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Purification of a dimethyladenosine compound from silkworm pupae as a vasorelaxation substance

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Abstract

To identify the active substance in the male silkworm pupae that strengthens men's vitality, the vasorelaxation activity was determined by measuring the vascular endothelial nitric oxide (eNO) produced in calf pulmonary artery endothelial (CPAE) cells treated with extracts from the pupae. Dried silkworm male pupae were extracted with ethanol and suspended in water, then partitioned with hexane, chloroform, ethylacetate, and butanol, sequentially. Among these fractions, the aqueous fraction had maximal NO production (156.87 μM/200 μl well, 10 mg/ml) and minimal cytotoxicity (IC_{50} 362.3 mg/ml). The vasorelaxation substances (VAS) from the aqueous fraction were isolated by a combination of gel filtration and anion-exchange chromatography on DEAE Sephadex A-25 and reverse phase-HPLC. Their chemical structures were determined on the basis of their spectroscopic parameters of EI-MS, MALDI-TOF MS, ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, and GC–MS spectral data. The active substance was subsequently identified as a dimethyladenosine and dimethyladenosine-5′-L-arabinose that has phosphodiesterase (PDE) inhibition activity. This compound was shown to inhibit PDE4 activity in a dose-dependent manner. Also, it inhibited the PDE5 activity of cyclic-GMP-specific PDE5 enzyme. These results imply that dimethyladenosine may be a lead compound for the development and improvement of vasculogenic impotence drugs through phosphodiesterase inhibition and NO production in endothelial cells.

Keywords: Nitric oxide; Silkworm; Vasorelaxation

1. Introduction

Of the crude insect drugs in Oriental medicine, unmated silkworm male moths are known as a remedy to treat erectile dysfunction to strengthen men's vitality. Silkworm larvae and pupae are currently registered as a food source and 14 days after metamorphosis the pupae can be substituted for the silkworm moth with same efficacy (Ryu et al., 2002). An ethanol extract of the pupae possessed a tonic effect that increased testosterone level in serum by 19% by 3-week repeated treatment (Ryu et al., 2002). Yet, the main component responsible for the improvement in erectile dysfuction and its detailed mechanism remains uncertain.

The mechanism of sildenafil citrate (Viagra) and other similar drugs for the treatment of erectile dysfunction is well known. When a man is sexually stimulated, either physically or psychologically, nitric oxide (NO) is released from non-cholinergic, non-adrenergic neurons in the penis, as well as from endothelial cells. NO diffuses into cells, where it activates soluble guanylyl cyclase, the enzyme that converts GTP to cGMP (Toda et al., 2005). The cyclic nucleotide then stimulates protein kinase G (PKG), which initiates a protein phosphorylation cascade. This results in a decrease in intracellular levels of Ca^{2+} ions, ultimately leading to dilation of the arteries that bring blood to the penis and compression of the spongy corpus cavernosum. A PDE5 inhibitor that inhibits enzymatic hydrolysis of cGMP in the human corpus cavernosum can cause the same outcome (Kukreja et al., 2004). Despite the pressing need to develop selective PDE inhibitors as therapeutic drugs, only the cAMP-specific PDE4 inhibitors are currently available (Sung et al., 2003).

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Food preparations (capsules) containing male silkworm pupae extract such as Nuegra® are currently commercially available in Korea and male silkworm pupae are considered a candidate nutraceutical agent or supplement to enhance masculine function. Thus, the active substances in male silkworm pupae extract need to be purified and characterized. Our study was designed to isolate and identify the most active vasculogenic and least cytotoxic substances from silkworm pupae in order to develop a pharmaceutical candidate for treating vasculogenic impotence.

2. Materials and methods

2.1. Chemicals

Male silkworm (*Bombyx mori* L.) pupae, 14 days after metamorphosis, were reared and supplied by the Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Korea. DEAE Sephadex A-25 purchased from Sigma Chemicals (St. Louis, MO). Cyclic-(8-^{3}H)-AMP and cyclic-(8-^{3}H)-GMP were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Snake venom of *Crotalus atrox* was from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture and solutions

Investment of NO production and cytotoxicity performed on calf pulmonary artery endothelial (CPAE) cells (ATCC CCL-209, Manassas, VA, USA) in Dulbecco's modified Eagle medium (GIBCO, New York, USA) supplemented with 10% newborn calf serum (GIBCO), 1 mM/l L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Furthermore investment of NO production also performed on human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, VA, USA) in endothelial cell basal medium (EBM)-2 with EGM™-2 singlequots (Cambrex, Walkersville, USA) at 37 °C, 5% CO_2 incubator.

The cytotoxicities of the purified fractions were tested against CPAE cell line using XTT {sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate} kit solution (Boehringer Mannheim), as described previously (Geldof et al., 1999).

2.3. Nitrite assay

The production of NO was measured as nitrite accumulated in the culture medium by using a colorimetric reaction with the Griess reagent. In brief, samples were collected 24 h after the treatment of calf pulmonary artery endothelial (CPAE)/HUVEC cells. The absorbance at 540 nm was measured with a VERSAmax microplate reader (Molecular Devices, Menlo Park, CA, USA). The nitrite concentration was determined from a sodium nitrite standard curve (Nims et al., 1995).

2.4. Extraction and isolations of vasorelaxation substances

The dried male silkworm pupae (500 g) were soaked and extracted three times with EtOH by ultrasonification for 30 min. The extracts obtained were dried on a rotary evaporation; the residue was suspended in water and successively extracted with hexane, chloroform, ethylacetate and *n*-butanol. H_2O fraction subjected to gel filtration and anion exchange chromatography.

2.5. Purification of vasorelaxation substances (VAS)

VAS was isolated by a combination of gel filtration and anion exchange chromatography. The aqueous fraction after *n*-BuOH fractions (400 mg) was loaded onto a DEAE Sephadex A-25 gel chromatography column (30 cm × 1.6 cm) equilibrated with the 50 mM phosphate buffer (pH 7.4). The non-interacting solutes were washed from the column with the equilibration buffer. The bound fractions were eluted using a linear sodium chloride gradient from 0 to 2.5 M NaCl in phosphate buffer (pH 7.4) at a flow rate of 20 ml/h. Desalting step was conducted by BioGel P10 gel filtration chromatography using water as an eluant. Fractions showing maximal NO activity and non-cytotoxicity were pooled and concentrated as previously described.

2.6. Reverse phase-high performance liquid chromatography (RP-HPLC)

Semipreparative separations were carried out on Luna C^{18} column (25 cm × 1.0 cm, I.D.) (Phenomex, USA). The Bio Gel-P10 VAS concentrated fractions were eluted using an isocratic HPLC apparatus that equipment consisted of a Thermo Spectra Products (San Jose, USA) liquid chromatographic system (UV 3000 photodiode UV–vis detector) for 30 min with 70% methanol, at a flow rate of 0.8 ml/min. The fractions were collected according to the 6 peaks (I–VI) in Fig. 1C and freeze-dried, and then used as the NMR/MS samples. The retention time was matched as followers: 14.5 min: RPC Fr II, 15 min: RPC Fr III and 16 min: RPC Fr IV.

2.7. Adenosine identification of MALDI mass and EI mass spectrometry

Matrix-Assisted Laser Desorption Ionization (MALDI) Mass spectrometer (Voyager-DE STR, Applied Biosystems, Germany) MS analysis was performed using a High resolution Tandem Mass spectrometer (Micromass Autospec OA-TOF, Manchester, UK) using an EI (electron ionization)-mode, Electron 70 eV DIP (Direct Inlet Probe) mode in National instrumentation center for environmental management of Seoul National University. All MS data were processed using in the mass databases by the webbook program (NIST, Gaithersburg, MD).

2.8. General instruments

NMR spectra were obtained at 400 MHz (^{1}H) and 600 MHz (^{13}C) on a high-resolution spectrophotometer (Avance 600 FT, Bruker, Germany) using TMS as an internal standard.

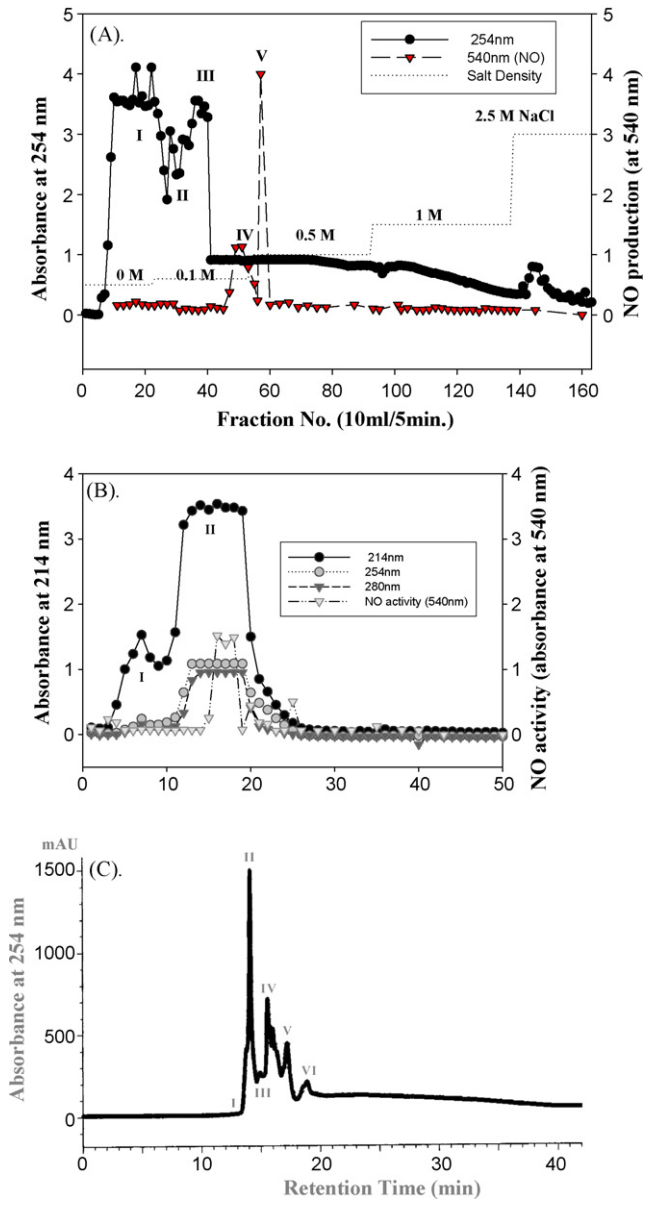

Fig. 1. Fractionation of male silkworm pupae extract. Chromatography using DEAE Sephadex A-25 chromatography liquid column (1A), BioGel P10 gel, desalting using gel filtration chromatography (1B) RPC-HPLC on Luna C^{18} column (1C).

UV spectra were measured on a JASCO V-550 UV/Vis spectrophotometer and IR spectra using a Jasco FT/IR-3300 spectrophotmeter on KBr plate, and melting points were determined on a Büchi B-540 melting point apparatus.

2.9. Sugar moiety identification by HPLC

After the co-incubation of the RPC IV fraction (10 mg/ml, 500 μl) with 0.1 N HCl (100 μl), or 2% endoglycosidase F for 18 h (Sigma) for enzymatic digestion, the sugar moieties were detected using an HPLC-AU electrochemical detector (Dionex submmit HPLC, Sunnyvale, USA) with a sugar analytic column. The sugar moieties were eluted using a linear gradient over 50 min (from 5 to 55 min), with 95% solvent A (16 mM Sodium hydroxide) to 80% solvent B (200 mM Sodium hydroxide). The flow-rate was 1 ml/min for the sugar analytic column (Carbopac^{TM} PA1, 4 mm × 250 mm, Dionex, Sunnyvale, USA). The isolated sugar moieties were identified according to their retention times by a carbohydrate kit [D(−)-arabinose, D(−)-ribose, D(+)-xylose, etc.] (Sigma Co., USA), using and L(+)-arabinose (Fluka, Swiss) as a standard reference.

2.10. Sample preparation for gas chromatography mass spectrometry

For gas chromatography-mass (GC–MS) spectrometry analysis, we used a 16 TMS (trimethylsilyl) standard (Sigma): L(+)-arabinose, β-D(−)-fructose, D(+)-galactose, α-D(+)-glucose, β-D(+)-glucose, glycerol, meso-inositol, D(+)-mannitol, β-D(+)-mannose, L(+)-rhamnose, D(−)-ribose, D(−)-sorbitol, sucrose, D(+)-trehalose, xylitol and D(+)-xylose. The single trimethylsilane (TMS) – standards were diluted at 10 μl/ml in chloroform, and the standard solutions were removed at 100 μl each to make the standard mixture in a vial. To induce trimethylsilylation, 100 μl of 3 samples (1 mg) was hydrolyzed by 1 N HCl for 10 min, and dried with N_2 gas. The evaporated samples were added to pyridine (200 μl) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (120 μl), reacted for 30 min at 65 °C, and then injected into GC–MS. To confirm the target components, we made the TMS standard mixture, and blended the dried standard mixture with a derivative sample.

2.11. GC–MS analysis

We carried out GC-EI (ion impact)–MS analysis with an Agilent 6890 GC coupled to an Agilent 5973 N mass selective detector, and then the analysis process was regulated with HP 3365 Chem Station software (HP, Palo Alto, CA, USA). A HP 5 MS capillary column (5% PH ME siloxane, 30 m × 0.25 mm, 0.25 μm, USA) was used; the oven was heated for 3 min at 130 °C increased to 200 min at a rate of 5 °C/min and held for 10 min, heated to 300 °C at a rate of 20 °C/min and remained for 15 min. A 0.2 μl portion of the samples or the standard mixture was injected, and the split ratio was 100:1. The mass spectrometry was set up with an ion source temperature of 230 °C, a quadrupole temperature of 150 °C, a filament emission current at 34.6 μA, an ionization volt at 70 eV, a hydrogen flow rate of 1.0 ml/min as the carrier gas.

2.12. Phosphodiesterase (PDE) activity assay

The enzymatic activity was assayed using a commercially available PDE scintillation proximity (SPA) assay kit (Amersham product #TRKQ 7090 for cAMP kit, #TRKQ 7100 for cGMP kit) with [^{3}H] cAMP as the substrate depending on the PDE of interest: type IV (snake venom of *Crotalus atrox*, Sigma, USA) at 5 mg/ml, or diluted (1:20) phosphodiesterase V [cGMP-specific, bovine, recombinant, *Spodera frugiperda* (Calbiochem, LA Jolla, CA)] at 10 μl. The manufacturer's protocol was followed explicitly. Ten microliters of each

Table 1
Measurement of cytotoxicity and NO production of solvent fractions of silkworm male pupae in CPAE cells

Fraction	1	2	3	4	5	6	7
Cytotoxicity IC_{50} (mg/ml)	>100	>100	362.3	2.31	1.35	3.73	>100
NO production (μM/10 mg)	–	0.94	156.87	21.06	17.98	64.53	3.87

(1) Control; (2) $CHCl_3$ fraction; (3) H_2O fraction; (4) *n*-BuOH fraction; (5) *n*-hexane fraction; (6) *n*-hexane residue fraction; (7) EtOAc fraction, was treated 10 mg/ml in CPAE cells, respectively.

fraction was added to assay buffer containing 0.05 μCi/10 μl [^{3}H] cAMP or 0.5 μM [^{3}H] cGMP (about 10,000 cpm/assay), respectively, for 20 min (with inhibitors) at 30 °C in a final volume of 0.15 ml. [^{3}H] nucleotide monophosphate, preferentially bound to the SPA beads, excited the scintillant embedded in the beads, and was quantified on a liquid scintillation counter (Microbeta Plus, PerkinElmer, Turku, Finland). The activity in the samples that received the test compound was calculated as a percent of the control activity measured in the samples that only received the vehicle (Rotella et al., 2000; Xin et al., 2003; White et al., 2004). Furthermore PDE activity was assayed (with minor modifications) in combination with commercial phosphodiesterase inhibitors [8-methoxymethyl-IBMX (PDE type I inhibitor) 15 μM, 10 μl; Trequinsin, hydrochloride (PDE type III inhibitor) 10 mM, 10 μl; Rolipiram (PDE type IV inhibitor) 10 mM, 10 μl; 4-{[3′,4′-(methylenedioxy) benzyl] amino}-6-methoxyquinazoline (PDE type V inhibitor) 10 mM, 10 μl] that were purchased from Calbiochem (EMD sciences, San Diego, CA), to evaluate potential as a combination drug with the commercial inhibitors.

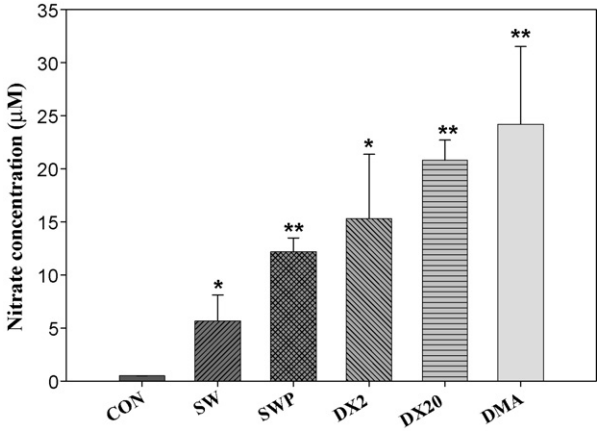

Fig. 2. Effects of the silkworm pupae purified fractions on nitric oxide production activity in HUVEC cells.
CON: Treated with PBS buffer; SW: silkworm ethanol extract 0.8 mg; SWP: silkworm male pupae ethanol extract 0.8 mg; DX: DX 2: DEAE Sephadex A-25 fraction V 0.04 mg; DX 20: DEAE Sephadex A-25 fraction V 0.4 mg; DMA: BioGel P10 gel filtration II with dimethyladenosine-arabinose 0.2 mg.
Values represent mean ± S.D. Significantly different from the untreated controls ($^*P<0.05$; $^{**}P<0.01$).

3. Results

3.1. Purification

The total EtOH extract of male silkworm pupae was partitioned into *n*-hexane, $CHCl_3$, EtOAC, *n*-BuOH, and H_2O fractions. To identify the active principles, we evaluated the NO production activity and the cytotoxicity of these organic solvent soluble fractions in CPAE cells. The H_2O fraction showed the greatest NO production activity (156.87 μM/200 μl well, 10 mg/ml) and the least cytotoxicity (IC_{50} 362.3 mg/ml) (Table 1).

Vasorelaxation substances (VAS) were further isolated by a combination of gel filtration and anion-exchange chromatography. The purification of the active compound from the H_2O fraction was achieved by a combination of three steps. Following *n*-BuOH extraction, the remaining aqueous fraction (400 mg) was loaded onto an anion-exchange chromatography (DEAE Sephadex A-25) column (30 cm × 1.6 cm) equilibrated with 50 mM phosphate buffer (pH 7.4). Fractions showing maximal NO activity and minimal cytotoxicity were pooled and concentrated (Fig. 1A). The anion-exchange chromatography on DEAE Sephadex A-25 gel chromatography yielded a fraction (V) with an NO production activity around 1.9 times greater than the previous aqueous fraction (Fig. 1A). Gel filtration chromatography using BioGel P10 was then conducted to desalt and fractionate (Fig. 1B). Finally, desalted fraction (II) was further purified by HPLC-RPC (Fig. 1C).

Six bound fractions were observed in the reverse phase chromatography (RPC). Of the six, two HPLC-RPC fractions (II and IV) produced more NO oxides than the other fractions and showed almost no cytotoxic effects on CPAE cells and HUVEC cells (Table 2, Fig. 2). These results suggested that these purified fractions might elicit vasorelaxation through the direct release of endothelium-derived NO (Tanner et al., 1999).

3.2. Identification of compound 1

Compound 1 – white powder, mp 188–189 °C, R*f* = 0.61 on silica gel (CH_3Cl_3-MeOH-H_2O, 5:4:1), MALDI-TOF MS: $C_{17}H_{27}N_5O_8$ *m/z* 432.929 $[M+H]^+$; UV (MeOH) λ_{max} (log ε): 206 nm (2.8); IR ν_{max} cm^{-1} (KBr): 3522(OH), 3441(NH),

Table 2
Measurement of cytotoxicity and NO production of silkworm male pupae HPLC-RPC fractions in CPAE cells

Fraction	I (Con)	II	III	IV	V	VI
NO production (μM/5 mg)	–	2.34	147.78	149.31	86.67	83.79
Cell viability (%)	82.4	23.3	93.6	86.9	107.0	93.9

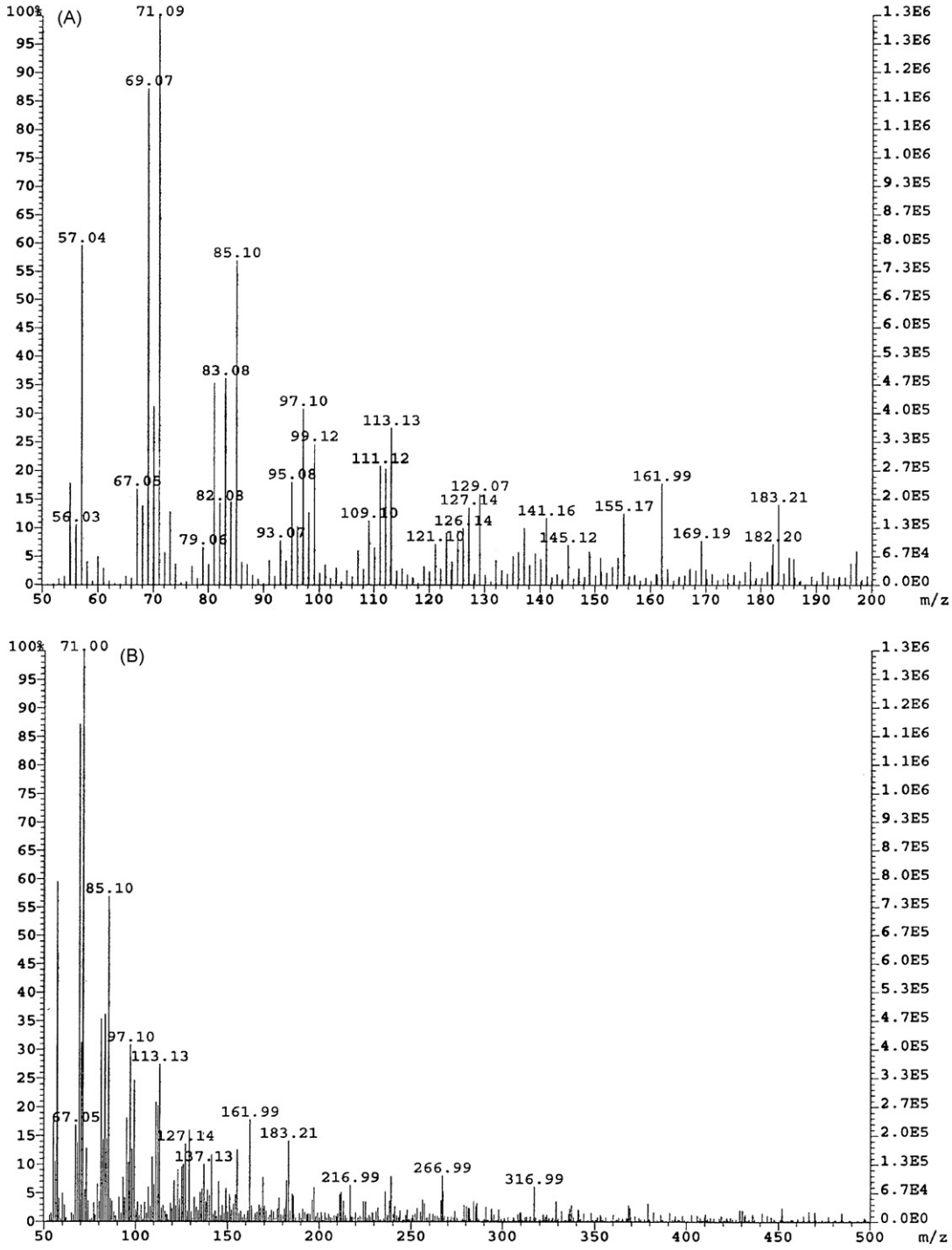

Fig. 3. (A and B) Full mass spectrometric scan (50–500 amu) product, ion spectra of RPC Fr(II), compound **1**.

2955(CH-CH_2), 2361(CH_3CO), 1572(C=O), 1141(glycosidic CO); EI-MS m/z: 295$[M]^+$ (Fig. 3); ^{1}H NMR (D_2O, 600 MHz) and ^{13}C NMR ((D_2O, 600 MHz): Their chemical structures were determined on the basis of their spectroscopic parameters of MADI-TOF-MS, EI-MS, ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY and HMBC spectral data.

^{1}H NMR (600 MHz, D_2O) δ: 2.26 (1H, dd, J = 10.3, 15.3 Hz, H-2′a), 2.30 (6H, s, 2 × N-C$\underline{H_3}$), 2.57 (1H, dd, J = 2.9, 15.4 Hz, H-2′b), 3.91 (1H, br t, J = 3.5 Hz, H-5′a), 4.20 (1H, dd, J = 2.9, 10.3 Hz, H-3′), 4.27 (1H, br s, H-4′), 4.41 (1H, dd, J = 3.5, 4.9 Hz, H-5′b), 6.04 (1H, d, J = 5.95 Hz, H-1′), 8.16 (1H, s, H-8), 8.51 (1H, s, H-2).

^{13}C NMR (150 MHz, D_2O) δ: 34.9 (N-$\underline{C}H_3$), 43.4 (C-2′), 63.2 (C-5′), 71.1 (C-3′), 85.4 (C-1′), 87.5 (C-4′), 116.6 (C-5), 141.0 (C-8), 151.6 (C-4), 153.6 (C-2), 156.4 (C-6), VAS 1 was found to be 5-{6-(dimethylamino)-9H-9-yl}-tetrahydro-

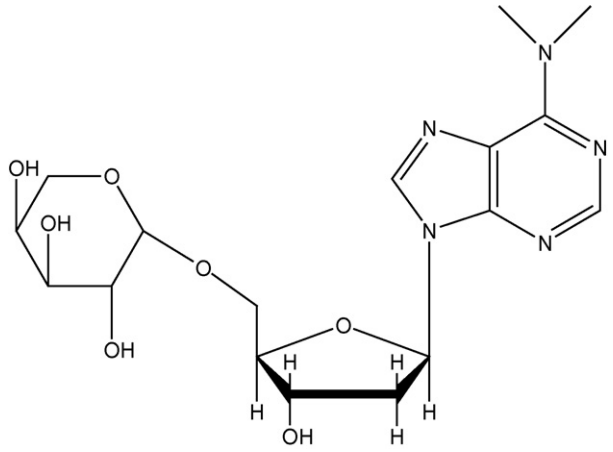

Fig. 4. Structure of 5-{6-(dimethylamino)-9H-9-yl}-tetrahydro-4′-methylfuran-3′-ol-5′-o-tetrafyro-2H-pyran-3″, 4″, 5″-triol.

4′-(hydroxymethyl)furan-3′-ol as a dimethyladenosine, having sugar moiety (Fig. 4).

3.3. Identification of compound 2

Compound 2 – Yellow brown powder, mp 172–173 °C, R*f* = 0.65 on silica gel (CH_3Cl_3-MeOH-H_2O, 5:4:1), MALDI-MS: $C_{17}H_{29}N_5O_9$, *m/z* 451.009 $[M+H]^+$, EI-MS *m/z*: 295$[M]^+$. Compound **2** was hydrate form of compound **1**. The IR spectrum revealed absorptions assigned to hydroxyl group (3495 cm^{-1}), imine group (1657 cm^{-1}), aromatic C=C (1385 cm^{-1}) and glycosidic C–O (1145 cm^{-1}), showing similarity to compound **1**. In the ^1H NMR spectrum, two methyl signals at δ 2.30 (6H, s, 2 × N-CH_3) appeared. The signal at δ 6.04 (1H, d, *J* = 5.95 Hz) indicated the presence of a sugar moiety in this compound. In the ^{13}C NMR spectrum, the signals for sugar moiety at δ 43.4, 63.2, 71.1, 85.4, 87.5 suggested the presence of ribose. With the above spectral data, the structure of compound **2** was identified, as a new compound, [5-{6-(dimethylamino)-9H-9-yl}-tetrahydro-4′-methylfuran-3′-ol-5′-o-tetrafyro-2H-pyran-3″, 4″, 5″-triol (Fig. 4)·hydrate].

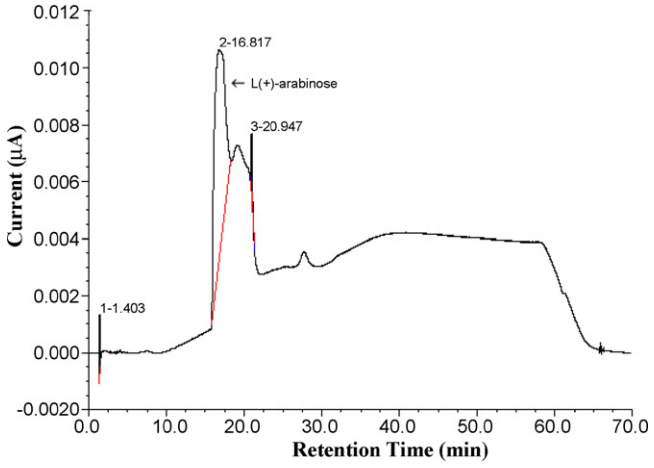

Fig. 5. Identification of purified adenosine derivatives with monosaccharide moiety (right) lysed by 0.1 N HCl.

3.4. Digestion of sugar moiety, HPLC, GC–MS analysis

After digestion of RPC IV fraction by 0.1 N HCl, the sugar moiety was detected using HPLC-AU electrochemical detector using a linear sodium hydroxide gradient. Isolated sugar moieties were identified according to the retention time of standard reference carbohydrates. The standard of arabinose showed the same retention time (16.82 min) as that of compound **2** (Fig. 5). As a result, the compound **2** may be a new compound [5-{6-(dimethylamino)-9H-9-yl}-tetrahydro-4′-methylfuran-3′-ol-5′-o-tetrafyro-2H-pyran-3″, 4″, 5″-triol·hydrate.

In total ion current of GC–MS, 16 of TMS monosaccharide standards were separated, and each of them can be identified by its extracted ion chromatogram. Of TMS standards, although arabinose, ribose, and xylose had same derivatized molecular weight, and similar fragmentation pattern, we can easily describe each other by the retention time. The EI mass of L(+)-arabinose showed specific ion fragmentation such as *m/z* 217 [TMSO=CH–CH=CH–OTMS], 204 [TMSOCH=CHOTMS], and 191 [TMSO–CH=O$^+$TMS]. As suggested in another report (Bleton et al., 1996), this study examined the common ions in TMS derivatives, *m/z* 73 [Si+$(CH_3)_3$], and *m/z* 147 [$(CH_3)_3$SiOSi+$(CH_3)_2$]. The difference between L(+) and D(+)-arabinose in the GC-MS chromatogram was confirmed by the retention time and total ion current: L(+)-arabinose (13.0 min, one peak) and D(+)-arabinose (13.0 and 13.6 min, two ion peaks).

The 16 min sample of three samples only had a specific component, and it corresponded with the fragmentation pattern of TMS – L(+)-arabinose.

3.5. Effect on cAMP-specific PDE4/cGMP-specific PDE5 inhibition

The purified adenosine derivatives inhibited PDE4 activity in a dose-dependent manner (Fig. 6A). The IC_{50}s of the DEAE sephadex A25 fraction (V) and the purified adenosine derivatives (HPLC-RPC Fr IV) were 2.8 ng/ml and 880 ng/ml, respectively (Table 3). Also, it inhibited PDE5 activity on cyclic-GMP-specific PDE5 enzyme showing lower case than PDE4's (Fig. 6A and B). A combination treatment of the purified VAS and each of four commercial phosphodiesterse inhibitor: 8-methoymethyl-IBMX (PDE1 inhibitor) Trequinsin, hydrochloride (PDE3 inhibitor), Rolipiram (PDE4 inhibitor), and 4-{[3′,4′-(methylenedioxy) benzyl]amino}-6-methoxyquinazoline (PDE5 inhibitor), more effectively inhibited phosphodiesterse than VAS treatment alone in a dose dependent manner (Fig. 6B).

Table 3
NO production and inhibitory effects of silkworm male pupae purified fractions on phosphodiesterase (PDE) four inhibitions

Purification steps	NO production (μM/mg)	PDE4 IC_{50} (μg/ml)
DEAE Sephadex	17.90	2.8×10^{-3}
HPLC-RPC Fr II	0.47	8.63
HPLC-RPC Fr IV	29.86	0.88

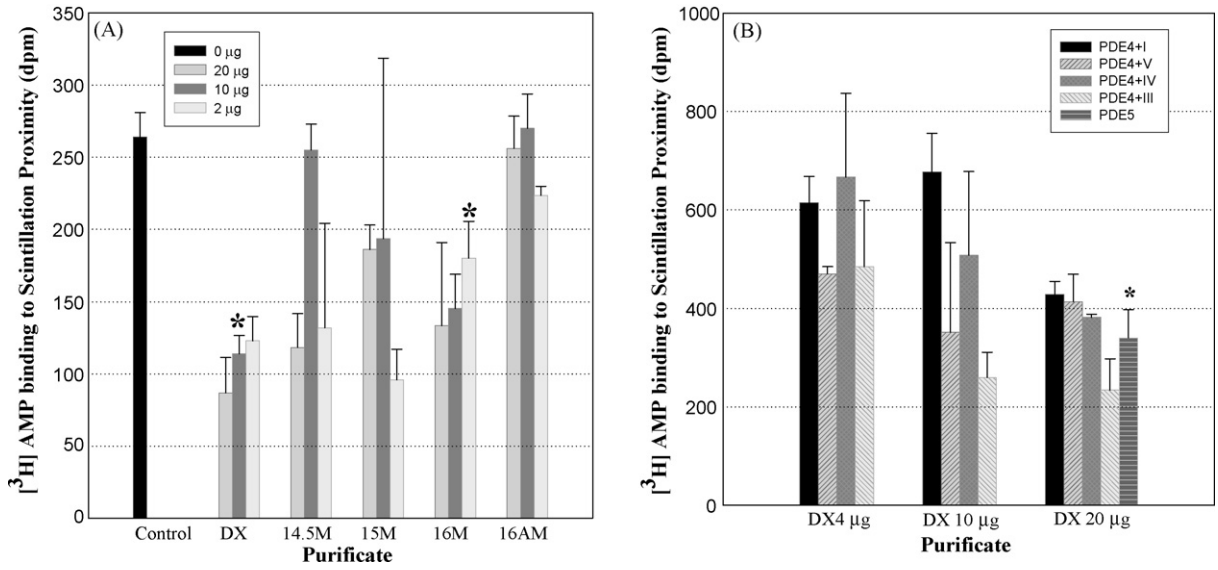

Fig. 6. (A) Phosphodiesterase [^{3}H] cAMP SPA assay according to purified substance concentration on PDE4. DX: DEAE Sephadex A-25 fraction V; 14.5M: RPC Fr II; 15M: RPC Fr III; 16 M: RPC Fr IV; 16 AM: RPC Fr V.
Values represent mean ± S.D. *Significantly different from the untreated controls ($P < 0.05$). (B) PDE5 enzyme inhibition and PDE4 inhibition level of the purified substance compared with other commercial PDE inhibitors (I, V, IV, III).
I: 8-Methoxymethyl-IBMX; III: Trequinsin, hydrochloride; IV: Rolipiram; V: 4-{[3′,4′-(Methylenedioxy)benzyl]amino}-6-methoxyquinazoline; DX: DEAE sepahadex A-25 fraction V.
Values represent mean ± S.D. *Significantly different from the untreated controls ($P < 0.05$).

4. Discussion

In oriental Asia, male silkworm pupae extract is known for its effectiveness in enhancing male stamina and improving vitality (Ryu et al., 2002). Its main ingredients are reported as protein (51%); fatty acid (29%); saccharide (2%); cholesterol (3%); chitin and vitamins A; B_2 and D (Ahn et al., 2007).

However, its active component that affects endothelial vasorelaxation was unknown until now. In this study, we purified the active vasorelaxation substances occurring in male silkworm pupae by organic solvent extraction and isolations, using three chromatographic steps: anion-exchange, gel filtration, and HPLC-RPC (Table 1).

The identified compounds (**1**, **2**) showed similar characteristic bands at 3522 (OH), 3441 (NH), 2955 (CH–CH_2), 2361 (CH_3CO), 1572 (C=O), and 1141 (glycosidic CO) cm^{-1} in their IR spectra. In the UV spectra, compound I exhibited strong absorption peaks at 206 nm and 260 nm, suggesting that it was a purine nucleoside (Son et al., 1991). This result was further supported by the EI mass spectra of the compounds (**1**, **2**). The mass spectrum of compound 1 showed a molecular ion at *m/z* 267, along with a base peak at *m/z* 162 (dimethyladenine). Other important peaks with high relative abundance at *m/z* 317, 239, and 197, were characteristic of adenosine and its analogs. The direct comparison with an authentic sample (^{1}H NMR and ^{13}C NMR) further confirmed the assignment of structure 1 as dimethyladenosine. Dimethyladenosine is found in plants and animals, and has been synthesized as an antitumor agent (Takamura et al., 1989). However, the isolation of dimethyladenosine derivatives in the silkworm pupae is rare. There is an earlier report indicating that adenosine has vasoconstrictor and vasodilator effects due to preferential activation on the A1 and A2 adenosine receptors (Hansen et al., 2005). According to MALDI-TOF data of the RPC fraction (IV), there was a molecular weight of 150 Da for some moiety other than dimethyladenosine as proved by EI Mass. The moiety confirmed the presence of L(+)-arabinose, as compared to HPLC-AU ECD and GC-EI–MS monosaccharide standards, by glycosidase digestion or acid hydrolysis of the RPC fraction (IV).

The above findings indicate that the vasorelaxation substance in silkworm pupae, which leads to remarkable NO production in endothelial cells, is a 5-{6-(dimethylamino)-9H-9-yl}-tetrahydro-4′-methylfuran-3′-ol-5′-o-tetrafyro-2H-pyran-3″,4″,5″-triol·hydrate with phosphodiesterase inhibition activity.

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References

Ahn, M.Y., Heo, J.E., Ryu, J.H., Jeong, H.K., Chung, W.T., 2007. Antioxidant activity of cholesterol derived from silkworm pupae. Natural Product Science 13, 220–224.

Bleton, J., Mejanelle, P., Sansoule, J., Goursaud, T.S., Tchapla, A., 1996. Characterization of neutral sugars and uronic acids after methanolysis and trimethylsilylation for recognition of plant gums. Journal of Chromatography A 720, 27–49, SSDI 0021-9673(95)00308-8.

Geldof, A.A., Mastbergen, S.C., Henrar, R.E., Faircloth, G.T., 1999. Cytotoxicity and neurocytotoxicity of new marine anticancer agents evaluated using *in vitro* assays. Cancer Chemotherapy and Pharmacology 44, 312–318, doi:10.1007/s002800050983.

Hansen, P.B., Hashimoto, S., Oppermann, M., Huang, Y., Brigger, J.P., Schnermann, J., 2005. Vasoconstrictor and vasodilator effects of adenosine in the mouse kidney due to preferential activation of A1 or A2 adenosine receptors. The Journal of Pharmacology and Experimental Therapeutics 315, 1150–1157, doi:10.1124/jpet.105.091017.

Kukreja, R.C., Ockaili, R., Salloum, F., Yin, C., Hawkins, J., Das, A., Xi, L., 2004. Cardioprotection with phosphodiesterase-5 inhibition a novel preconditioning strategy. Journal of Molecular and Cellular Cardiology 36, 165–173, doi:10.1016/j.yjmcc.2003.11.001.

Nims, R.W., Darbyshire, J.F., Saavedra, J.E., 1995. Colorimetric methods for the determination of nitric oxide concentration in neutral aqueous solutions. Methods 7, 48–54, doi:10.1006/met.1995.1007.

Rotella, D.P., Sun, Z., Zhu, Y., Krupinski, J., Pongrac, R., Seliger, L., Normandin, D., Macor, J.E., 2000. N-3-substituted imidazoquinazolinones: potent and selective PDE5 inhibitors as potential agents for treatment of erectile dysfunction. Journal of Medicinal Chemistry 43, 1257–1263, doi:10.1021/jm000081+s0022-2623(00)0081-9.

Ryu, K.S., Ahn, M.Y., Lee, H.S., Kim, I., Kim, J.W., Kim, S.H., Choi, J.H., 2002. The tonic effect of the extract from male silkworm (*Bombyx mori* L.) pupae on rats. International Journal of Industrial Entomology 5, 123–126.

Son, K.H., Do, J.C., Kang, S.S., 1991. Isolation of adenosine from rhizomes of *Polygonatum sibiricum*. Archives of Pharmacal Research 14, 193–194.

Sung, B.J., Hwang, K.Y., Jeon, Y.H., Lee, J.I., Heo, Y.S., Kim, J.H., Moon, J.H., Yoon, J.M., Hyun, Y.L., Kim, E., Eum, S.J., Park, S.Y., Lee, J.O., Lee, T.G., Ro, S., Cho, J.M., 2003. Structure of the catalytic domain of human phosphodiesterase 5 with bound drug molecules. Nature 425, 98–102, doi:10.1038/nature01914.

Takamura, T., Kato, T., Arakawa, E., Ogawa, S., Suzumura, Y., Kato, T., 1989. Syntheses and antitumor activities of N6, N6-dimethyladenosine carboxylate analogues. Chemical & Pharmaceutical Bulletin 37, 2828–2831.

Tanner, M.A., Bu, X., Steimle, J.A., Myers, P.R., 1999. The direct release of nitric oxide by gypenosides derived from the herb *Gynostemma pentaphyllum*. Nitric Oxide 3, 359–365, doi:10.1006/niox.1999.0245.

Toda, N., Ayajiki, K., Okamura, T., 2005. Nitric oxide and penile erectile function. Pharmacology & Therapeutics 106, 233–266, doi:10.1016/j.pharmthera.2004.11.011.

White, B., Thompson, W.J., Pittler, S.J., 2004. Characterization of 3′,5′ cyclic nucleotide phosphodiesterase activity in Y79 retinoblastoma cells: absence of functional PDE6. Molecular Vision 10, 738–749.

Xin, Z.C., Kim, E.K., Lin, C.S., Liu, W.J., Tian, L., Yuan, Y.M., Fu, J., 2003. Effects of icariin on cGMP-specific PDE5 and cAMP-specific PDE4 activities. Asian Journal of Andrology 5, 15–18.