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Original article

Anti-nitric oxide production activity of isothiocyanates correlates with their polar surface area rather than their lipophilicity

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ABSTRACT

There is increasing demand for novel anti-inflammatory drugs with different mechanisms of action. We synthesized a series of isothiocyanates **2b**–**h** based on 6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) found in the pungent spice *Wasabia japonica*. Inhibitory activities against *in-vitro* growth of tumor cells and production of nitric oxide (NO) using the mouse macrophage-like cell line J774.1 were noted. All isothiocyanates were optimized by Hartree-Fock/3-21G model, and the log *P* values and the polar surface area (PSA) values were calculated. Substitution of the methylsulfinyl group ($CH_3S(=O)$–R) in 6-MITC with a formyl (CHO–R), a methylsulfanyl (CH_2S–R) or a methyl (CH_3–R) group reduced the activities of the parent isothiocyanate. Substitution with a formyl group resulted in lower lipophilicity (log *P* value) whereas substitution with a methylsulfanyl or methyl group resulted in a lower PSA value. The inhibitory activity of isothiocyanates showed better correlation with their PSA values rather than their partition coefficient (log *P*) values. Isothiocyanates with higher PSA values and some degree of log *P* value may have potent biological activity.

1. Introduction

Accumulating evidence from epidemiologic and clinical studies indicates that chronic inflammatory disorders harbor an increased risk of cancer, arthritis, heart attacks, and Alzheimer's disease [1].

Sulforaphane [4-(methylsulfinyl)butyl isothiocyanate] is a natural isothiocyanate present in cruciferous vegetables such as broccoli and cabbage. It possesses potent anti-inflammatory and chemopreventive activities [2]. The compound 6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) is found in the pungent spice *Wasabia japonica* (wasabi) used in Japan [3,4] and is thought to have anti-inflammatory and chemopreventive activities [5–7].

The relationship between structure and the biological activity of alkyl isothiocyanates reveals that an increase in the length of the carbon chain correlates with augmented induction of the activity of NADPH:quinone oxidoreductase and its mRNA [8]. Morimitsu et al. [5] and Prawan et al. [9] reported that the phase-II detoxifying/antioxidant enzyme inducer potency of sulforaphane and its analogs is influenced by the number of methylene ($-CH_2-$) groups in the bridge linking the functional group and isothiocyanate moieties. Talalay et al. [10] suggested in structure–biological activity studies of various alkyl and aromatic isothiocyanates that at least one hydrogen on the carbon adjacent to the isothiocyanate moiety (which leads to tautomerization of the methylene–isothiocyanate moiety ($-CH_2-N=C=S$) to a structure resembling an α, β-unsaturated thioketone) may be important for the inductive ability of quinone reductase and glutathione *S*-transferases. The oxidation state of sulfur in the methylsulfinyl functional group appears to be important for the inducer activity of sulforaphane and its analogs [5], and the type of the functional group may be more important than the number of methylene groups [9]. Morpholine and methylenedioxybenzene groups have been reported to have superior inducer activity than other functional groups [9]. Differences in physicochemical properties (e.g., solubility, acid–base properties) may also influence the biological activity of isothiocyanates. Jiao et al. [11] reported that inhibitory activities of isothiocyanates against 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis show better

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correlation with their high lipophilicity and low reactivity against glutathione (making conjugations with thiol groups).

Clarification of the biological roles of the methyl sulfinyl group of alkyl isothiocyanates is the subject of this contribution. A series of new isothiocyanate derivatives replaced the methyl sulfinyl group with other functional groups, though the six-carbon-chain framework of 6-MITC was maintained. Structure–biological activity relationships of the newly synthesized isothiocyanates were evaluated for their inhibitory activities against *in-vitro* growth of tumor cells and production of nitric oxide (NO) using the mouse macrophage-like cell line J774.1.

2. Results and discussion

2.1. Chemistry

Chemical structures of 6-MITC and analogs are illustrated in Fig. 1. Four isothiocyanates (**2b**, **2c**, **2d** and **2e**) were prepared from 1,6-hexanediol via alkyl halide **1a** [12] and their isothiocyanato (–NCS) group formed by adopting the method of Ding et al [13] (Scheme 1). Two isothiocyanates (**2f** and **2g**) and thiocyanate **4** were prepared from 1,6-dichlorohexane (Scheme 2). The isothiocyanato group of **2f** and **2g** was formed by adopting the method of Ding et al [13], and the thiocyanato (–SCN) group of **4** was synthesized by nucleophilic substitution of the thiocyanate anion with alkyl halide **1c**. The structures of all isothiocyanates (**2b**, **2c**, **2d**, **2e**, **2f** and **2g**) and thiocyanate (**4**) were confirmed by ^{1}H NMR and IR spectral data, and all new compounds gave satisfactory HRMS data. Isothiocyanates (R–N=C=S) showed strong and broad absorption at about 2200 cm^{-1} to 2100 cm^{-1}, whereas thiocyanate (R–S–C≡N) showed strong and sharp absorption at about 2150 cm^{-1} in IR spectra. In addition, *n*-hexyl isothiocyanate (**2h**) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

In addition, all isothiocyanates were optimized by Hartree-Fock/3-21G model, and the log *P* values and the polar surface area (PSA) values were calculated by using of Spartan '06 program (Wavefunction, Incorporated, CA, USA).

2.2. Inhibitory activities against in-vitro growth of tumor cells and NO production

First of all, we examined inhibitory activity of 6-MITC on NO production by mouse peritoneal macrophages. Table 1 shows 6-MITC inhibited the NO production in a dose-dependent manner.

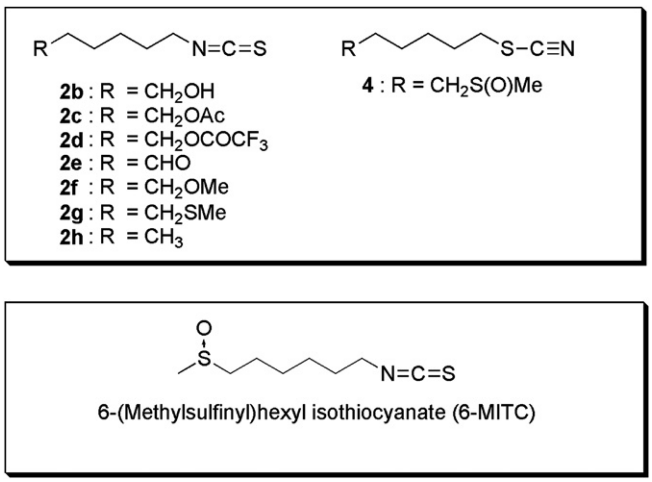
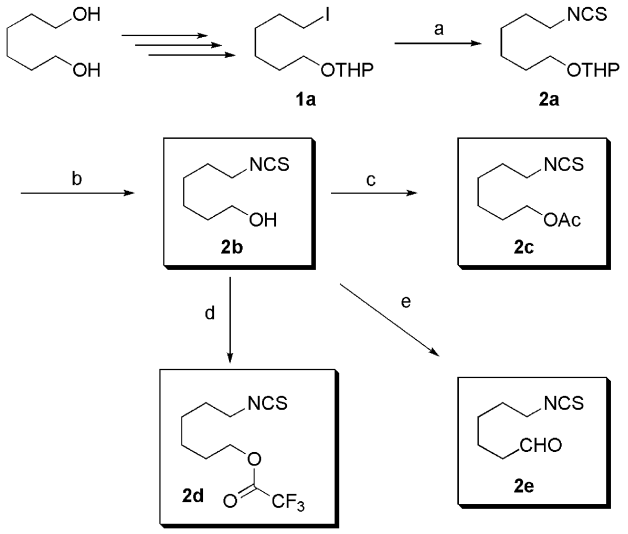


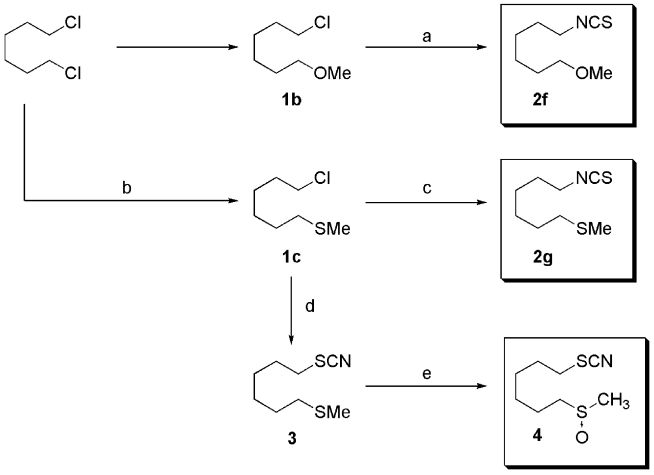
Fig. 1. Chemical structures of 6-MITC and analogues used in this study.



Scheme 1. *Reagents & Conditions*: (a) i. NaN_3, DMF, reflux, 3 h, 79%; ii. Ph_3P, ether, 12 h, rt., then CS_2, reflux, 2 h, 82%; (b) TsOH, MeOH, 3 h, rt, 71%; (c) Ac_2O, pyr., 16 h, rt, 56%; (d) $(CF_3CO)_2O$, pyr., 18 h, rt, 32%; (e) Dess-Martin periodinane, CH_2Cl_2, 18 h, rt., 27%.

This indicates 6-MITC inhibits one of functions of normal macrophages, and suggests a possibility that 6-MITC would inhibit the function of macrophages *in vivo* as well. According to this result, we selected a mouse macrophage-like cell line J774.1 to test biological activities of isothiocyanate compounds, instead of peritoneal exudates macrophages because of their inconvenience in preparation.

The newly synthesized isothiocyanates **2b**–**g**, purchased **2h** and thiocyanate **4** were screened for their anti-proliferative and anti-NO production activities *in vitro* using J774.1 cells. The half-maximal inhibitory concentration (IC_{50}) values of compounds **2b**–**h** and thiocyanate **4** are listed in Table 2. Thiocyanate **4** had no activity, indicating that the inhibitory activities of 6-MITC were entirely dependent on its isothiocyanato group. This observation is in accordance with that of Morimitsu et al. [5]. That is, the methylthioalkyl isothiocyanate but not the methylthioalkylamine has the induction potency of glutathione *S*-transferase (GST) activity, indicating that the isothiocyanate moiety is essential for the



Scheme 2. *Reagents & Conditions*: (a) i. NaN_3, DMF, reflux, 3 h, 100%; ii. Ph_3P, ether, 12 h, rt., then CS_2, reflux, 2 h, 83%; (b) $(CH_3S)_2CO$, $^nBu_4NBr$, 30% aq. NaOH, 0.5 h, reflux, 67%; (c) i. NaN_3, DMF, reflux, 3 h, 100%; ii. Ph_3P, ether, 12 h, rt., then CS_2, reflux, 2 h, 80%; (d) NaSCN, MeOH, reflux, 4 h, 39%; (e) 30% H_2O_2, AcOH, 0.5 h, rt., 64%.

Table 1
Effect of 6-MITC on nitric oxide (NO) production by mouse peritoneal exudate macrophages.

6-MITC (μM)	NO (μM)[a]	Inhibition (%)
0	8.6 ± 0.3	0
0.01	7.7 ± 0.4	10
0.1	8.1 ± 0.3	6
1	6.0 ± 0.3	30
5	0.7 ± 0.2	92

[a] Mean ± SE ($n = 6$).

induction of GST activity. 6-MITC and *N*-acetyl cysteine have been reported to spontaneously make conjugates in aqueous solution [14]. Compounds **2b**, **2c, 2d** and **2f** had almost identical anti-proliferative and anti-NO production activities as those of 6-MITC. Compounds **2e**, **2g** and **2h** were less active than 6-MITC.

Hou et al. [8] reported that an increase in the length of the carbon chain in alkyl isothiocyanates correlates with augmentation of their biological activities. Increasing the carbon-chain length appears to increase their log *P* values. The biological activity of isothiocyanates may therefore increase with increment of their log *P* values. An inverse correlation between log *P* and the activities of each compound was noted, i.e., both activities tend to decrease with increment in log *P* values, though the correlation was poor (Fig. 2). A better correlation between PSA and the activities of each compound were observed (Fig. 3). The low biological activities of compounds **2h** and **2g** could therefore be due to their low PSA values. Compound **2e** showed weaker activity than compound **2f**, though the PSA value of compound **2e** was larger than that of compound **2f**. This could be because the aldehyde group of compound **2e** tends to be readily attacked by nucleophiles and its log *P* value is quite low. A certain degree of the log *P* value of isothiocyanates is necessary for their effective activity. As mentioned above, the biological activities of isothiocyanates should be through binding of their isothiocyanato groups with the SH groups of target molecules. It may be hypothesized that the PSA of isothiocyanates facilitates formation of isothiocyanate–SH conjugations.

3. Experimental protocols

3.1. Chemistry

^{1}H and ^{13}C NMR data were measured by a JNM-AL300 spectrometer (Jeol, Tokyo, Japan) using $CDCl_3$ as a solvent. Chemical shifts are given in δ unit (ppm). MS data were measured with a Jeol JMS-700 spectrometer in EI and FAB modes, and IR spectra were recorded on a JASCO FT/IR-5300 spectrometer (Jasco, Tokyo, Japan). Data are given in ν max (cm^{-1}). Preparative TLC work was carried out using plates coated with Kieselgel 60 F_{254} (0.75-mm thick; Merck KGaA, Darmstadt, Germany). All chemicals and solvents were of reagent grade, and the latter were distilled and dried before use. Jiao et al. [11] suggested that the application of log *P* (partition coefficients between 1-octanol and aqueous phases) values is suitable for measurement of the relative lipophilicity of isothiocyanates, and that the log *P* values measured for isothiocyanates are consistent with those predicted on the basis of their chemical structures. We adopted the Spartan '06 program (Wavefunction, Incorporated, CA, USA) for calculation of log *P* from Crippen model and polar surface area (PSA) values in $Å^2$ of newly synthesized isothiocyanates.

Table 2
In vitro NO production and tumor growth inhibitory activities of isothiocyanates **2b-h** and thiocyanate **4.**

Compounds	Inhibition (IC_{50}, μM)[a]		PSA($Å^2$)[b]	log *P*[b]
	NO production	Growth		
6-MITC	5.7 ± 0.5	8.0 ± 0.6	19.9	1.24
4 (thiocyanate)	>200	>200	–	–
Isothiocyanates				
2b	6.0 ± 1.2	4.4 ± 0.3	23.1	1.41
2c	6.6 ± 1.2	4.1 ± 0.2	23.8	1.64
2d	9.1 ± 1.0	5.6 ± 0.4	23.2	2.78
2f	11.5 ± 5.9	8.1 ± 0.6	10.2	1.36
2e	25.5 ± 5.9	19.7 ± 1.1	17.5	0.94
2g	30.4 ± 6.3	14.8 ± 0.9	2.7	2.08
2h	66.8 ± 13.5	47.9 ± 10.8	2.5	2.61

Data represent means and standard errors of 5-11 independent experiments.
[a] 50% inhibition.
[b] PSA (polar surface area) and log *P* value were obtained with Spartan '06 program.

3.1.1. Synthesis of 2-(6-Isothiocyanatohexyloxy)-tetrahydro-2H-pyran (**2a**)

A solution of 2-(6-iodohexyloxy)-tetrahydro-2*H*-pyran (1.10 g, 5 mmol) and sodium azide (400 mg, 6.0 mmol) in DMF (10 mL) was stirred at reflux temperature for 2 h. The reaction mixture was poured into water (100 mL) and extracted with *n*-hexane (50 mL, 3 times). Combined extracts were washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated under reduced pressure. A solution of dry residue (650 mg, containing 3.8 mmol of 2-(6-azidohexyloxy)-tetrahydro-2*H*-pyran) and PPh_3 (1.23 g, 4.7 mmol) in Et_2O (10 mL) was stirred at room temperature for 8 h. After removing the solvent under diminished pressure, 1.5 mL of CS_2 was added to the reaction system. The resulting mixture was refluxed for another 2 h. The reaction mixture was poured into water (100 mL) and extracted with Et_2O (30 mL, 3 times). Combined extracts were washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated. The residue was chromatographed over silica gel and eluted with *n*-hexane/benzene (2:1 = v/v) to give 2-(6-isothiocyanatohexyloxy)-tetrahydro-2*H*-pyran (**2a**, 530 mg, 2.2 mmol, 44% yield from 2-(6-chlorohexyloxy)-tetrahydro-2*H*-pyran) as a colorless oil. IR ν_{max} (film) cm^{-1}: 2940, 2865, 2180, 2105, 1454, 1348, 1123, 1076, 1034. ^{1}H NMR ($CDCl_3$) δ: 1.35–1.90 (14H, m), 3.40 (1H, dt, $J = 9.7$, 6.4 Hz), 3.46–3.56 (1H, m), 3.52 (2H, t, $J = 6.6$ Hz), 3.75 (1H, dt, $J = 9.5$, 6.6 Hz), 3.82–3.92 (1H, m), 4.55–4.60 (1H, m). ^{13}C NMR($CDCl_3$) δ: 19.8, 25.5, 25.6, 26.4, 29.5, 29.9, 30.8, 45.0, 62.5, 67.3, 99.0 (carbon of the isothiocyanato group was not observed). HREI-MS: Found: 243.1282, calcd. for $C_{12}H_{21}NO_2S$ (M^+): 243.1293.

3.1.2. Synthesis of 6-Isothiocyanatohexan-1-ol (**2b**)

A mixture of 2-(6-isothiocyanatohexyloxy)-tetrahydro-2*H*-pyran (**2a**, 530 mg, 2.2 mmol) and a catalytic amount of *p*-TsOH in dry MeOH (10 mL) was stirred for 3 h at room temperature under an atmosphere of nitrogen. The reaction mixture was then concentrated *in vacuo*. The residue was purified by preparative TLC (*n*-hexane-AcOEt = 4:1) to give pure 6-isothiocyanatohexan-1-ol (**2b**, 250 mg, 1.7 mmol, 71% yield) as a colorless oil. IR ν_{max} (film) cm^{-1}: 3348, 2935, 2860, 2361, 2106, 1456, 1346, 1163, 1053, 945, 902, 729. ^{1}H NMR ($CDCl_3$) δ: 1.35–1.55 (4H, m), 1.55–1.65 (2H, m), 1.70–1.80 (2H, m), 3.52(2H, t, $J = 6.4$ Hz), 3.65 (2H, t, $J = 6.4$ Hz). ^{13}C NMR($CDCl_3$) δ: 25.0, 26.4, 29.9, 32.4, 45.0, 62.7 (carbon of the isothiocyanato group was not observed). HREI-MS: Found: 159.0722, calcd. for $C_7H_{13}NOS$ (M^+): 159.0718.

3.1.3. Synthesis of 6-Isothiocyanatohexyl acetate (**2c**)

A solution of a mixture of **2b** (122 mg, 0.8 mmol) in Ac_2O (1 mL) and pyridine (1 mL) was stirred at room temperature for 16 h. The reaction was quenched with methanol, and the reaction

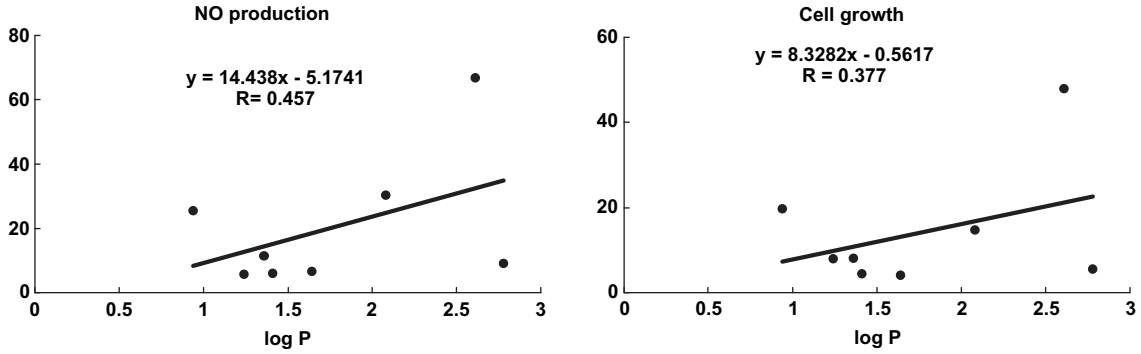


Fig. 2. Linear regression curves for the interactions of log *P* with 50% inhibition of NO production and cell growth.

mixture concentrated *in vacuo*. The dry residue was purified by silica gel vacuum flash column chromatography eluting with *n*-hexane/EtOAc (2:1 = v/v) to give pure 6-isothiocyanatohexyl acetate (**2c**, 86 mg, 0.43 mmol, 56% yield) as a colorless oil. IR ν_{max} (film) cm^{-1}: 2941, 2860, 2361, 2339, 2179, 2106, 1738, 1456, 1365, 1238, 1037, 889, 731, 669. ^1H NMR ($CDCl_3$) δ: 1.35–1.55 (4H, m), 1.60–1.80 (4H, m), 2.05 (3H, s), 3.53 (2H, t, $J = 6.6$ Hz), 4.08 (2H, t, $J = 6.6$ Hz). ^{13}C NMR($CDCl_3$) δ: 21.0, 25.3, 26.3, 28.4, 29.8, 44.9, 64.21, 171.1 (carbon of the isothiocyanato group was not observed). HREI-MS: Found: 201.0831, calcd. for $C_9H_{15}NO_2S$ (M^+): 201.0823.

3.1.4. Synthesis of 6-Isothiocyanatohexyl 2,2,2-trifluoroacetate (**2d**)

A solution of a mixture of **2b** (107 mg, 0.67 mmol) in trifluoroacetic anhydride (1 mL) and pyridine (1 mL) was stirred at room temperature for 18 h. The reaction was quenched with methanol, and the reaction mixture concentrated *in vacuo*. The dry residue was purified by preparative TLC (*n*-hexane/EtOAc = 2:1) to give pure 6-isothiocyanatohexyl 2,2,2-trifluoroacetate (**2d**, 55 mg, 0.22 mmol, 32% yield) as a colorless oil. IR ν_{max} (film) cm^{-1}: 2943, 2864, 2361, 2339, 2183, 2108, 1786, 1556, 1456, 1404, 1350, 1221, 1161, 937, 891, 777, 731, 669. ^1H NMR ($CDCl_3$) δ: 1.35–1.55 (4H, m), 1.65–1.85 (4H, m), 3.53 (2H, t, $J = 6.4$ Hz), 4.38 (2H, t, $J = 6.4$ Hz). ^{13}C NMR($CDCl_3$) δ: 24.9, 26.2, 28.0, 29.8, 44.9, 67.9, 116.4, 157.8 (carbon of the isothiocyanato group was not observed). HREI-MS: Found: 255.0543, calcd. for $C_9H_{12}F_3NO_2S$ (M^+): 255.0541.

3.1.5. Synthesis of 6-Isothiocyanatohexanal (**2e**)

Dess–Martin periodinane (810 mg, 1.9 mmol) was added in one portion to a stirred solution of **2b** (120 mg, 0.75 mmol) in dichloromethane (8 mL) at room temperature. After 18 h, the reaction mixture was filtered. The filtrate was concentrated *in vacuo* to give a colorless residue, which was purified by preparative TLC to give pure 6-isothiocyanatohexanal (**2e**, 35 mg, 0.22 mmol, 27% yield) as a colorless oil. IR ν_{max} (film) cm^{-1}: 2928, 2361, 2339, 2104, 1722, 1456, 1348. ^1H NMR ($CDCl_3$) δ: 1.45–1.55 (4H, m), 1.60–1.75 (4H, m), 2.49 (2H, dt, $J = 1.5$, 7.3 Hz), 3.53 (2H, t, $J = 6.6$ Hz), 9.80 (1H, t, $J = 1.5$ Hz). ^{13}C NMR ($CDCl_3$) δ: 21.2, 26.1, 29.8, 43.6, 44.8, 201.9 (carbon of the isothiocyanato group was not observed). HREI-MS: Found: 157.0566, calcd. for $C_7H_{11}NOS$ (M^+): 157.0561.

3.1.6. Synthesis of 1-Isothiocyanato-6-methoxyhexane (**2f**)

A solution of 1-chloro-6-methoxyhexane [15] (**1b**, 950 mg, 6.3 mmol) and sodium azide (600 mg, 9.2 mmol) in DMF (10 mL) was stirred at reflux temperature for 2 h. The reaction mixture was poured into water (100 mL) and extracted with *n*-hexane (50 mL, 3 times). The combined extracts were washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated under diminished pressure. The residue was used for the next step without purification. A solution of dry residue (1.0 g, containing 6.3 mmol of 1-azido-6-methoxyhexane) and PPh_3 (2.07 g, 7.9 mmol) in Et_2O (10 mL) was stirred at room temperature for 8 h. After removing the solvent under diminished pressure, 2 mL of carbon disulfide (CS_2) was added to the reaction system. The resulting mixture was refluxed for another 2 h. The reaction mixture was poured into water (100 mL) and extracted with Et_2O (50 mL, 3 times). Combined extracts were washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated. The residue was chromatographed over silica gel and eluted with *n*-hexane/benzene (2:1 = v/v) to give 1-isothiocyanato-6-methoxyhexane (**2f**, 820 mg, 4.7 mmol, 75% yield from 1-chloro-6-methoxyhexane) as a colorless oil. IR ν_{max} (film) cm^{-1}: 2936, 2861, 2180, 2106, 1454, 1348, 1120. ^1H NMR ($CDCl_3$) δ: 1.32–1.51 (4H, m), 1.54–1.64 (2H, m), 1.65–1.77 (2H, m), 3.34 (3H, s), 3.38 (2H, t, $J = 6.4$ Hz), 3.53 (2H, t, $J = 6.6$ Hz). ^{13}C NMR ($CDCl_3$) δ: 25.4, 26.4, 29.4, 29.8,

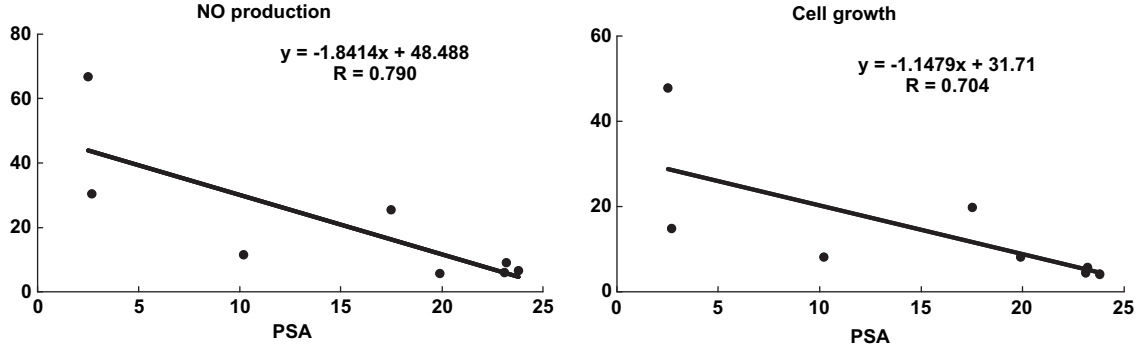


Fig. 3. Linear regression curves for the interactions of PSA with 50% inhibition of NO production and cell growth.

44.9, 58.6, 72.5 (carbon of the isothiocyanato group was not observed). HREI-MS: Found: 173.0877, calcd. for $C_8H_{15}NOS$ (M^+): 173.0874.

*3.1.7. Synthesis of (6-Chlorohexyl) (methyl) sulfane (**1c**)*

A solution of 1,6-dichlorohexane (1.55 g, 10 mmol), *S,S′*-dimethyl dithiocarbonate (730 mg, 6.0 mmol) and 50 mg of tetra *n*-butyl ammonium bromide in 30% aqueous KOH (10 mL) was stirred vigorously at reflux temperature for 30 min. The reaction mixture was poured into water (100 mL) and extracted with diethyl ether (30 mL, 3 times). Combined extracts were washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated under diminished pressure. The residue was chromatographed over silica gel and eluted with *n*-hexane/benzene (4:1 = v/v) to give (6-chlorohexyl) (methyl) sulfane (**1c**, 1.45 g, 8.7 mmol, 87% yield). IR ν_{max} (film) cm^{-1}: 2934, 2857, 1435, 1271, 1044, 723. ^1H NMR ($CDCl_3$) δ: 1.36–1.54 (4H, m), 1.56–1.68 (2H, m), 1.72–1.86 (2H, m), 2.10 (3H, s), 2.50 (2H, t, $J = 7.2$ Hz), 3.54 (2H, t, $J = 6.6$ Hz). ^{13}C NMR($CDCl_3$) δ: 15.6, 26.5, 28.0, 29.0, 32.5, 34.2, 45.0. HREI-MS: Found: 166.0587, calcd. for $C_7H_{15}ClS$ (M^+): 166.0583.

*3.1.8. Synthesis of 1-Isothiocyanato-6-(methylthio)hexane (**2g**)*

A solution of (6-chlorohexyl) (methyl) sulfane (**1c**, 740 mg, 4.4 mmol) and sodium azide (400 mg, 6.0 mmol) in DMF (10 mL) was stirred at reflux temperature for 2 h. The reaction mixture was poured into water (100 mL) and extracted with *n*-hexane (50 mL, 3 times). Combined extracts were washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated under diminished pressure. A solution of dry residue (650 mg, containing a 3.8 mmol) of 1-azido-6-methoxyhexanel and PPh_3 (1.23 g, 4.7 mmol) in Et_2O (10 mL) was stirred at room temperature for 8 h. After removing the solvent under diminished pressure, 1.5 mL of CS_2 was added to the reaction system. The resulting mixture was refluxed for another 2 h. To the reaction mixture was poured into water (100 mL) and extracted with Et_2O (30 mL, 3 times). Combined extracts were washed with water and brine, dried over anhydrous $CaCl_2$, and concentrated. The residue was chromatographed over silica gel and eluted with *n*-hexane/benzene (2:1 = v/v) to give 1-isothiocyanato-6-(methylthio)hexane (**2g**, 530 mg, 2.8 mmol, 64% yield from **7**) as a colorless oil. IR ν_{max} (film) cm^{-1}: 2936, 2857, 2183, 2102, 1453, 1346. ^1H NMR ($CDCl_3$) δ: 1.40–1.50 (4H, m), 1.50–1.78 (4H, m), 2.10 (3H, s), 2.51 (2H, t, $J = 7.5$ Hz), 3.52 (2H, t, $J = 6.4$ Hz). ^{13}C NMR ($CDCl_3$) δ: 15.5, 26.2, 27.9, 28.8, 29.8, 34.1, 45.0 (carbon of the isothiocyanato group was not observed). HREI-MS: Found: 189.0651, calcd. for $C_8H_{15}NS_2$ (M^+): 189.0646.

*3.1.9. Synthesis of 1-(Methylthio)-6-thiocyanatohexane (**3**)*

Sodium thiocyanate (NaSCN, 810 mg, 10 mmol) was added to a stirred solution of (6-chlorohexyl)(methyl)sulfane (**1c**, 1.1 g, 6.6 mmol) in methanol. The reaction mixture was heated at reflux temperature for 4 h. After dilution with chloroform, the mixture was filtered with silica gel, and the filtrate concentrated. The dry residue was chromatographed over silica gel and eluted with *n*-hexane/benzene (2:1 = v/v) to give 1-(methylthio)-6-thiocyanatohexane (**3**, 490 mg, 3.2 mmol, 39% yield). ^1H NMR ($CDCl_3$) δ: 1.40–1.50 (4H, m), 1.50–1.70 (2H, m), 1.75–1.90 (2H, m), 2.10 (3H, s), 2.50 (2H, t, $J = 7.2$ Hz), 2.95 (2H, t, $J = 7.1$ Hz). ^{13}C NMR ($CDCl_3$) δ: 15.5, 27.5, 27.9, 28.8, 29.8, 33.9, 34.0, 112.3. IR ν_{max} (film) cm^{-1}: 2934, 2857, 2153, 1460, 1435. HREI-MS: Found: 189.0642, calcd. for $C_8H_{15}NS_2$ (M^+): 189.0646.

*3.1.10. Synthesis of 1-(Methylsulfinyl)-6-thiocyanatohexane (**4**)*

An aqueous solution (30%) of hydrogen peroxide (50 μL, 0.45 mmol) was added to a stirred solution of 1-(methylthio)-6-thiocyanatohexane (**3**, 57 mg, 0.3 mmol) in acetic acid (1.0 mL) at room temperature. After 30 min, the reaction mixture was diluted with dichloromethane and neutralized by adding potassium carbonate. The mixture was filtered and purified by preparative TLC to give pure 1-(methylsulfinyl)-6-thiocyanatohexane (**4**) with quantitative yield. IR ν_{max} (film) cm^{-1}: 2935, 2861, 2153, 1460, 1300, 1028. ^1H NMR ($CDCl_3$) δ: 1.44–1.57 (4H, m), 1.75–1.94 (4H, m), 2.58 (3H, s), 2.68 (1H, dt, $J = 12.8$, 7.9 Hz), 2.73 (1H, dt, $J = 12.8$, 7.3 Hz), 2.96 (2H, t, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$) δ: 22.4, 27.5, 28.1, 29.6, 33.8, 38.6, 54.4, 112.2. HRFAB-MS: Found: 206.0676, calcd. for $C_8H_{16}NOS_2$ ($M+1^+$): 206.0673.

3.2. Biological activities

3.2.1. Preparation of peritoneal exudates cells (PEC)

Five-7week-old BALB/c mice received an intraperitoneal (i.p.) injection of 3 mL of 2.4% thioglycolate broth. After 4–5 d, BALB/c mice were sacrificed by heart punctuation after chloroform anesthetization, and PEC were aseptically obtained from the mice by injecting i.p. twice 5 mL cold phosphate-buffered saline solution containing 0.1% fetal calf serum (FCS), heparin (10 U/mL) and 10 mM glucose, massaging and collecting the cells immediately thereafter. The cells were washed and resuspended in 10% FCS-RPMI 1640 medium containing 5×10^{-5} M 2-mercaptoethanol and gentamicin sulfate (10 μg/mL). Next, 200 μL cell suspension (5×10^5 cells/mL) was added to flat-bottomed 96-well microplate (Greiner). Non-adherent cells were removed with a Titer mixture MB-1 (Japan Tirika Co.) after 2 h incubation at 37 °C in a humidified CO_2 (5%) atmosphere. Adherent cells comprised 90–95% of the plated PEC and consisted of over 95% macrophages, by morphological definition (Giemusa staining). Thus, the numbers of initial plated PEC are represented as macrophage number in this study. All animal experiments in this paper followed the Guidelines for Animal Experimentation of Aomori University.

3.2.2. Inhibition of in vitro production of NO

Production of NO in peritoneal macrophages and J774.1 cells (measured as NO_2 concentration) was determined by Griess reagent as described previously [17] except for FBS concentration; the culture medium for J774.1 cells was RPMI-1640 containing 10% FBS and gentamicin sulfate (10 μg/mL). In brief, peritoneal macrophages were prepared as described above, and J774.1 cells (4×10^4/0.2 mL) in each well of a 96-well flat-bottomed microplate were cultured at 37 °C for 1 d. The culture medium was replaced with freshly prepared warm (37 °C) 10% FCS-RPMI 1640 medium. After incubation for 30 min, 10 μL serial twofold diluted solution of test compounds and 10 μL interferon gamma (2 U/mL) + lipopolysaccharide (10 *n*g/mL) solution were added to each well. The microplates were further incubated at 37 °C. After 20–24 h, 0.1 mL culture fluid from each well was withdrawn and mixed with 0.1 mL Griess reagent. Mixtures were incubated for 10 min at room temperature. Absorbance of each well, 540–655 nm, was measured with a Bio-RAD model 550 microplate reader. NO_2 concentrations were measured using sodium nitrite as a standard.

3.2.3. Inhibition activity of in-vitro cell growth

J774.1 cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and passaged every 4 d. The culture medium was RPMI-1640 containing 5% fetal bovine serum (FBS) and gentamicin sulfate (10 μg/mL). J774.1 cells (1×10^4/0.2 mL) in each well of a 96-well flat-bottomed microplate (Greiner) were cultured at 37 °C for 1 d. After addition of 10 μL serial twofold diluted solution of test compounds, microplates were further incubated at 37 °C for 1 d. Cell viability was determined by a [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] (MTT) assay (Sigma–Aldrich) [16].

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