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Identification of Steroidal Compounds as Acetates in a Lysate of "Depressor-I," an Antihypertensive Phospholipid Occurring in Acetone-Soluble Fraction of Bovine Brain

KENKICHI TAKAUCHI,[a] AKIRA TOKUMURA,[a] JUN-ICHI YOSHIDA,[b] TAKEHISA IWAMA,[a] YASOMI HANDA,[c] HIROYASU TATSUMICHI[a] and HIROAKI TSUKATANI*,[a]

Faculty of Pharmaceutical Sciences, The University of Tokushima,[a] Shomachi, Tokushima 770, Japan, School of Medicine, The University of Tokushima,[b] Kuramoto-cho, Tokushima 770, Japan and Faculty of Pharmaceutical Sciences, Tokushima Bunri University,[c] Yamashiro-cho, Tokushima 770, Japan

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The alkaline hydrolysate of "Depressor-I," an antihypertensive phospholipid occurring in the acetone-soluble fraction of bovine brain, was [^{3}H]acetylated and applied to an alumina column. Various [^{3}H]acetylated compounds were eluted. Among the major fractions, the eluates with hexane and hexane–benzene (9:1, 7:3 and 5:5, v/v) mixtures contained labile compounds which decomposed on standing for one month. Gas chromatography-mass spectrometry was carried out with the corresponding sample obtained in a "cold" experiment, and diosgenin acetate, a sapogenin, was detected besides cholesteryl acetate. A new class of phospholipid containing steroidal components is proposed.

Keywords——Depressor-I; hypotensive phospholipid; bovine brain; alkaline lysate; cholesteryl acetate GC-MS; diosgenin acetate GC-MS; platelet activating factor-like phospholipid; [^{3}H]acetyl sterol; sapogenin-containing phospholipid

Introduction

The occurrence of certain hypotensive phospholipids has been reported in lipid fractions of bovine brain[1-4] and a peritoneal dialysate from dogs,[5-7] and they have been tentatively named "Depressor-I" (D-I) and "Peritoneal Dialysate Depressor-I" (PD·D-I), respectively. They were purified to homogeneity on thin-layer chromatography (TLC), and found to be different from known active substances, though their chemical structures have not yet been clarified. On the other hand, many papers have been published concerning platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) since the determination of its chemical structure.[8,9] PAF has a unique structure, and is considered to be a new class of lipid chemical mediator. Besides causing platelet activation, it shows certain other biological effects, such as polymorphonuclear leucocyte-activating,[10] cardiovascular,[11,12] spasmodic[13,14] and antispasmodic[15] effects. Various biological effects of lysophosphatidic acid(1-acyl-2-lyso-*sn*-glycero-3-phosphoric acid, LPA) have also been reported recently. It causes platelet activation,[16,17] enhancement of neutrophil chemotaxis[18] and smooth muscle stimulation[19,20] and shows animal species-dependent vasoactivaties.[21,22] The new active phospholipids D-I and PD·D-I resembled lysolecithin chemically and showed a short-term antihypertensive effect in anesthetized rats, guinea pigs, cats and rabbits. In addition, the similarity of D-I and PD·D-I to PAF in terms of biological effects attracted our attention, though there

are some differences. In the course of a study on D-I, it was found that certain steroidal compounds exist in its alkaline lysate, and two of them were identified as diosgenin and cholesterol from an alkaline lysate of a phospholipid preparation with antihypertensive activity obtained from bovine brain.

Materials and Methods

Preparation of D-I from Bovine Brain——D-I was prepared as reported.[2] In brief, the total lipid fraction of bovine brain obtained by the extraction and washing procedures described by Folch *et al.*[23] was reextracted with acetone. The acetone extract was partitioned between 70% ethanol and hexane, and the polar fraction was dried and applied to a silicic acid (Mallinckrodt AR 100 mesh) column. Material was eluted with chloroform–methanol mixtures with stepwise-increasing polarity. The hypotensive effects of the fractions were estimated in rats anesthetized with pentobarbital and expressed in units (U) relative to the activity of acetylcholine. The hypotenisve factor was purified to homogeneity on TLC by rechromatography on silicic acid with a chloroform–methanol system and gel filtration twice on Sephadex LH-20 with chloroform–methanol (1 : 1, v/v) and acetone–ethanol (1 : 1, v/v) mixtures. The antihypertensive phospholipid preparation showed similar mobility on silica gel TLC plates to L-α-lysolecithin (palmitoyl, Sigma) in three different solvent systems.

[^{3}H]Acetylation of Alkaline Lysate of D-I and Alumina Column Chromatography——The hypotensive phospholipid preparation D-I (corresponding to 5 kg of wet tissue) was hydrolyzed with alkali,[22] mixed with ethyl acetate, passed through Amberlite CG 50 resin (HR-form) and evaporated *in vacuo*. The residue was dissolved in ether containing 10^{-4} M HCl, kept for 4 h, washed with 5% Na_2CO_3 solution and water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The syrup was dissolved in pyridine, treated with [^{3}H]acetic anhydride (New England Nuclear, specific activity 50 mCi/mmol, diluted 50 fold with non-radioactive acetic anhydride), allowed to stand overnight at room temperature, and added a small volume of water. The mixture was made up to 50% ethanol and extracted with 5 vol. of ethylene dichloride. The extract was washed with 50% ethanol and water, successively, and dried *in vacuo*. In the "cold" experiment, acetylation was carried out in a similar manner. Next, 40% of the [^{3}H]acetylated product was applied to an alumina column (ICN Pharmaceuticals GmbH, Aluminiumoxide W 200 neutral, grade II, 4 g). Material was eluted with 20 ml each of hexane, hexane–benzene (9 : 1, 7 : 3, 5 : 5 and 3 : 7, v/v) mixtures, benzene, benzene–chloform (9 : 1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8 and 1 : 9, v/v) mixtures, chloroform, chloroform–methanol (9 : 1, 8 : 2, 7 : 3 and 5 : 5, v/v) mixtures and methanol with ingradient increments in the polarity of the solvent. Fractions of 5 ml each were collected in test tubes. An aliquot of each fraction was transferred into a vial, and dried *in vacuo*, then 10 ml of liquid scintillation fluid (Nakarai) was added, and the radioactivity of [^{3}H]acetyl derivatives was counted by a Tricarb scintillation counter (Fig. 1(1)). The same sample was chromatographed on an alumina column in the same way after one month of standing at −20 °C (Fig. 1(2)). Rechromatography of fraction A [eluates with hexane and hexane–benzene (9 : 1, 7 : 3 and 5 : 5, v/v) mixtures, Fig. 1(1)] on alumina was also performed after one month of standing at −20 °C (Fig. 2). TLC of [^{3}H]acetyl compounds in fraction A was carried out on silica gel plates (Merck TLC plate Silica gel 60, 0.25 mm thickness) with chloroform–ethanol (95 : 5, v/v) and hexane–ether–acetic acid (90 : 10 : 1, v/v). Standard cholesteryl acetate and diosgenin acetate were also spotted on the plates. Radioactivity in each zone was measured after extraction in the same way as described above.

Mass Spectrometric Measurement——For gas chromatography-mass spectrometry (GC-MS), a sample corresponding to fraction A was prepared by acetylation of the alkaline lysate of D-I preparation, followed by alumina column chromatography. The fraction corresponding to A [eluates with hexane, and hexane–benzene (9 : 1, 7 : 3 and 5 : 5, v/v) mixtures] was further purified by preparative TLC on silica gel plates (Merck) with hexane–ether–acetic acid (90 : 10 : 1, v/v). The zones of the plate corresponding to authentic cholesteryl acetate and diosgenin acetate were scraped off, transferred to a test tube and extracted successively with chloroform and chloroform–methanol (1 : 1, v/v). The extracts were combined, and dried *in vacuo*, then the residue was redissolved in ethyl acetate and an aliquot was injected into a gas chromatograph. GC-MS was carried out in a JEOL JMS-D 300 mass spectrometer coupled with a gas chromatograph (Nihon Denshi, Japan). The column used was a glass spiral tube (2 mm × 2 m) packed with 2% OV 17 on Chromosorb W(AW-DMCS), 80—100 mesh. Helium gas was used at 60 ml/min. The temperatures of the injection port and separator were 290 and 280 °C, respectively. The column temperature was programmed from 260 °C to 295 °C at 5 °C/min. Electron impact-mass spectrometry (EI-MS) was carried out at an ionizing potential of 20 eV, ionizing current of 300 μA, accelerating voltage of 3.0 kV and ion source temperature of 260 °C. Mass number calibration was carried out with perfluorokerosene as a standard.

Results

Alumina Column Chromatography of [^{3}H]Acetyl Derivatives of the Lysate of D-I

Previously, it was demonstrated that the factor was a phospholipid, probably lysolecithin

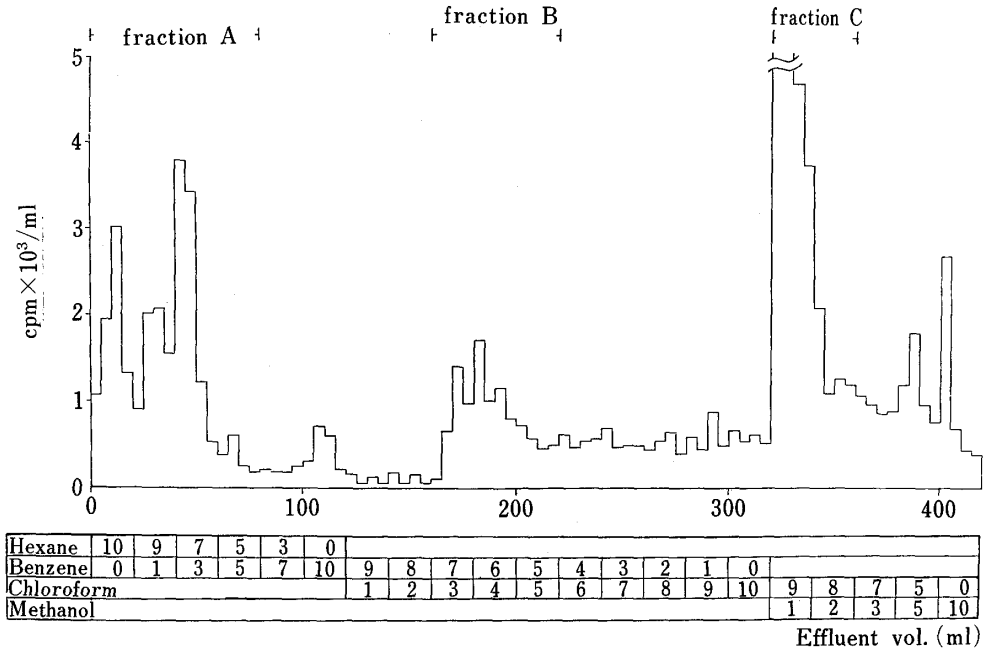

Fig. 1(1). Alumina Column Chromatography of [^{3}H]Acetyl Derivatives of an Alkaline Lysate of the Antihypertensive Phospholipid D-I

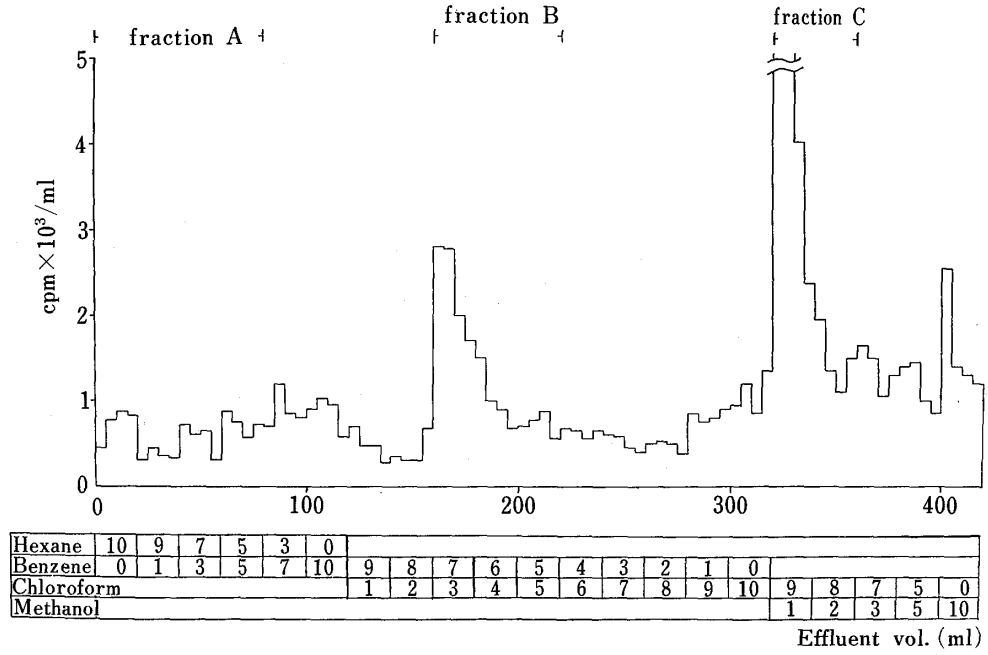

Fig. 1(2). Alumina Column Chromatography of the Lysate after One Month of Standing at −20 °C

(1-*O*-acyl-2-lyso-*sn*-glycero-3-phosphocholine, LPC) or its analog, judging from the TLC data, and analysis of phosphorus, fatty acid, glycerol and choline. However, no synthetic LPC caused such remarkable hypotension[25] in a comparison on the basis of phosphorus content. The fatty acid of D-I was mostly unsaturated (*ca.* 70%), *e.g.* oleic acid.[2] During the study, it was found that certain unknown substances other than those described above were also present as minor components in the alkaline lysate obtained by Dawson's procedure.[24] In order to clarify the nature of these minor components, we carried out [^{3}H]acetylation of the

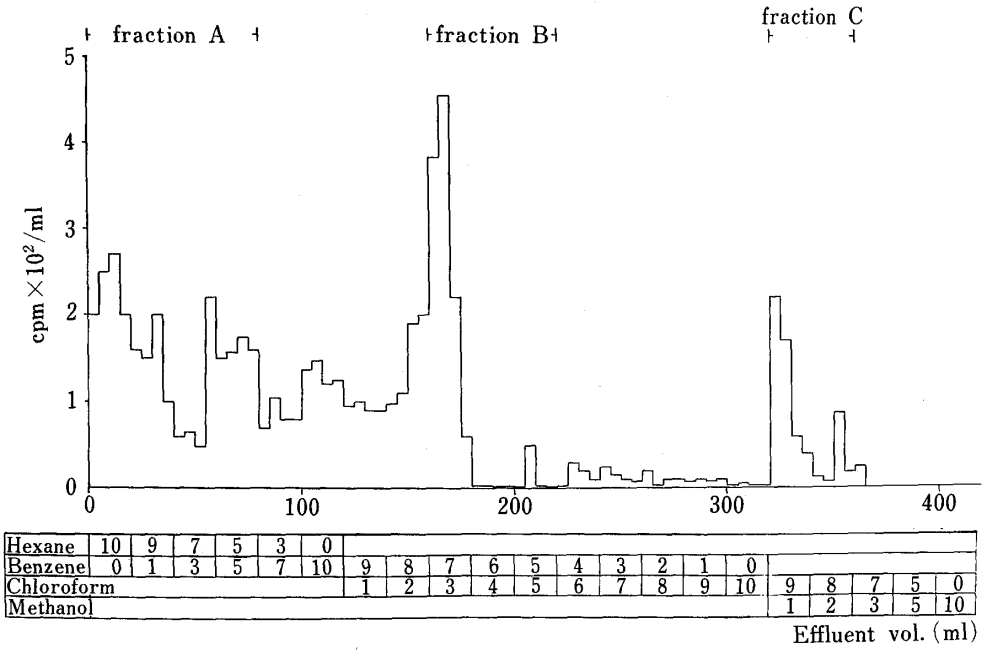

Fig. 2. Alumina Column Rechromatography of Fraction A after One Month of Standing at −20 °C

A half of the fraction A eluted in the first chromatography (1) was applied.

compounds in the lysate. After alkaline treatment and passage through Amberlite CG 50 resin, the eluate was [^{3}H]acetylated. The mixture of [^{3}H]acetates was applied to an alumina column, and developed with a hexane–benzene–chloroform–methanol system with increasing polarity of the solvent. Figure 1(1) shows the elution profile of ^{3}H-labelled acetyl derivatives of D-I alkaline lysate; three major fractions were obtained. Peaks were detected in the eluates with hexane and hexane–benzene (9 : 1, 7 : 3 and 5 : 5, v/v) mixtures (fraction A). The eluates with benzene–chloroform (7 : 3, 6 : 4 and 5 : 5, v/v) mixtures contained the second major fraction (fraction B). Finally the third major fraction was eluted with chloroform–methanol (9 : 1 and 8 : 2, v/v) mixtures (fraction C). To test the stability of the acetates, the same [^{3}H]acetylated product was examined in the same manner after one month of standing at −20 °C as shown in Fig. 1(2). Almost all the radioactivity of the [^{3}H]acetyl radical of fraction A seems to have been transferred to the eluates with benzene–chloroform (7 : 3, 6 : 4 and 5 : 5, v/v) mixtures corresponding to fraction B, and a part to the eluates with chloroform–methanol (9 : 1 and 8 : 2, v/v) mixtures corresponding to fraction C. Next, the stability of each fraction A, B or C was tested on standing for one month. On rechromatography of fraction A after one month, the elution profile was considerably altered (Fig. 2). Approximately a half of the radioactivity of [^{3}H]acetyl radical (*ca.* 48%) had migrated to the eluates with hexane–benzene (3 : 7, v/v) mixture, benzene and benzene–chloroform (9 : 1, 8 : 2 and 7 : 3, v/v) mixtures, roughly corresponding to fraction B. In addition, a part of the residual radioactivity of fraction A (*ca.* 11%) had moved to the more polar fraction corresponding to C. In the case of fraction B, a part of the radioactivity of the fraction was transferred to the more polar fraction corresponding to fraction C (*ca.* 14%) on rechromatography, though the change was not great. In the rechromatography of fraction C, the radioactivity of the fraction was recovered almost completely in the original location. It was assumed that some of the compounds involved in fraction A are labile and yield more polar compounds, either by intramolecular rearrangement, or by cleavage of some portions of the molecules, generating new polar groups such as OH or C=O. Similar changes might take place in fraction B.

Fraction A was further examined by TLC, and a significant part of [^{3}H]acetyl radioactivity was located in the zone corresponding to cholesteryl acetate with hexane–ether–acetic acid (90 : 10 : 1, v/v, *Rf* 0.48) or chloroform–ethanol (95 : 5, v/v, *Rf* 0.85) as a solvent. Therefore, the unlabelled derivatives were next examined by GC-MS.

Occurrence of Steroidal Compounds in Lysate of D-I

A fraction corresponding to A from the acetylated lysate of the hypotensive phospholipid D-I was examined by GC-MS; a sample corresponding to fraction A (obtained in a "cold" exaperiment by alkaline hydrolysis, acetylation and alumina column chromatography as described above, and further purified by preparative TLC) was subjected to GC-MS. Two compounds were identified. An intense peak at 8.5 min in the gas chromatogram was assigned to cholesteryl acetate (Table I). Though the expected molecular ion peak of cholesteryl acetate ($C_{29}H_{48}O_2$) (a) or its desmethylated ion peak ($C_{28}H_{45}O_2$) could not be detected at *m/z* 428 or 413, an intense ion peak at *m/z* 368 (b) was observed as a base peak corresponding to the $[M-60]^{+\cdot}$ ion formed by removal of CH_3COOH. Standard cholesteryl acetate showed the same spectrum and fragmentation pattern. Next, another potent peak (11 min) detected in the gas chromatogram of the fraction corresponding to A was examined. Figure 3 shows the EI mass spectrum of the second intense peak. High-resolution mass spectroscopic analysis of the peak was also carried out (Table II). The peaks at *m/z* 139 (h) and 115 (i) were O-containing and were determined to be $C_9H_{15}O$ and $C_6H_{11}O_2$ from the high-resolution mass spectral data. Because it is well known that these ion peaks are specific and useful diagnostic peaks for

TABLE I. High-Resolution Mass Spectral Data for Major Ions in the EI Mass Spectrum of a Peak at 8.5 min in the Gas Chromatogram of Acetyl Derivatives of an Alkaline Lysate of the Hypotensive Phospholipid D-I

m/z	Elemental composition	Observed mass	Calculated mass	Relative intensity (%)
(a) 428	$C_{29}H_{48}O_2$	Not detected	428.3656	—
(b) 368	$C_{27}H_{44}$	368.3426	368.3445	100
(c) 353	$C_{26}H_{41}$	353.3201	353.3210	25.2
(d) 260	$C_{19}H_{32}$	260.2523	260.2506	24.3
(e) 255	$C_{19}H_{27}$	255.2134	255.2114	26.5
(f) 247	$C_{18}H_{31}$	247.2461	247.2427	29.2
(g) 213	$C_{16}H_{21}$	213.1651	213.1644	22.3
(h) 160	$C_{12}H_{16}$	160.1262	160.1252	16.0
(i) 147	$C_{11}H_{15}$	147.1181	147.1175	51.2
(j) 145	$C_{11}H_{13}$	145.1036	145.1018	41.2

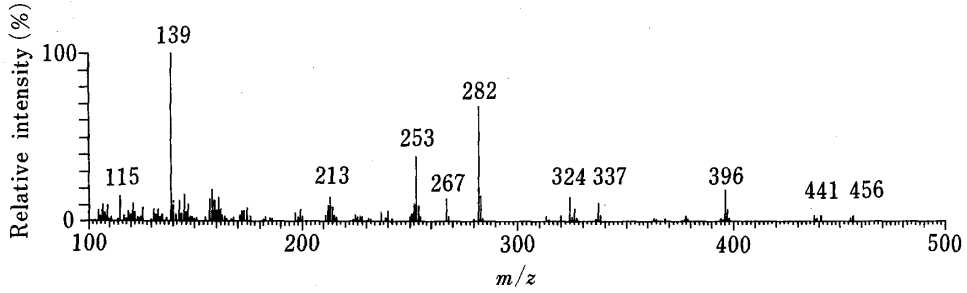

Fig. 3. EI Mass Spectrum of the Peak at 11 min in the Gas Chromatogram of Acetyl Derivatives of an Alkaline Lysate of the Antihypertensive Phospholipid D-I

TABLE II. High-Resolution Mass Spectral Data for Major Ions in the EI Mass Spectrum of a Peak at 11 min in the Gas Chromatogram of Acetyl Derivatives of an Alkaline Lysate of the Hypotensive Phospholipid D-I

m/z	Elemental composition	Observed mass	Calculated mass	Relative intensity (%)
(a) 456	$C_{29}H_{44}O_4$	456.3335	456.3241	3.8
(b) 441	$C_{28}H_{41}O_4$	441.2904	441.3006	3.9
(c) 396	$C_{27}H_{40}O_2$	396.3039	396.3030	18.8
(d) 282	$C_{21}H_{30}$	282.2379	282.2349	67.2
(e) 267	$C_{20}H_{27}$	267.2070	267.2114	13.7
(f) 253	$C_{19}H_{25}$	253.1948	253.1958	38.2
(g) 213	$C_{16}H_{21}$	213.1599	213.1644	14.7
(h) 139	$C_9H_{15}O$	139.1120	139.1124	100
(i) 115	$C_6H_{11}O_2$	115.0162	115.0759	15.7

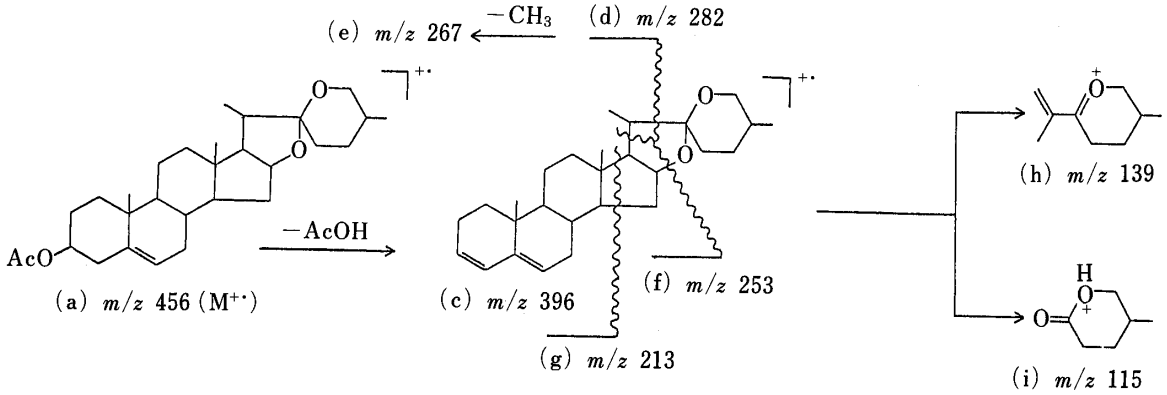

Chart 1. Proposed Pathway for the Formation of Major Ion Peaks in the EI Mass Spectrum of Diosgenin Acetate

spiroketal compounds,[26] it was assumed that the compounds might be steroidal sapogenins containing spiroketal structure. The highest mass-number ion at *m/z* 456 (a) in this spectrum might represent the molecular species. The following high mass-number ions, *m/z* 441 (b) and 396 (c), were assigned to $[M-CH_3]^+$ and $[M-CH_3COOH]^{+\cdot}$ ions, respectively. The ion peaks at *m/z* 282 (d), 267 (e) and 253 (f) were assumed to be formed by removal of CH_3COOH (60) and successive fission of ring E in the skeletal structure of the steroidal sapogenin, based on the work of Budzikiewiez *et al.*[26] on free diosgenin, desoxytigogenin and others. Considering these findings, it was strongly suggested that the peak in question may be possibly diosgenin acetate. The peak in question gave a retention time and EI mass spectrum very similar to those of standard diosgenin acetate. A proposed pathway for the formation of major ion peaks in the EI mass spectrum of diosgenin acetate is shown in Chart 1, referring to the discussion by the above authors.[26] Thus, GC-MS analysis indicated that D-I isolated from bovine nervous tissue unexpectedly contains diosgenin (about 1.5 μg/kg wet tissue) as well as cholesterol. Cholesterol and diosgenin appear to exist in bound forms, becoming detectable after hydrolysis of D-I with alkali.

Discussion

Potent biological activities of certain phospholipids such as PAF or LPA have been reported recently. On the other hand, D-I has been characterized as one of a class of new

antihypertensive phospholipids, though its chemical structure has not yet been defined. This active factor D-I was once considered to be LPC or an analog, though this possibility has now been excluded. The similarity of D-I to PAF, a new class of lipid chemical mediator, attracted our attention, and the question of whether D-I might be identical with PAF was raised. However, D-I shows some marked differences from the above substances in pharmacological and chemical properties as follows. a) A mixture of synthetic LPCs with equivalent fatty acid composition to D-I on the basis of phosphorus content showed no such potent hypotensive activity.[25] The minimum effective dose of D-I was approximately 7.8×10^{-7} g/kg as phosphorus in urethane-anesthetized rats.[4] b) The D-I preparation is rather labile and tends to lose its hypotensive activity even on storage as a syrup in the cold under an atmosphere of nitrogen, unlike PAF. c) Purified D-I preparation shows different behavior towards several TLC staining reagents such as 2,4-dinitrophenylhydrazine reagent. d) D-I shows no smooth muscle stimulating effect, in contrast to PAF, but rather has a relaxing effect.[27] e) PAF has not been reported in the mammalian nervous tissue as far as we know.

Chloesterol and diosgenin were detected in the alkaline lysate of the new antihypertensive phospholipid D-I. As lipo-soluble forms of cholesterol and other steroids in mammalian tissues or fluids, free and fatty acyl esters are well known. Sulfate conjugates are the main water-soluble forms of steroids. However, free or fatty acyl esters of the steroids could not have contaminated the hypotensive preparation of D-I, because the factor was purified through Folch's method,[23] involving a washing procedure, several solvent fractionations, and two silicic acid and two Sephadex LH-20 column chromatography procedures, and was concentrated about 5×10^4-fold compared to the starting total lipid fraction of bovine brain. The active lipid is a polar one and is located near LPC on column chromatographies and TLC, being fundamentally different from free or fatty acyl steroids. Additionally, the steroids became detectable only after exposure of D-I to rather severe alkaline hydrolytic conditions. No sugar moiety was detectable. Therefore, cholesterol or diosgenin did not seem to exist as a free form in the antihypertensive phospholipid preparation, but rather as a bound form other than a glycoside or fatty acyl ester. The occurrence of diosgenin was surprising, because this sapogenin is known in plants, but is unknown not only in the nervous tissue but also in other tissues or body fluids of mammals. At present, it is not clear whether these steroidal compounds were directly responsible for the short-term antihypertensive action of D-I, or whether all acetylated compounds in the fraction were volatilized. Other sterol acetates may exist in other fractions in the eluate from the alumina column.

Diosgenin is a steroidal sapogenin, and is distributed widely in the plant kingdom as an aglycone of saponins. In general, saponins are very potent emulsifiers, exhibiting toxic and hemolytic actions. However, little information on their cardiovascular activity is available. Guseinov and Iskenderov isolated two new saponins, ruscosides A and B, from *Ruscus hycanus*;[28] these saponins have antisclerotic and hypotensive effects, but the effect of their sapogenin, ruscogenin, is unknown. As regards animal sources, steroidal sapogenins have been found in asteroids,[29-31] and triterpenoid sapogenins from trepang,[32] but none has been reported from mammals. Thus, the detection of diosgenin as well as cholesterol in the hypotensive phospholipid from bovine brain seems to be of considerable significance. As regards phospholipids involving steroids, Ramsammy and Brockerhoff[33] reported the formation of a complex between LPC and cholesterol. Oertel reported the isolation of lipophile phosphatide or sulfatide from human serum, and showed that it consisted of dehydroepiandrosterone, glycerol, inorganic acid (phosphoric acid + sulfuric acid) and fatty acid in a ratio of 1 : 1 : 1 : 2;[34] he also synthesized them.[35] However, D-I seems to be different in nature from them. Thus, the hypotensive principle might belong to a new class of phospholipid containing steroids. At present, the biological significance of the presence of diosgenin in the brain is unknown, and the pathway of biosynthesis of diosgenin is also

unclear.

Further work on this interesting steroidal component-containing factor from bovine brain is necessary.

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