Investigation of Vasodilatory Substances in Diesel Exhaust Particles (DEP): Isolation and Identification of Nitrophenol Derivatives

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Vasodilatory compounds in the weak acidic fraction of a benzene extract of diesel exhaust particles (DEP) were fractionated by column chromatography through silica gel, and the chemical structures of these compounds were analyzed using GC-MS and ^{1}H-NMR. The compounds in DEP that cause vasodilation — 3- and 4-nitrophenol, 2-methyl-4-nitrophenol, 3-methy-4-nitrophenol, and 4-nitro-3-phenylphenol — were isolated and identified. All five of these nitrophenols had vasodilatory activities (10^{-4} to 10^{-6} M) in rat thoracic artery assays, and 4-nitro-3-phenylphenol was the most potent vasodilator among these compounds. In addition, nine other alkylnitrophenols were isolated from the benzene extract of DEP and characterized.

Key words ——— diesel exhaust particles, nitrophenols, vasodilation

INTRODUCTION

Diesel exhaust particles (DEP) have a carbon core and a vast number of organic compounds such as polyaromatic hydrocarbons, nitroaromatic hydrocarbons, heterocycles, quinones, aldehydes, and aliphatic hydrocarbons.[1–4] DEP can have hazardous effects on human health including lung cancer,[5,6] allergic rhinitis,[7,8] bronchial asthma-like disease,[9,10] and disruption of endocrine function.[11–13]

Previously it was reported that DEP extracts could induce arrhythmia and cardiovascular mortality in guinea pigs.[14] Furthermore, Toda *et al.*, have reported that DEP cause low blood pressure and arrhythmia in rats.[15] These findings suggest that DEP contain various chemical substances that affect cardiovascular function, but no such chemical compounds, have yet been isolated.

We recently reported on the successive fractionation of DEP with hexane, benzene, dichloromethane, methanol, ammonia, and HCl.[16] The hexane extract was further fractionated, and several fractions, that showed estrogenic and anti-estrogenic activities,[17] were successfully isolated and characterized.[18,19]

As a continuation of that research, we have turned our attention to the investigation of the benzene extract.

Here we report the isolation of nitrophenols from this DEP extract and the evaluation of the isolated compounds as vasodilators.[20]

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MATERIALS AND METHODS

DEP ——— DEP were collected, as described previously, from the diesel exhaust of a 4JB1-type engine manufactured by Isuzu Automobile Company, Tokyo, Japan.[21] The particles were produced from the diesel exhaust of light oil containing 0.05% sulfur. The DEP were kept in a sealed bottle at –20°C in the dark.

Reagents ——— Nitrophenols, 3-nitrophenol (**7**),

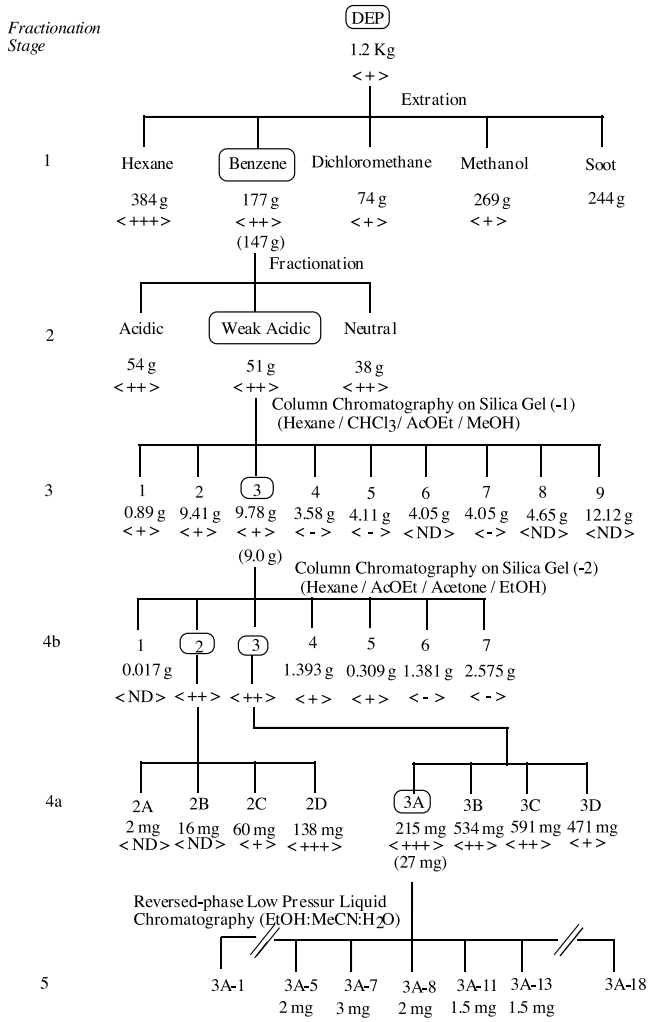

Fig. 1. Separation of Fractions Containing Compounds Positive for Vasodilation Activity
Vasodilatory activity, positive, +, ++, +++; negative, –; ND, not determined.

4-nitro-phenol (**1**), 2-methyl-4-nitrophenol (**3**) and 3-methyl-4-nitrophenol (**2**) used as authentic samples for GC-MS and ^{1}H-NMR measurement were purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. All other reagents used in this study were of HPLC or analytical reagent grades.

Isolation and Identification of Nitrophenols —— DEP (1.2 kg) were extracted successively with hexane, benzene, dichloromethane, and methanol, as described previously.[16] The benzene extract showed vasodilation activity. A 147 g aliquot of the benzene extract was fractionated into acidic, weak acidic, and neutral portions, following to the method described previously.[17] The compounds in the weak acid fraction (51 g) were further fractionated.

The weak acidic fraction was first subjected to gravity column chromatography through silica gel (900 g; Wakogel C-200, 75–150 μm, Wako Pure Chemical Industries Ltd., Osaka, Japan; column, 55 (w) × 700 (L) mm; volume of solvent, 3600 ml/fraction [Fr.]) with hexane (Fr. 1), 60% $CHCl_3$ in hexane (Fr. 2), 80% $CHCl_3$ in hexane (Fr. 3), $CHCl_3$ (Fr. 4), 10% ethyl acetate in $CHCl_3$ (Fr. 5), 30% ethyl acetate in $CHCl_3$ (Fr. 6), 60% ethyl acetate in $CHCl_3$ (Fr. 7), ethyl acetate (Fr. 8), and methanol (Fr. 9) as solvents. (Fig. 1, fractionation stage 3)

Fraction 3 (9.0 g) was further fractionated by gravity column chromatography (column, 60 × 1000 mm) through silica gel (900 g; Silica gel 60, 40–63 μm, Merck, Germany), and compounds were eluted successively with hexane (2600 ml), 10% ethyl acetate in hexane (Fr. 1A–D, 3600 ml), 30% ethyl acetate in hexane (Fr. 2A–D, 3600 ml), 50% ethyl acetate in hexane (Fr. 3A–D, 3600 ml), 70% ethyl acetate in hexane (Fr. 4A–D, 3600 ml), ethyl acetate (Fr. 5, 5000 ml), acetone (Fr. 6, 5000 ml) and

ethanol (Fr. 7, 6000 ml). (Fig. 1, fractionation stage 4b)

Finally, reversed-phase low-pressure liquid chromatography ([LiChroprep RP-18, 40–63 μm, size B [25 × 310 mm], Merck]), was applied to subfraction 3A. The low-pressure liquid chromatography was conducted using isocratic elution with ethanol: acetonitrile: water (2 : 3 : 5, v/v/v) with a flow rate of 2.0 ml/min for fractions 1–12 and 4.0 ml/min for the rest of fractions.

HPLC analyses of subfraction 3A were performed using a reversed-phase Shim-pack PREP-ODS (H) column (4.6 × 250 mm, Shimadzu, Kyoto, Japan), and isocratic elution with ethanol: acetonitrile: water (3 : 3 : 4, v/v/v) at a flow rate of 0.40 ml/min (see Table 1).

GC-MS ——— GC-MS was performed by using a GCMS-QP5000 (Shimadzu) instrument, equipped with a fused silica gel column (0.25 mm × 30 m; DB-1, J & W Scientific, CA, U.S.A.).

NMR ——— ^{1}H- and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively, in $CDCl_3$ (EX 400, JEOL, Tokyo, Japan). Chemical shifts are given in δ relative to tetramethylsilane (TMS, Tokyo Kasei Kogyo Co. Ltd.) as the internal standard.

Synthesis of 4-Nitro-3-Phenylphenol (4) ——— To a stirred aqueous solution of sulfuric acid, prepared by addition of 2.0 g (20 mmol) sulfuric acid to 4.7 g ice, we added 0.85 g (10.1 mmol) sodium nitrate followed by 5 ml ether. To the resulting mixture, which was cooled in an ice-bath, we added a solution of 1.70 g (10.0 mmol) 3-phenylphenol in 15 ml ether, at such a rate that the temperature was maintained at 0–4°C. Then a catalytic amount of sodium nitrate 17 mg, (0.25 mmol), was added to the mixture. After being stirred at an ice-bath temperature for 2 hr, the reaction was allowed to rise to ambient temperature, and the stirring was continued overnight. The reaction mixture was poured into 15 ml of ice-cold water, extracted with three 15 ml aliquots of ether, washed with 10 ml ice-cold aqueous 2% sodium bicarbonate solution, and dried over anhydrous magnesium sulfate. Evaporation of the solvent left 2.17 g of a thick dark- brown oil, which upon column chromatography through silica gel with hexane : ethyl acetate (8 : 2) as an eluent yielded 717 mg (yield, 33%) of 4-nitro-3-phenylphenol as a viscous pale-yellow oil. This product had the following characteristics: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.92 (d, J = 8.79 Hz, 1H), 7.39 (m, 3H), 7.27 (td, J = 1.95, 2.93, 7.81 Hz, 2H), 6.84 (dd, J = 2.93, 8.79 Hz, 1H), 6.79 (d, J = 2.93 Hz, 1H), 6.60 (br. S, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 159.4, 141.9, 139.8, 137.9, 128.5, 128.1, 127.7, 127.3, 118.6, 114.6; MS (EI) *m/z* 215 (M^+, 79), 198 (75), 187 (61), 170 (35), 159 (100), 139 (83), 131 (44), 115 (69), 89 (17), 77 (17), 77 (21), 63 (23); HRMS (EI) (M^+) calculated 215.0582, actual 215.0582; HRMS (EI) of fragment ions, $C_{12}H_8NO_2$ $(M\text{-}OH)^+$ calculated 198.0555, found 198.0578; $C_{11}H_9NO_2$ $(M\text{-}CO)^+$ calculated 187.0634, found 187.0624; $C_{11}H_8NO$ $(M\text{-}CO\text{-}OH)^+$ calculated 170.0606, actual 170.0604; $C_{12}H_8O$ $(M\text{-}OH\text{-}NO)^+$ calculated 168.0575, actual 168.0562; $C_{10}H_9NO$ $(M\text{-}2CO)^+$ calculated 159.0684, actual 159.0682; $C_{11}H_7$ $(M\text{-}OH\text{-}NO\text{-}CHO)^+$ calculated 139.0548, actual 139.0540; C_9H_7 $(M\text{-}OH\text{-}NO\text{-}CO\text{-}C_2H)^+$ calculated 115.0548, actual 115.0543.

Table 1. Fractionation of the Fraction 3A by Low- Pressure Liquid Chromatography[a)]

fraction	eluate (ml)	weight (mg)	identification of major component
3A-1	10	—	—
3A-2	40	1	—
3A-3	30	—	—
3A-4	40	1	—
3A-5	50	2	**1**
3A-6	2	—	—
3A-7	60	3	**2**
3A-8	40	2	**3**
3A-9	20	—	—
3A-10	20	—	—
3A-11	40	1.5	**4**
3A-12	55	—	—
3A-13	190	1.5	**5a–c**, **6**
3A-14	100	—	—
3A-15	100	—	—
3A-16	100	—	—
3A-17	90	—	—
3A-18	1000[b)]	14	alkanes

a) Sample loaded: 27 mg. Column: Lober Lichroprep, RP-18 (size B, 25 mm × 310 mm, Merck). Eluent: EtOH/MeCN/H_2O (2 : 3 : 5, v/v/v). Flow Rate: 2.0 ml/min for fractions 3A-1 through 3A-12, and 4.0 ml/min for fractions 3A-13 through 3A-17. UV detection: 320 nm and 254 nm. *b*) Eluent for this fraction ranged from EtOH/MeCN [1 : 1, v/v (600 ml)] to EtOH/MeCN [3.5 : 5, v/v (400 ml)] and flow rate was 0.75 ml/min.

Measurement of Vascular Relaxation ——— Vasodilatory activity was measured by relaxation of rat thoracic artery. Seven-month-old SPF F344 male rats (JCL: F344, CLEA Japan Inc., Tokyo, Japan) were used. The rats were maintained in a controlled room in terms of temperature (22 ± 1°C), humidity (50 ± 5%) and ventilation (25–30 times/hr). Lights

were cycles at 12 hr light-dark intervals. Food (CE-2 commercial diet, CLEA Japan Inc.) and water were given *ad libitum*.

The rat was anesthetized by pentobarbital sodium (50 mg/kg, *i.p.*). After bleeding, the thoracic artery of each rat was excised and cut into 3 mm rings. The artrial ring was located in a magmus tube with 1 g of tomus and incubated in Locke-Ringer's solution (NaCl, 153.8 mM; KCl, 5.63 mM; $CaCl_2$, 3.17 mM; glucose, 5.55 mM; $NaHCO_3$, 2.38 mM; pH 7.4) at 37°C under aeration with 95% O_2, 5% CO_2 for approximately 1 hr. After contraction of the thoracic artery rings with 10^{-6} M phenylephrine (PE), the nitrophenols (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) dissolved in PBS containing 0.05% Tween 80 were accumulatively added into an organ bath. The changes in tension were amplified (FD pickup TB-612T transducer and Multichannel amplifier MEG-6108, Nihon Kohden, Tokyo, Japan) and recorded (Phoenix, DKK-TOA Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Isolation of Nitrophenols from the Benzene Extract

The benzene extract was fractionated into acidic, weak acidic, and neutral fractions (Fig. 1).

The weak acidic fraction was subjected to gravity column chromatography to furnish three less polar fractions, denoted fractions 1, 2, and 3, which exhibited vasodilatory activity, whereas the remaining fractions had no effect on vasodilation. (Fig. 1, fractionation stage 3)

Fraction 3 was further fractionated through silica gel, and elution with a stepwise gradient of hexane-ethyl acetate (from 7 : 3 to 5 : 5), yielded six major subfractions, 2C, 2D and 3A–D, that were vasodilatory. (Fig. 1, factionation stage 4) Of these, the most potent fractions were 2D and 3A. The ^{1}H-NMR spectrum of fraction 3A was well-resolved and well-defined, suggesting the presence of phenolic compounds with an electron-withdrawing group substituted in the *p*-position. Therefore we selected fraction 3A for the isolation of the compounds responsible for vasodilation.

A typical HPLC chromatogram of fraction 3A is demonstrated in Fig. 2.

Fraction 3A was separated into 18 subfractions (3A-1 through -18) by monitoring their distinct UV absorptions using a low-pressure reversed-phase liquid chromatography with an isocratic elution with ethanol: acetonitrile: water (2 : 3 : 5, v/v/v). (Fig. 1, fractionation stage 5) The result of the fractionation was summarized in Table 1.

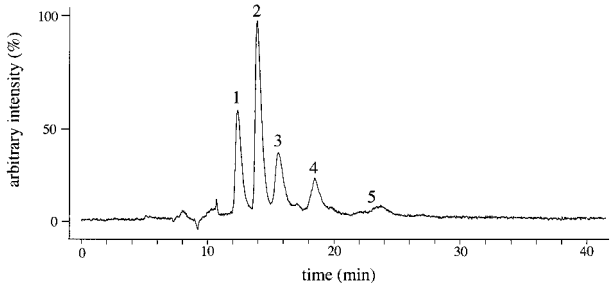

Fig. 2. HPLC Chromatogram of Fraction 3A

Peak 1 represents 4-nitrophenol (fraction 3A-5); peak 2, 3-methyl-4-nitrophenol (fraction 3A-7); peak 3, 2-methyl-4-nitrophenol (fraction 3A-8); peak 4, 4-nitro-3-phenylphenol (fraction 3A-11); peak 5, 4-nitro-3-tolyl-phenols (fraction 3A-13). Conditions: column, Shim-pack prep-ODS (4.6 × 250 mm); flow rate, 0.40 ml/min; solvent, EtOH/MeCN/water (3 : 3 : 4, v/v/v); UV detection at 320 nm.

Identification of Nitrophenols in Fractions 3A-5, 3A-7, 3A-8, and 3A-11

Fractions 3A-5, 3A-7, and 3A-8 were identified as 4-nitrophenol (**1**), 3-methyl-4-nitrophenol (**2**), and 2-methyl-4-nitrophenol (**3**) through direct comparison of the MS and ^{1}H-NMR spectra, and chromatographic behaviors with those of purchased standards.

Fraction 3A-11 was identified as 4-nitro-3-phenylphenol (**4**) by direct comparison of the MS characteristics, ^{1}H-NMR spectrum, and chromatographic behavior with those of the synthesized sample.

Nitrophenols Identified in Fraction 3A-13

The ^{1}HNMR and mass spectroscopic studies showed that fraction 3A-13 consists of three prominent phenylnitrophenols, 3-(2-methyl-phenyl)-4-nitro-phenol (**5a**), 3-(3-methyl-phenyl)-4-nitro-phenol (**5b**), 3-(4-methyl-phenyl)-4-nitrophenol (**5c**), and 5-methyl-4-nitro-3-phenylphenol (**6**) as a constrained isomer.

The structural assignment for **5a–c** and **6** was made by the followings; The ^{1}H-NMR spectrum of fraction 3A-13 showed three prominent sets of signals of approximately equal integration, with a constrained set of signals that strongly resembled those of 4-nitro-3-phenylphenol (**4**). *i.e.*, three doublet signals at δ 8.05 (J = 8.79 Hz), 7.91 (J = 9.28 Hz) and 7.89 (J = 9.28 Hz) in a 1.3 : 4.5 : 2.3 ratio as compared with the integration of the doublet at δ 6.59

Table 2. The Molecular Ions and Diagnostic Fragment Ions Observed in the GC-MS Spectra of the Fraction 3A-11 and 3A-13

Peak No.	RT. (min)/ GC-MS	M	molecular and fragment ions observed, *m/z* (%)					
			(M-CH_3)	(M-O)	(M-OH)	(M-CO)	(M-NO)	(M-CO-OH)
1[a]	20.33	215 (78)	—	199 (24)	198 (87)	187 (49)	185 (38)	170 (36)
2[b]	20.29	229 (61)	214 (3)	213 (11)	212 (57)	201 (8)	199 (24)	184 (35)
3[b]	20.66	229 (25)	214 (13)	213 (14)	212 (30)	201 (1)	199 (24)	184 (19)
4[b]	21.09	229 (22)	214 (17)	213 (7)	212 (35)	201 (47)	199 (46)	184 (36)
5[b]	21.28	229 (77)	214 (14)	213 (13)	212 (46)	201 (10)	199 (36)	184 (60)

Peak No.	molecular and fragment ions observed, *m/z* (%)						identification
	(M-NO_2)	(M-2CO)	(M-2CO-H)	(M-OH-NO-CHO)	C_7H_7	C_6H_5	of compound
1[a]	169 (9)	159 (100)	158 (24)	139 (93)	91 (11)	77 (39)	**4**
2[b]	183 (22)	173 (12)	172 (43)	153 (28)	91 (14)	77 (31)	**5a**[c]
3[b]	183 (14)	173 (7)	172 (11)	153 (18)	91 (8)	77 (10)	**6**[c]
4[b]	183 (21)	173 (23)	172 (90)	153 (15)	91 (18)	77 (23)	**5b**[c]
5[b]	183 (17)	173 (12)	172 (68)	153 (15)	91 (18)	77 (26)	**5c**[c]

a) Detected in the fraction 3A-11. *b*) Detected in the fraction 3A-13 and the base peak of these peaks was the ion of *m/z* 43. *c*) Tentative identification.

(*vide infra*), and three blocks of doublet peaks at δ 6.84 (J = 8.79, 2.93 Hz), 6.82 (J = 9.28, 2.44 Hz) and 6.81 (J = 8.79, 2.93 Hz), together with overlapping peaks of apparent doublets at around δ 6.72 ppm, indicating that it seems reasonable to interpret the spectrum as originated from three 3-substituted-4-nitrophenols that are present in the fraction as major components. In addition, there were four predominant singlets due to aromatic methyl groups at δ 2.39, 2.37, 2.31, and 2.08 in an approximate ratio of 2.6 : 4.8 : 1.3, which roughly corresponds to that of the signals in the aromatic region described above. Thus the structure of the three compounds are assigned to **5a**, **5b** and **5c**. Two separate peaks of doublets at δ 6.65 (J = 2.44 Hz) and 6.59 (J = 2.44 Hz) of equal integration were assigned to be the two protons *ortho* to the hydroxyl group of a 3,4,5-substituted-phenol. These two peaks of doublets and the multiplets near δ 7.37, which resembled those due to the phenyl protons of **4**, in conjunction with a signal of methyl protons, strongly suggested that the fourth component of the fraction is 5-methyl-4-nitro-3- phenylphenol (**6**).

In support of these above assignments, the mass spectra exhibited the characteristic ion peaks that were expected for the compounds **5a–c** and **6**. The selected data of the spectra are presented in Table 2, peak numbers 2–5. Thus, the presence of molecular ions at *m/z* 229, and ion peaks at *m/z* 214 (M-CH_3), 212 (M-OH), 199 (M-NO), 184 (M-CO-OH), and 183 (M-NO_2) common to the four spectra, demonstrates the validity of the identifications.

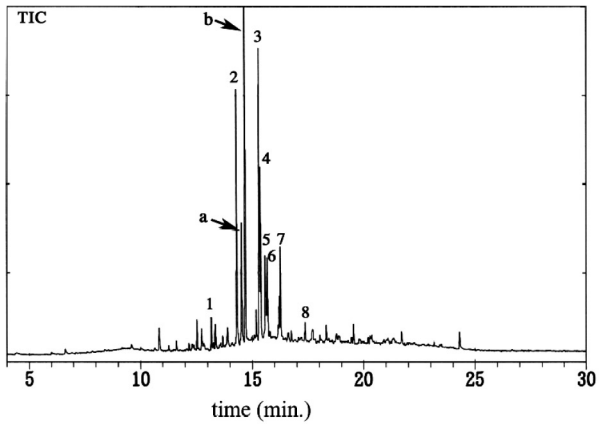

Fig. 3. GC-MS Total Ion Current Chromatogram of the Fraction 2D

The peak numbers correspond to those of Table 2. The peaks a and b were identified as 3-methyl-4-nitrophenol (**2**) and 2-methyl-4-nitrophenol (**3**), respectively.

Nitrophenols Identified and Detected in Fraction 2D

The ^{1}H-NMR spectrum of the fraction 2D was too complex to be analyzed, however, the GC-MS total ion chromatogram was well resolved to the analysis of the peaks as shown in Fig. 3. Eight prominent peaks observed in the chromatogram gave satisfactory MS spectra, the selected data of which are given in Table 3. The compound of the peak 1 (retention time of 13.19 min) was identified as 3-nitrophenol (**7**) by comparison of mass spectral data with those of an authentic sample measured under identical conditions; *m/z* 139 (M^+, base peak), *m/z* 123 (5%, M-O), *m/z* 109 (11%, M-NO), and *m/z* 93

Table 3. The Molecular Ions and Diagnostic Fragment Ions Observed in the GC-MS Spectra of the Fraction 2D

Peak No.	RT. (min)/	molecular and fragment ions observed, *m/z* (%)					
	GC-MS	M	(M-CH_3)	(M-O)	(M-OH)	(M-NO)	(M-CO-OH)
1	13.19	139 (100)	123 (5)	122 (< 1)	109 (11)	—	94 (4)
2	14.33	167 (100)	152 (1)	151 (12)	150 (98)	137 (11)	122 (17)
3	15.34	167 (20)	152 (2)	151 (14)	150 (100)	137 (2)	122 (25)
4	15.41	167 (63)	152 (2)	151 (13)	150 (100)	137 (5)	122 (20)
5	15.6	167 (69)	152 (2)	151 (16)	150 (100)	137 (9)	122 (13)
6	15.7	167 (98)	152 (100)	151 (5)	150 (14)	137 (20)	122 (5)
7	16.29	181 (16)	166 (2)	165 (13)	164 (100)	151 (2)	136 (21)
8[c)]	17.38	195 (12)	180 (1)	179 (12)	178 (84)	165 (4)	150 (10)

Peak No.	molecular and fragment ions observed, *m/z* (%)					identification
	(M-NO_2)	(M-NO-CO)	(M-NO-CHO)	C_7H_7	C_6H_5	of compound
1	93 (47)	81 (22)	—	—	—	**7**[a)]
2	121 (8)	109 (4)	108 (5)	91 (43)	77 (67)	**8a**[b)]
3	121 (6)	109 (3)	108 (13)	91 (23)	77 (38)	**8b**[b)]
4	121 (6)	109 (3)	108 (5)	91 (45)	77 (61)	**8c**[b)]
5	121 (6)	109 (3)	108 (3)	91 (49)	77 (74)	**8d**[b)]
6	121 (9)	109 (4)	108 (2)	91 (29)	77 (45)	**9**[b)]
7	135 (5)	123 (4)	122 (14)	91 (20)	77 (33)	**10**[b)]
8[c)]	149 (11)	137 (5)	136 (41)	91 (17)	77 (24)	**11**[b)]

a) The structure of this compound was confirmed by comparison of the GC-MS spectrum of an authentic sample. *b*) Tentative assignment. *c*) The base peak was the ion of *m/z* 43.

(91%, M-NO_2-CO).

The compound in peak 2 (*Rt.*, 14.33 min) was characterized as 3-methyl-2-nitrophenol with an additional methyl group substituted in either the 4-, 5-, or 6-position (**8a**). Other three compounds represented by peaks 3–5 (15.34 min, 15.41 min, and 15.60 min) were assigned as dimethylnitrophenols (**8b–d**) in which one of the methyl groups occupies *ortho* of the nitro group. The mass spectra of peaks 2, 3, 4, and 5 showed the distinct molecular ion peaks at *m/z* 167 with various intensities, the ion of peak 2 being the base peak.

In conjunction with the other characteristic ion peaks presented in Table 3, the compounds in these four peaks were characterized as the constitutional isomers of dimethyl-nitro-phenols. The pronounced peak at *m/z* 150 (loss of OH) in each spectrum, which appeared as the base peak except for that (98%) of peak 2, was highly diagnostic to indicate that one of the methyl groups is *ortho* to the nitro group, since the *M*-17 peak is often the base peak of nitrocresols in which the methyl and nitro groups are allocated in an *ortho* position, whereas the peak is very weak or unobservable in the spectra of nitrocresols substituted differently. Thus the compounds in peak 2–5 were characterized as **8a–d**.

The compound of peak 6 was assigned to ethylnitrophenol (**9**). In the mass spectrum, peak 6 showed the molecular ion peak at *m/z* 167 (98%) as those of peaks 2–5, however, the *M*-15 peak (loss of CH_3) was the base peak. These findings, in conjunction with the other fragment ions, clearly designated the structure of an ethylnitrophenol (**9**).

Peak 7 gave rise to the molecular ion peak at *m/z* 181 (16%) followed by the *M*-17 peak (loss of OH) as the base peak showed the compound to be trimethylnitrophenol (**10**). The fragmentation pattern of the spectrum was similar to those of peaks 2–5, supporting the structure of trimethylnitrophenol (**10**) in which one of the methyl groups is positioned at *ortho* to the nitro group.

The mass spectrum of peak 8 showed the molecular ion peak at *m/z* 195 (12%) and the second most intense peak at *m/z* 178 (84%, M-OH). Both of which, coupled with the similarity of the fragmentation pattern to those of peaks 2–5 and 7, appointed a tetramethylnitrophenol (**11**), in which a methyl group is necessarily allocated *o*-position of the nitro group.

The structures of the nitrophenols identified in the present study are summarized in Fig. 4.

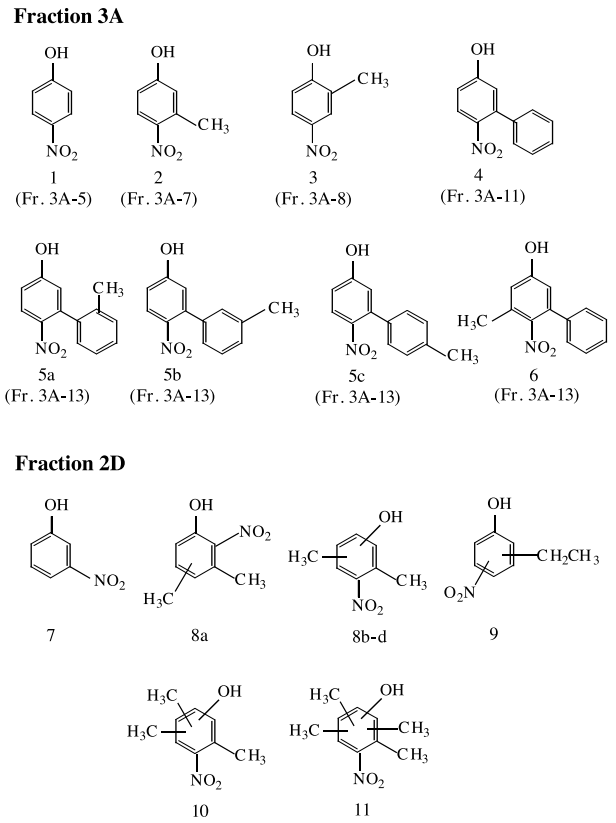

Fig. 4. Nitrophenols Identified or Detected in Fraction 3A and 2D

Measurement of Contents of Nitrophenol Derivatives

The contents of **1**, **2**, **3**, and **4** were estimated by GC-MS as 15, 28, 34, and 15 mg/kg DEP, respectively. Although we were unable to measure the content of compound **7** , it was much less than those of compounds **1–4**.

Vasodilation Activity of Nitrophenols

Table 4 shows the lowest concentration at which the nitrophenols we isolated from DEP cause vasodilation in the rat thoracic artery assay.

4-Nitro-3-phenylphenol (**4**) had the highest vasodilation activity (10^{-6} M), whereas, 4-nitrophenol (**1**), 2-methyl-4-nitrophenol (**3**) and 3-methyl-4-nitrophenol (**2**), exhibited vasodilatory activity (in the range of 10^{-5} M), and 3-nitrophenol (**7**) showed weak activity (10^{-4} M).

However, those activities were 10^{2}–10^{4} times weaker than acethylcoline (Ach).

No relaxation was observed with the vehicle containing 0.05% Tween 80. The magnitude of relaxation of the artery caused by nitrophenols were not dependent on cytotoxic effect, because after vasodilation with this compound, the artery was constricted again by the addition of PE or KCl.

Table 4. The Lowest Concentration for Vasodilation by Nitrophenols Isolated from DEP or Acetylcholine

Compounds	Concentration (M)
3-nitrophenol	10^{-4}
4-nitrophenol	10^{-5}
3-methyl-4-nitrophenol	10^{-5}
2-methyl-4-nitrophenol	10^{-5}
4-nitro-3-phenylphenol	10^{-6}
Ach	10^{-8}

Ach: acethylcholine.

Ours is the first report on the isolation from DEP of compounds responsible for vasodilation, *i.e.*, five nitrophenols (**1–4**, and **7**).

In their epidemiology studies, Dockery *et al.* found that cardiovascular mortality and morbidity are associated with exposure concentration of particulate matter in air.[22,23] Most of the particulate matter in urban ambient air pollutants in Japan is thought be due to DEP. Like others, we believe that the nitrophenol compounds we isolated from DEP could adversely affect human health by impairing cardiovascular functions.

Nishioka *et al.* reported that nitrophenols present in the particulate matter of air are mutagenic compounds.[24] 3-Methyl-4-nitrophenol is a known degradation product of the insecticide fenitrothion,[25] which is used widely in many countries and is being accumulated in air.[24,26] Furthermore, 4-nitrophenol is a degradation product of the insecticide parathion.[27]

The results of our present study indicate that as a result of diesel exhaust emission and the degradation of various pesticides, the accumulation of nitrophenols (including 3-methyl-4-nitrophenol) in the air and soil could have serious deleterious effects on human health because of disturbance of the cardiovascular system.

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