively) (Table I). Although compounds **[8a, 10a, 2b, 3b]** potently inhibited NO production by activated macrophages (Table I), they showed very weak tumor-specific cytotoxicity (TS= ><1.8, ><1.4, >2.3, ><1.8) (21). These data indicate that the biological activities of the trihaloacetylazulenes are not related to each other. Further studies are required to confirm the anti-inflammatory action of the trihaloacetylazulenes.

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