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Isolation and Identification of New Vasodilative Substances in Diesel Exhaust Particles

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Abstract

Goal, Scope, and Background We recently developed a new isolation method for diesel exhaust particles (DEP), involving successive extraction with H₂O, sodium bicarbonate, and sodium hydroxide, in which the sodium hydroxide extract was found to consist of phenolic components. Analysis of the extract revealed that vasodilative active nitrophenols are in DEP in significantly higher concentration than those estimated by an earlier method involving a combination of solvent extraction and repeated chromatography. These findings indicated that our new procedure offers a simple, efficient and reliable method for the isolation and identification of bioactive substances in DEP. This encouraged us to extend our work to investigating new vasodilatory substances in the sodium bicarbonate extract.

Materials and Methods DEP were collected from the exhaust of a 4JB1-type engine (ISUZU Automobile Co., Tokyo, Japan). GC-MS analysis was performed with a GCMS-QP2010 instrument (Shimadzu, Kyoto, Japan).

Results DEP dissolved in 1-butanol was successively extracted with water, sodium bicarbonate, and then aqueous sodium hydroxide. The sodium bicarbonate extract was neutralized and the resulting mixture of acidic components was subjected to reverse-phase (RP) column chromatography followed by RP-HPLC with fractions assayed for vasodilative activity. This led to the identification of telephthalic acid, *p*-hydroxybenzoic acid, isophthalic acid, phthalic acid, 3-hydroxy-4-nitrobenzoic acid, 4-hydroxy-3-nitrophenol, and 1,4,5-naphthalene tricarboxylic acid as components of DEP.

Discussion The sodium bicarbonate extract was rich in the acidic components. Repeated reverse-phase chromatography resulted in the successful isolation of several acidic substances including the new vasodilative materials, 4-hydroxy-3-nitrobenzoic acid and 3-hydroxy-4-nitrobenzoic acid.

Conclusion Our new fractionation method for DEP has made possible the isolation of new vasodilative compounds from the sodium bicarbonate extract.

Keywords Acidic components; Diesel exhaust particles; Fractionation; 3-Hydroxy-4-nitrobenzoic acid; 4-hydroxy-3-nitrobenzoic acid; Separation method; Vasodilatory compounds

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Fig. 1

2.2 Reagents

Authentic samples of terephthalic acid (**1**), 4-hydroxybenzoic acid (**2**), isophthalic acid (**3**), dimethyl phthalate, dimethyl 4-methylphthalate, methyl 4-hydroxy-3-nitrobenzoate, methyl 3-hydroxy-4-nitrobenzoate, and trimethyl 1,4,5-naphthalenetetracarboxylate for GC-MS analysis were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). All other reagents were of analytical reagent grade.

2.3 Instrumentation

GC-MS analysis was performed at 70 eV with a GCMS-QP2010 instrument (Shimadzu, Kyoto, Japan) equipped with a DB-5ms capillary GC column (0.25 mm $\times$ 30 m; Shimadzu, Kyoto, Japan). Analysis was carried out at a column temperature of 80–200°C. NMR — ^1H - and ^{13}C -NMR spectra were recorded at 400 MHz, in CDCl_3 (EX 400, JEOL, Tokyo, Japan). Chemical shifts are given in δ relative to tetramethylsilane (TMS, Tokyo Kasei Kogyo Co., Ltd. Tokyo, Japan) as the internal standard.

2.4 Measurement of Vascular Relaxation —Measurement of vasodilatory activity was made by using seven-month-old SPF F344 male rats (JCL:F344, CLEA Japan, Inc., Tokyo, Japan), which were maintained in a controlled room in terms of temperature ($22 \pm 1^\circ\text{C}$), humidity ($50 \pm 5\%$) and ventilation (25–30 times/h). Lights were cycles at 12 h light-dark intervals. Food (CE-2 commercial diet, CLEA Japan, Inc.) and water were given *ad libitum*. The rats were 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 arterial ring was located in a magnum tube with 1 g of tonus and incubated in Locke-Ringer's solution (NaCl, 153.8 mM; KCl, 5.63 mM; CaCl₂, 3.17 mM; glucose, 5.55 mM; NaHCO₃, 2.38 mM; pH 7.4) at 37 °C under aeration with 95% O₂ and 5% CO₂ for approximately 1 h. After contraction of the thoracic artery rings with 10⁻⁶ M phenylephrine (PE), substrates (4-methylphthalic acid, 4-hydroxy-3-nitrobenzoic acid and 3-hydroxy-4-nitrobenzoic acid), dissolved in PBS (10⁻⁶, 10⁻⁵, 10⁻⁴, 10⁻³, 10⁻² and 10⁻¹ M) 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) (cf. Mori et al. 2003a).

2.5 Extraction procedure from diesel exhaust particles (DEP)

DEP (10 g) was dissolved in 1-butanol (300 ml), and was first washed with water, then extracted with a saturated aqueous solution of sodium bicarbonate (NaHCO₃; 100 ml × 5), and subsequently extracted with 10% aqueous NaOH (100 ml × 3).

The combined NaHCO₃ extracts were neutralized with 10% HCl, and then the solution was extracted with dichloromethane (100 ml × 3). After drying over anhydrous sodium sulfate, the organic solution was evaporated to give an oily residue (1.6 g). The resulting oil was subjected to preparative HPLC (Inertsil ODS-3, GL Science) with water containing 0.1 % formic acid, acetonitrile, and 1-butanol (70 : 15 : 15), to give 12 fractions (F1 – F12) (9.6 ml each). Samples from each fraction were tested for activity in the vascular relaxation assay. The fractions 3 (F3) (22.7 mg) and 4 (F4) (25.2 mg), that revealed significant vascular relaxation activity, were re-fractionated by HPLC (water containing 0.1 % formic acid and acetonitrile = 70 : 30) with monitoring of the UV-absorption at 254 nm. Thus, F3 was fractionated into eight subfractions; F3.1 (2.0 mg), F3.2 (2.2 mg), F3.3 (1.5 mg), F3.4 (1.1 mg), F3.5 (4.9 mg), F3.6 (1.4 mg), F3.7 (3.7 mg), F3.8 (3.1 mg). Similarly, F4 was separated into six subfractions; F4.1 (1.2 mg), F4.2 (1.4 mg), F4.3 (1.9 mg), F4.4 (0.6 mg), F4.5 (2.5 mg), F4.6 (7.3 mg) (Figure 2). Preliminary assays revealed that F3.5, F4.1, and F4.3 possessed vasodilative activity.

3 Results

Spectroscopic studies (LC-MS, GC-MS, ¹H-NMR) of each subfraction revealed that F3.1 was a mixture of telephthalic acid (**1**) and 4-hydroxybenzoic acid (**2**), F3.2, F3.3, and F3.5 were ascribed to isophthalic acid (**3**), phthalic acid (**4**), and 4-methylphthalic acid (**5**), respectively (Figure 3). Compounds **1**, **2**, and **3** were identified by comparison of their ¹H-NMR spectra and chromatographic behaviors with those of the authentic samples: ¹H-NMR of **1** (CD₃OD) δ: 8.09 (4H, s, H-2, H-3, H-5 and H-6), ¹H-NMR (CD₃OD) of **2** δ: 6.79 (2H, dt, *J* = 8.6, 2.3 Hz, H-3 and H-5), 7.86 (2H, dt, *J* = 8.6, 2.3 Hz, H-2 and H-6)), F3.2, F3.3, and ¹H-NMR (CD₃OD) of **3** δ: 7.58 (1H, t, *J* = 7.5 Hz, H-5), 8.21 (2H, dd, *J* = 1.8, 7.5 Hz, H-4 and H-6), 8.64 (1H, t, *J* = 1.8 Hz, H-2). The structures of phthalic acid (**4**, ¹H-NMR (CD₃OD) δ: 7.56 (2H, dd, *J* = 5.7, 3.5 Hz, H-3 and H-4), 7.72 (2H, dt, *J* = 5.7, 3.5 Hz, H-2 and H-5)) and 4-methylphthalic acid (**5**, ¹H-NMR (CD₃OD) δ: 2.41 (3H, s, C⁴-CH₃), 7.37 (1H, d, *J* = 8.0 Hz, H-5), 7.49 (1H, s, H-3), 7.66 (1H, d, *J* = 8.0 Hz, H-6)) were confirmed by comparison of the GC-MS data of their methyl ester derivatives (**6** and **7**) with those of the authentic samples: MS for **6** (*m/z*); 194 (*M*⁺, 10 %), 183 (base peak), **7** (*m/z*); *M*⁺, 208 (15%), 177 (base peak). Similarly, F4.1, F4.3, and F4.6 were identified as 4-hydroxy-3-nitrobenzoic acid (**8**) (¹H-NMR (CD₃OD) δ: 7.19 (1H, d, *J* = 8.6 Hz, H-5), 8.17 (1H, dd, *J* = 1.7, 8.6 Hz, H-6), 8.65 (1H, d, *J* = 1.7 Hz, H-2)), 3-hydroxy-4-nitrobenzoic acid (**9**) (¹H-NMR (CD₃OD) δ: 7.57 (1H, dd, *J* = 1.7, 8.6 Hz, H-6), 7.71 (1H, d, *J* = 1.7 Hz, H-2), 8.07 (1H, d, *J* = 8.6 Hz, H-5)), and 1,4,5-naphthalenetetracarboxylic acid (**10**), respectively (Figure 4) by comparison of the GC-MS spectra of their methyl esters (**11**, **12**, **13**) with those of authentic samples (Figure 5, 6, 7). The result of the fractionation was summarized in Table 1. 4-Hydroxy-3-nitrobenzoic acid (**8**), and 3-hydroxy-4-nitrobenzoic acid (**9**) clearly demonstrated vasodilative activity. 4-Methylphthalic acid (**5**) showed vasodilative activity, though the effect was only transient and weaker than **8** and **9**. The lowest concentrations of 4-methylphthalic acid (**5**), 4-hydroxy-3-nitrobenzoic acid (**8**), and 3-hydroxy-4-nitrobenzoic acid (**9**) causing vasodilatory activity were estimated as ~10⁻³ M (Figure 8).

4 Discussion

Although a number of studies have demonstrated epidemiologically important results, none of the investigations focused on a systematic separation and characterization of the bioactive components of DEP until our initial work on the isolation and identification of such entities by using a method involving successive solvent extractions, and subsequent repeated column chromatography. In spite of poor fractionation and a significant lack of accuracy and reproducibility associated with this method, such isolated compounds as phenanthrenes (Mori et al. 2003b), dibenzothiyophenes (Mori et al. 2003c), alkylphenols (Taneda et al. 2004c), hydroxyphthalic acids (Mori et al. 2003d), and vasodilative active nitrophenols (Mori et al. 2003a) suggest that the constituents of DEP can be broadly classified into the following groups: (i) aromatic carboxylic acids, (ii) phenol derivatives, (iii) non-combustible neutral compounds. They are thus amenable to separation by using a conventional acid-base extraction procedure.

Based on this concept, we have developed the new fractionation scheme used for this work (Noya et al. 2008). The sodium bicarbonate extract obtained by this procedure has been shown to contain various aromatic carboxylic acids including the new vasodilatory nitro-compounds, 4-hydroxy-3-nitrobenzoic acid (**8**) and 3-hydroxy-4-nitrobenzoic acid (**9**). 4-Methylphthalic acid (**5**) revealed vasodilative activity, but the effect was only temporary and feeble, suggesting that different pharmacological mechanism might be involved in **5** from that of nitrophenols.

Although vascular relaxation effects of the newly isolated 4-hydroxy-3-nitrobenzoic acid (**8**) and 3-hydroxy-4-nitrobenzoic acid (**9**) are much weaker ($\sim 10^{-3}$ M) than those reported for nitrophenols (active at threshold concentrations of $\sim 10^{-4}$ M), the contents of the newly isolated vasodilatory components are as high as those of nitrophenols (Table 1). These findings may be important from the environmental perspective, as remarkable amounts of DEP are exhausted into the air; for example, 58902 t in Japan (Japan Environmental Agency 1998), 111 530 t in the United States (United States Environmental Protection Agency 1999), 37 000 t in England (Airborne Particles Expert Group 1999), and 240 000 t in Europe (Petroleum Energy Center Japan 1999). Many automobile companies either in Japan or European countries are competitively developing modern Diesel cars equipped with different types of exhaust treatment such as a particulate trap/filter to detoxify diesel exhaust. Swiss researchers very recently investigated the secondary effects of catalytic diesel particulate filters on the conversion of polyaromatic hydrocarbons (PAHs) versus formation of nitro-polyaromatic hydrocarbons (nitro-PAHs), whereby PAHs were reduced by using filters, while they noticed the formation of selected nitro-PAHs (Norbert V. Heeb et al. 2008). Although our present investigation was performed on the DEP collected from an engine without using a filter, we have demonstrated significant amounts of vasodilative nitro compounds are consisted in DEP, and their hazardous effects on human health cannot be ignored because of the vast amount of DEP emitted into the environment.

5 Conclusions

Our present work reports new vasodilative active nitro substances in DEP, which are released in significant amounts from Diesel engines into the air. Because of the simple and systematic isolation procedure, the present analytical method, involving a combination of acid-base extraction and subsequent chromatography with monitoring of bioactivity, would serve as a facile and efficient method for identification of such bio-hazardous substances as vasodilator, carcinogen, and in DEP.

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References

- Airborne Particles Expert Group (1999) Source apportionment of airborne particulate matter in the Unnited Kingdom. United Kingdom Department of Environment, Transport and Regions, The Welsh Office, the Scottish Office and the Department of Environment (north Ireland) 9–37.
- Bayona JM., Markides KE, Lee M (1988) Characterization of polar polycyclic aromatic compounds in a heavy-duty diesel exhaust particulate by capillary column gas chromatography and high-resolution mass spectrometry. *Environ Sci*

Technol 22:1440–1447

- Draper WM (1986) Quantitation of nitro- and dinitropolycyclic aromatic hydrocarbons in diesel exhaust particulate matter. *Chemosphere* 15:437–447
- Erdinger L, Durr M, Hopker KA (2005) Correlations between mutagenic activity of organic extracts of airborne particulate matter, NO_x, and sulphur dioxide in southern Germany – Results of a two-year study. *Env Sci Pollut Res* 12:10–20
- Furuta C, Li CM, Taneda S, Suzuki AK, Kamata K, Watanabe G, Taya K (2005) Immunohistological study for estrogenic activities of nitrophenols in diesel exhaust particles. *Endocrine* 27:33–36
- Furuta C, Suzuki AK, Taneda S, Kamata K, Hayashi H, Mori Y, Li C, Watanabe G, Taya K (2004) Estrogenic activities of nitrophenols in diesel exhaust particles. *Biol Report* 70:1527–1533
- Hayatsu M, Hirano M, Tokuda S (2000) Involvement of two plasmids in fenitrothion degradation by *Burkholderia* sp. strain NF100. *Appl Environ Microbiol* 66:1737–1740
- Ichinose T, Yajima Y, Nagashima M, Takenoshita S, Nagamachi Y, Sagai M (1997) Lung carcinogenesis and formation of 8-hydroxy-deoxyguanosine in mice by diesel exhaust particles. *Carcinogenesis* 18:185–192
- Japan Environmental Agency (1998) The research on the total amount of emission and automobile exhaust source unit-the NO_x according to object automobile in the usual transit 200–203
- Kim TS., Kim JK, Choi K, Stenstrom MK, Zoh KD (2006) Degradation mechanism and the toxicity assessment in TiO₂ photocatalysis and photolysis of parathion. *Chemosphere* 62:926–933
- Li CM, Takahashi S, Taneda S, Furuta C, Watanabe G, Suzuki AK, Taya K (2006a) Impairment of testicular function in adult male Japanese quail (*Coturnix japonica*) after a single administration of 3-methyl-4-nitrophenol in diesel exhaust particles. *J Endocrinol* 189:555–564
- Li CM, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K (2006b) Anti-androgenic activity of 3-methyl-4-nitrophenol in diesel exhaust particles. *Eur J Pharmacol* 543:194–199
- Li CM, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K (2006c) Estrogenic and anti-androgenic activities of 4-nitrophenol in diesel exhaust particles. *Toxicol Appl Pharmacol* 217:1–6
- Li CM, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K (2006d) Effects of 3-methyl-4-nitrophenol in diesel exhaust particles on the regulation of testicular function in immature male rats. *J Androl* 28:252–258
- McClellan RO (1987) Health effects of exposure to diesel exhaust particles. *Annu Rev Pharmacol Toxicol* 27:279–300
- Mestres R (2005) Green chemistry – Views and strategies. *Env Sci Pollut Res* 12:128–132
- Miyabara Y, Ichinose T, Takano H, Lim HB, Sagai M (1998) Effects of diesel exhaust on allergic airway inflammation in mice. *J Allergy Clin Immunol* 102:805–812
- Mori Y, Kamata K, Toda N, Hayashi H, Seki K, Taneda S, Yoshino S, Sakushima A, Sakata M, Suzuki AK (2003a) Isolation of nitrophenols from diesel exhaust particles (DEP) as vasodilatation compounds. *Biol Pharm Bull* 26:394–395
- Mori Y, Taneda S, Kamata K, Sakushima A, Hayashi H., Suzuki AK, Sakata M, Yoshino S, Sagai M, Seki K (2003b) Identification of Phenanthrene and Related Compounds in Diesel Exhaust Particles by Gas Chromatography-mass Spectrometry. *Environmental Sciences* 10:187–192.
- Mori Y, Taneda S, Kamata K, Sakushima A, Hayashi H., Suzuki AK, Sakata M, Yoshino S, Sagai M, Seki K (2003c) Identification of Alkyldibenzothiophenes in Diesel Exhaust Particles by Gas Chromatography-mass Spectrometry. *Environmental Sciences* 10:157–164.
- Mori Y, Taneda S, Sakushima A, Hayashi H, Kamata K, Suzuki AK, Sakata M, Yoshino S, Sagai M, Seki K (2003d) Isolation and Characterization of Hydroxyphthalate Derivatives in Diesel Exhaust Particles. *Environmental Sciences* 10: 51–54
- Morville S, Scheyer A, Mirabel P, Millet M (2006) Spatial and geographical variations of urban, suburban and rural atmospheric concentrations of phenols and nitrophenols. *Env Sci Pollut Res* 13:83–89
- Muranaka M, Suzuki S, Koizumi K, Takafuji S, Miyamoto T, Ikemori R, Tokiwa H (1986) Adjuvant activity of diesel-exhaust particulates for the production of IgE antibody in mice. *J Allergy Clin Immunol* 77:616–623
- Norbert V. Heeb, Peter Schmid, Martin Kohler, Erika Gujer, Markus Zennegg, Daniela Wenger, Adrian Wichser, Andrea Ulrich, Urs Gfeller, Peter Honegger, Kerstin Zeyer, Lukas Emmenegger, Jean-Luc Petermann, Jan Czerwinski, Thomas Mosimann, Markus Kasper, and Andreas Mayer (2008) Secondary effects of catalytic diesel particulate filters: conversion of PAHs versus Formation of nitro-PAHs. *Environ. Sci. Technol* 42:3773–3779
- Noya, Y Mikami, Y Taneda, S Mori, Y Suzuki, A. K Ohkura, K Yamaki, K Yoshino, S, Seki K-I (2008) Improvement of a Facile and Efficient Separation Method for Chemicals in Diesel Exhaust Particles: Analysis of the Contents of Nitrophenols. *Env Sci Pollut Res* 15:318–321.
- Ona LF, Melinda A, Alberto P, Prudente JA, Sigua GC (2006) Levels of lead in urban soils from selected cities in a central region of the Philippines. *Env Sci Pollut Res* 13:177–183
- Petroleum Energy Center Japan (1999) Workshop report with the European experts 9-37.
- Sagai M, Furuyama A, Ichinose T (1996) Biological effects of diesel exhaust particles (DEP). III. Pathogenesis of asthma like symptoms in mice. *Free Radical Biol Med* 21:199–209
- Sagai M, Saito H, Ichinose T, Kodama M, Mori Y (1993) Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. *Free Radical Biol Med* 14:37–47
- Schuetzle D (1983) Sampling of vehicle emissions for chemical analysis and biological testing. *Environ Health Perspect* 47:65–80
- Schuetzle D, Lewtas J (1986) Bioassay-directed chemical analysis in environmental research. *Anal Chem* 58:1060A–1075A
- Seidel B, Alm M, Peters R, Kordell W, Schaffer A (2006) Safety evaluation for a biodiesel process using prion-contaminated animal fat as a source. *Env Sci Pollut Res* 13:125–130
- Takafuji S, Suzuki S, Koizumi K, Tadokoro K, Miyamoto T, Ikemori R, Muranaka M (1987) Diesel-exhaust particulates inoculated by the intranasal route have an adjuvant activity for IgE production in mice. *J Allergy Clin Immunol* 79:639–645
- Taneda S, Kamata K, Hayashi H, Toda N, Seki K, Sakushima A, Yoshino S, Yamaki K, Sakata M, Mori Y, Suzuki AK

- (2004a) Investigation of vasodilatory substances in diesel exhaust particles (DEP) Isolation and identification of nitrophenol derivatives. *J Health Sci* 50:133–141
- Taneda S, Mori Y, Kamata K, Hayashi H, Furuta C, Li C, Seki K, Sakushima A, Yoshino S, Yamaki K, Watanabe G, Taya K, Suzuki AK (2004b) Estrogenic and anti-androgenic activity of nitrophenols in diesel exhaust particles (DEP). *Biol Pharm Bull* 27:835–837
- Taneda S, Mori Y, Sakushima Akiy, Kamata K, Hayashi H, Seki K, Sakata M, Yoshino S, Yamaki K, Sagai M, and Suzuki AK, (2004c) Separation and characterization of alkyltrimethylbenzene derivatives in diesel exhaust particles (DEP). *Environmental Sciences*, 11, 171-178.
- Tsukue N, Toda N, Sagai M, Watanabe G, Taya K, Tsubone H, Suzuki AK (2002) Diesel exhaust particulate extract (DEPE)-induced abnormal parturition associated with increased myometrial contractility in C57BL mice. *Environ Sci* 9:355–367
- Tsukue N, Toda N, Tsubone H, Sagai M, Jin WZ, Watanabe G, Taya K, Birumachi J, Suzuki AK (2001) Diesel exhaust (DE) affects the regulation of testicular function in male Fischer 344 rats. *J Toxicol Environ Health A* 63:115–126
- United States Environmental Protection Agency (1999) Analysis of the impacts of control program on motor vehicle toxics emissions and exposure in urban areas and nationwide EPA420-R-99-029:120.
- Watanabe N, Oonuki Y (1999) Inhalation of diesel engine exhaust affects spermatogenesis in growing male rats. *Environ Health Perspect* 107:539–544
- Yoshida S, Sagai M, Oshio S, Umeda T, Ihara T, Sugamata M, Sugawara I, Takeda K (1999) Exposure to diesel exhaust affects the male reproductive system of mice. *Int J Androl* 22:307–315

Table 1. Representative components isolated from the sodium bicarbonate extract of DEP (mg/kg)

Compound (Fraction)	3 ^a (F3.2)	4 ^b (F3.3)	5 ^c (F3.5)	8 ^d (F4.1)	9 ^e (F4.3)	10 ^f (F4.6)
Content	220	150	490	120	190	730 mg

a) isophthalic acid, b) phthalic acid, c) 4-methylphthalic acid, d) 4-hydroxy-3-nitrobenzoic acid, e) 3-hydroxy-4-nitrobenzoic acid, f) 1,4,5-naphthalenetetracarboxylic acid.

Fig. 1 DEP collection procedure.

Fig. 2 Fractionation of NaHCO₃ extract by HPLC

Fig. 3 Compounds isolated from fraction 3

Fig. 4 Compounds isolated from fraction 4

Fig. 5 MS spectrum of **8** (**11**)

Fig. 6 MS spectrum for methyl ester of **9** (**12**)

Fig. 7 MS spectrum of **10** (**13**)

Fig. 8 Measurement of Vasodilatory activity for **8**, **9**, and **5**