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Identification of Fatty Acid Conjugates of Plaunotol, a New Anti-ulcer Agent, Formed in Rat Liver Homogenate System

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Plaunotol, a new anti-ulcer isoprenoid (C_{20}) which possesses two hydroxyl groups at the 1- and 18-positions, was readily conjugated with fatty acids by esterification in a rat liver homogenate system without added cofactors. The hydroxyl group at the 1-position was exclusively esterified *in vitro*. The fatty acid conjugates of plaunotol served as substrates for cholesterol esterase. After purification by thin layer chromatography and high performance liquid chromatography, esters with palmitic, oleic and stearic acids were identified by comparing the gas chromatography-mass spectrometry with those of synthesized authentic standards.

Keywords——plaunotol; isoprenoid; anti-ulcer agent; fatty acid conjugation; liver homogenate; rat

Plaunotol is the major component of CS-684 (Kelnac®), the new anti-ulcer agent extracted from the leaves of Plau-noi (*Croton sublyratus*) which is produced and used as a native drug in Thailand (Fig. 1). The major metabolic pathway of plaunotol consists of the oxidation of both carbons at the 1- and 16-positions to dicarboxylic acids and subsequent chain shortening by β-oxidation.[1,2] Although the amount is as low as about 9% of the administered dose, glucuronic conjugates of highly lipophilic metabolites which could not be explained by the metabolic pathway mentioned above were observed on thin layer chromatography (TLC) in bile samples from both rats and dogs.[2] These lipophilic metabolites were inert to lipase treatment but after treatment with cholesterol esterase, liberated unchanged plaunotol and small amounts of metabolites with the carboxylic group. We, therefore, tentatively identified these *in vivo* metabolites as plaunotol conjugated with fatty acids at the hydroxyl group at either the 1- or 18-position, then further conjugated with glucuronic acid and excreted into the bile.[2] In the present work, we isolated the fatty acid conjugates of plaunotol formed in a rat liver homogenate system by means of TLC and high performance liquid chromatography (HPLC) and identified their structures by comparing the gas chromatography-mass spectrometry (GC-MS) with those of authentic standards.

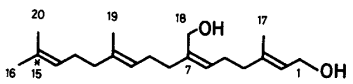

Fig. 1. Structure of [15-^{14}C]Plaunotol
*, position labeled with ^{14}C.

Materials and Methods

Labeled Compound——[15-^{14}C]Plaunotol was prepared in the Chemical Research Laboratories of Sankyo Co., Ltd. The specific radioactivity was 7.45 mCi/mmol (24.36 μCi/mg) and the radiochemical purity was over 99% by TLC using benzene–ethyl acetate–ethanol (20 : 4 : 4) as the developing solvent.

Reagents——Non-radioactive plaunotol was prepared in the Chemical Research Laboratories of Sankyo Co., Ltd.[3] Fatty acid chlorides (palmitoyl, palmitoleyl, oleyl and stearoyl chloride) were purchased from Sigma (St. Louis, U.S.A.). Precoated silica gel TLC plates were purchased from Merck (Darmstadt, FRG). Omnifluor (NEN, Boston, U.S.A.) was used as the fluorescent mixture for liquid scintillation counting. Pancreatic lipase and cholesterol esterase (*Pseudomonas* sp., EC 3.1.1.13) were purchased from Wako Pure Chemicals (Tokyo, Japan).

Preparation of Authentic 1- and 18-Monoesters of Plaunotol with Fatty Acids——A solution of plaunotol (2 g) in 10 ml of diethyl ether and 2 ml of pyridine was added to 1.2 mol eq of fatty acid chloride (palmitoyl, palmitoleyl, oleyl or stearoyl chloride) and kept overnight under constant ice-cooling and stirring. The reaction mixture was washed successively with dilute hydrochloric acid and 1% sodium bicarbonate solution in a separatory funnel. The organic layer was evaporated *in vacuo*. The residue was taken up in 5 ml of benzene and subjected to silica gel column chromatography. By increasing the ratio of ethyl acetate gradually in the eluting solvent (benzene base), plaunotol-1,18-diester, plaunotol-1-monoester, plaunotol-18-monoester and unreacted plaunotol were eluted in that order. The position esterified with fatty acid was identified by 90 MHz proton nuclear magnetic resonance (^{1}H-NMR); for example, in the case of palmitate esters the results were as follows. Plaunotol: 16, 17, 19 and 20-(–CH_3), 1.6 (6H, s) and 1.7 (6H, s); 4, 5, 8, 9, 12 and 13-(–CH_2–), 2.0—2.3 (12H, m); 18-(–CH_2OH), 4.1 (2H, s); 1-(CH_2OH), 4.15 (2H, d); 2, 6, 10 and 14-(=CH–), 5—5.5 (4H, m). Plaunotol-1-palmitate: same as plaunotol except for new peaks at 0.9 (m), 1.3 (s) and 2.3 (m) assigned to the palmitic acid moiety and with the peak of 1-(CH_2OH) shifted toward low magnetic field by 0.5, to 4.6 (2H, d). Plaunotol-18-monoester: same as plaunotol-1-monoester except for the peak of 18-(CH_2OH) shifted toward low magnetic field by 0.5, to 4.6 (2H, s) instead of the shift of the 1-(CH_2OH) peak. Plaunotol-1,18-diester: same as plaunotol monoesters except for both 1- and 18-(CH_2OH) peaks shifted toward low magnetic field by 0.5, to 4.63 (2H, s) and 4.6 (2H, d).

Formation of Fatty Acid Conjugates of Plaunotol in Liver Homogenate System——Livers of Donryu strain male rats were excised and homogenized in a Polytron homogenizer (Kinematica, Switzerland) in 3 volumes of potassium phosphate buffer (50 mM, pH 7.4) containing 1.15% potassium chloride. An ethanol solution of [^{14}C]plaunotol (1.53 mg/0.1 ml) was added to 100 ml of the liver crude homogenates and incubated for 30 min at 37°C (final 50 μM). The incubation mixture was extracted twice with 100 ml of diethyl ether. After gentle evaporation of the diethyl ether by a nitrogen gas stream, the residue was redissolved in 2.5 ml of ethanol, spotted onto three preparative silica gel TLC plates (0.5 mm of thickness) and developed with isopropyl ether–acetic acid (9 : 1). The spots developed at the solvent front were scraped off and extracted with ethyl acetate; each extract was evaporated *in vacuo* and the residue was redissolved in 0.5 ml of acetonitrile to serve as the fatty acid conjugate fraction of [^{14}C]plaunotol.

In the experiments using liver subcellular fractions, the liver homogenate was first centrifuged for 20 min at 9000 ***g*** in a refrigerated centrifuge (model RS-18GL, Tomy Seiko Co., Ltd., Tokyo, Japan). The supernatant fraction was centrifuged for 1 h at 105000 ***g*** in an ultracentrifuge (model L5-65, Beckman Instruments Inc., Palo Alto, U.S.A.) to sediment the microsomal fraction. The pellet at 105000 ***g*** (microsomal fraction) was washed once with the potassium phosphate buffer by centrifugation and resuspended in the original volume of potassium phosphate buffer. Then, 0.9 ml of the liver crude homogenate, 105000 ***g*** supernatant or microsomal fraction was supplemented with 10 μl of [^{14}C]plaunotol (50 μM) and incubated for 30 min at 37 °C with or without addition of palmitoyl-CoA (0.1 ml, 50 μM). After extraction of the incubation mixture twice with 4 ml of diethyl ether, the ether layer was evaporated and the residue was dissolved in 0.2 ml of ethanol. A 10 μl aliquot of the ethanol solution was subjected to silica gel TLC using isopropyl ether–acetic acid (9 : 1) and the amount of fatty acid conjugates of plaunotol was measured by counting the radioactivity of the scraped spots directly.

Hydrolysis of Fatty Acid Conjugates of Plaunotol by Cholesterol Esterase——A methanol solution (0.1 ml) of plaunotol fatty acid conjugate was added to 5 ml of 200 mM potassium phosphate buffer (pH 7.0, with 0.485% sodium cholate) containing 20 units of cholesterol esterase. After being incubated overnight at 37 °C, the mixture was acidified with 6 N hydrochloric acid to reduce the pH to below 2.0, and extracted twice with 5 ml of ethyl acetate. The organic layer was evaporated *in vacuo*, and the residue was dissolved in 0.3 ml of methanol and analyzed by two-dimensional TLC using benzene–ethyl acetate–ethanol (20 : 4 : 4) as the first solvent and isopropyl ether–acetic acid (9 : 1) as the second solvent. The spots were detected by autoradiography and iodine color development.

Separation of 1- and 18-Fatty Acid Esters of Plaunotol by TLC——Plaunotol-1-fatty acid esters were effectively separated from plaunotol-18-fatty acid esters by silica gel TLC using hexane–ethyl acetate (7 : 3) as the developing solvent (*Rf* values: 1-esters, 0.76; 18-esters, 0.6). This method was applied only to determine the esterified position. The brief separation of fatty acids according to chain length was carried out by HPLC as described below.

HPLC Methods to Separate the Conjugates of Plaunotol with Fatty Acids——Fatty acid conjugates of plaunotol were separated by HPLC (model 655A, Hitachi Co., Ltd., Japan) using an ERC-WP300-C8 separating column (Eruma Co., Ltd., Tokyo, Japan) and acetonitrile–water (95 : 5) as the eluting solvent at a flow rate of 1 ml/min. The effluent was continuously monitored at 220 nm. At the same time, effluent fractions were collected every 30 s and radioactivity was measured by a liquid scintillation counter (Packard model 460C) after the addition of 10 ml of liquid scintillation cocktail (500 ml of toluene, 500 ml of ethanol and 4 g of Omnifluor). Although the palmitate and oleate esters were eluted at the same retention time (20.6 min), palmitoleate (16.2 min) and stearate esters (26.8 min) were separated effectively. Good separation between palmitate and oleate esters was obtained by the following GC-

MS-method. Because the 1-ester and 18-ester of a given fatty acid were not separated from each other in this HPLC method, separation of these esters was carried out by the TLC method before HPLC analysis.

Identification of Fatty Acid Conjugates of Plaunotol by GC-MS——A Hewlett–Packard model 5985 computerized GC-MS system was used. A megabore column coated with DB-5 (0.53 mm i.d. × 15 m) was used. Oven temperature was controlled at 300 °C (350 °C at the injection port). Helium gas at the flow rate of 5 ml/min was used as the carrier gas. Ammonia gas was employed as the reagent gas for chemical ionization detection. Mass numbers from *m/z* 250 to 650 were scanned. The retention times of synthesized plaunotol esters were as follows: 18-palmitoleate, 6.9 min; 1-palmitoleate, 8.2 min; 18-palmitate, 7.1 min; 1-palmitate, 8.4 min; 18-oleate, 10.2 min; 1-oleate, 12.1 min; 18-stearate, 10.6 min; 1-stearate, 12.5 min. In each of the authentic esters, the 18-ester was detected 1—2 min earlier than the 1-ester.

Results

Isolation and Identification of Fatty Acid Conjugates of Plaunotol

Formation in Liver Homogenate System——After incubating [^{14}C]plaunotol (50 μM) with rat liver homogenate, highly lipophilic metabolites, which were identified as fatty acid conjugates of plaunotol as described in the following sections, were detected as one of the two major spots on two-dimensional TLC, amounting to 15.3% of total radioactivity. Another major spot was the metabolite in which the 1-position of plaunotol was oxidized to carboxylic acid. As shown in Table I, high activity for the fatty acid conjugation of plaunotol was observed in the microsomal fraction. The supernatant fraction was about 80% less active than the microsomal fraction. The addition of 50 μM palmitoyl-CoA to the incubation mixture did not increase the amount of fatty acid conjugates produced in this system. After treatment with cholesterol esterase, the lipophilic metabolites liberated plaunotol (results are not shown). Since authentic fatty acid mono-esters of plaunotol were hydrolyzed to plaunotol by cholesterol esterase treatment, the lipophilic metabolites were concluded to be fatty acid conjugates of plaunotol.

Separation by TLC——Plaunotol possesses two hydroxyl groups at the 1- and 18-positions which should not differ significantly from the chemical point of view. Authentic 1- and 18-monoesters of plaunotol could be separated from each other effectively by TLC using hexane–ethyl acetate (7 : 3) as the developing solvent, thus making identification of the esterified position possible. The fatty acid conjugates of plaunotol formed *in vitro* turned out to be exclusively 1-esters as shown in Fig. 2.

Separation by HPLC——Fatty acid conjugates of [^{14}C]plaunotol were separated into three fractions by HPLC (palmitoleyl ester, palmitoyl and oleyl esters and stearoyl ester) using a C8 reverse-phase column (Fig. 3). As described in the experimental section, the palmitate and oleate esters of plaunotol were not separated from each other and were eluted as a combined fraction by this method. In co-chromatography of fatty acid conjugates of [^{14}C]plaunotol with authentic compounds, the two ultraviolet (UV) peaks corresponding to palmitate, oleate combined esters and stearoyl ester completely coincided with the major radioactive peaks as shown in Fig. 3. Shortly after the peak of palmitoleyl ester, a minor

TABLE I. Formation of Fatty Acid Conjugates of Plaunotol in Liver Subcellular Fractions

Fraction	Fatty acid conjugates of plaunotol formed (nmol/30 min/fraction)	
	−Palmitoyl-CoA	+Palmitoyl-CoA
None	0.32	0.68
Crude homogenate	7.64	6.07
105000 *g* supernatant	1.42	1.09
Microsome	6.92	6.49

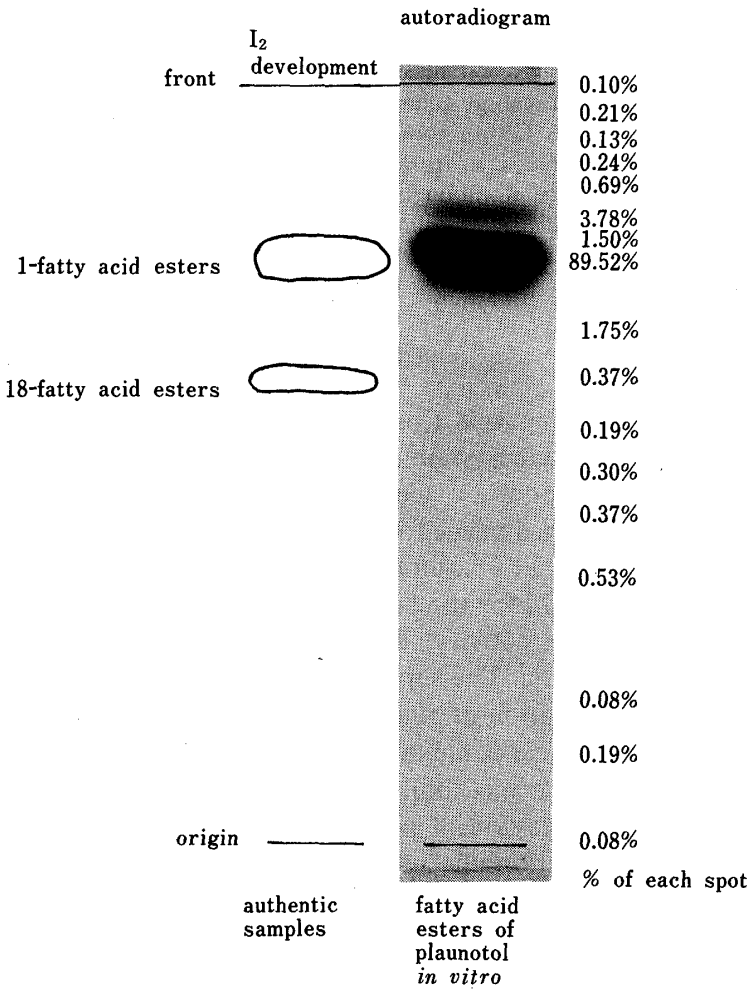

Fig. 2. Identification of 1-Fatty Acid Esters of [^{14}C]Plaunotol by TLC

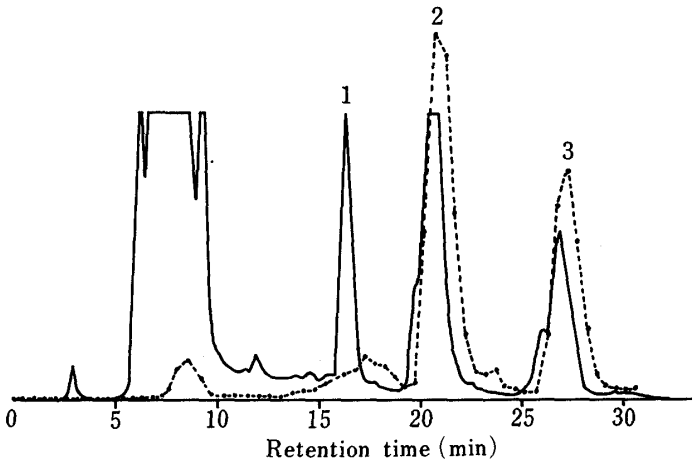

Fig. 3. Co-chromatogram of Fatty Acid Ester Fraction of [^{14}C]Plaunotol with Authentic Standards (Simultaneous Detection by UV and ^{14}C-RI)

——, UV; – – – –, RI. 1, 1- or 18-palmitoleate of plaunotol; 2, 1- or 18-palmitate and oleate of plaunotol; 3, 1- or 18-stearate of plaunotol.

radioactive peak was also observed. The relative amounts of these three radioactive peaks were 52.0%, 32.1% and 11.1% for palmitate and oleate esters, stearoyl ester and unknown fatty acid ester(s), respectively (average from duplicate experiments). In addition to the three major radioactive (RI) peaks, a small radioactive peak due to a polar metabolite was observed at the retention time of 9 min (4%).

GC-MS——The three major RI peaks were collected by repeated chromatography with-

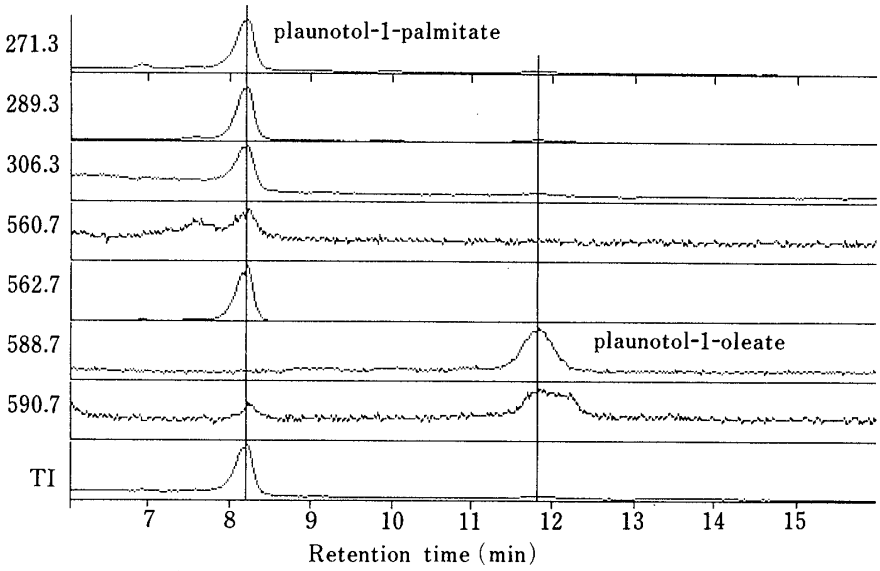

Fig. 4. GC-MS Chromatogram of 1-Palmitate and 1-Oleate Ester Fraction of [^{14}C]Plaunotol

TI: total ion.

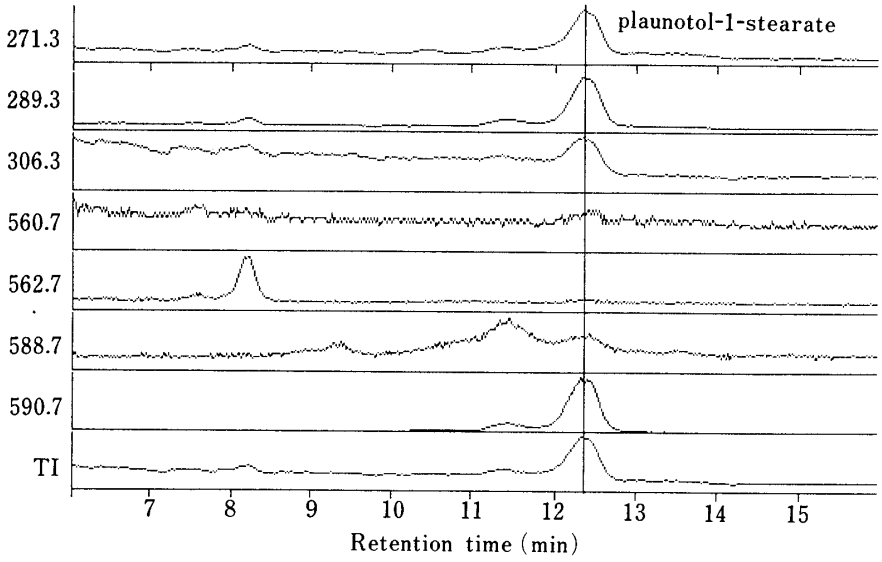

Fig. 5. GC-MS Chromatogram of 1-Stearate Ester Fraction of [^{14}C]Plaunotol

TI: total ion.

out authentic compounds and subjected to GC-MS analysis. In the mass chromatogram of the palmitate and oleate esters fraction, a substantial plaunotol-1-palmitate peak and a small but significant plaunotol-1-oleate peak were observed at the same retention times as the respective authentic standards, as shown in Fig. 4. Similarly, the plaunotol-1-stearate peak was observed in the mass chromatogram of the stearate ester fraction (Fig. 5). Probably because of the limited amount of the unknown fatty acid ester fraction, no significant peak was recognized. The mass spectra of the above three peaks also coincided with those of the respective authentic standards (Figs. 6—8). In the case of oleate ester, contaminating background peaks observed due to its low amount, as judged from the total ion mass chromatogram. On the basis of all these results, plaunotol was shown to be conjugated at the hydroxyl group at the 1-position but not at the 18-position with fatty acids

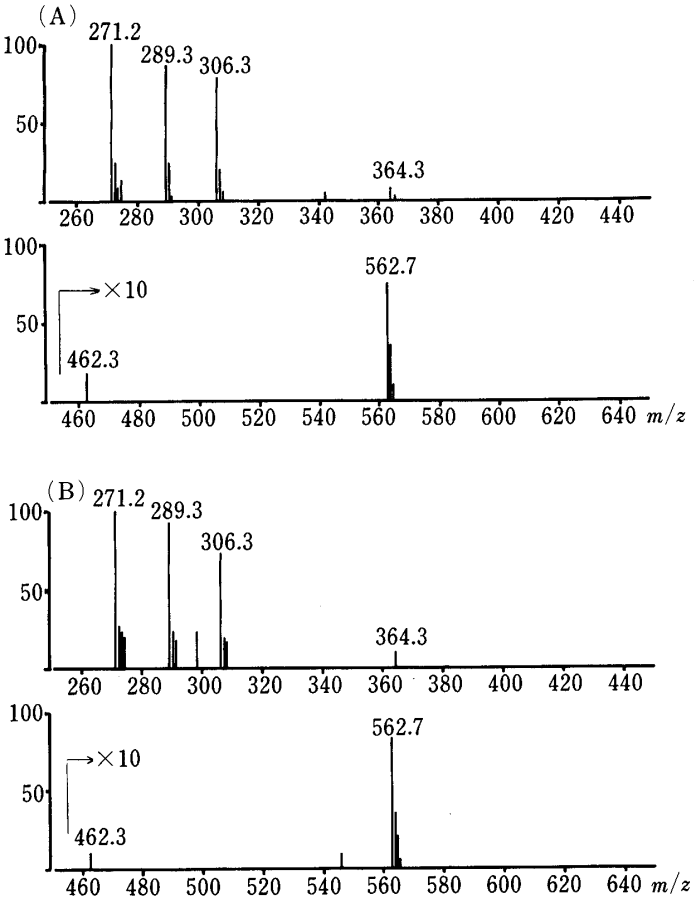

Fig. 6. GC-MS of Authentic (A) and *in Vitro* Formed (B) Plaunotol-1-palmitate

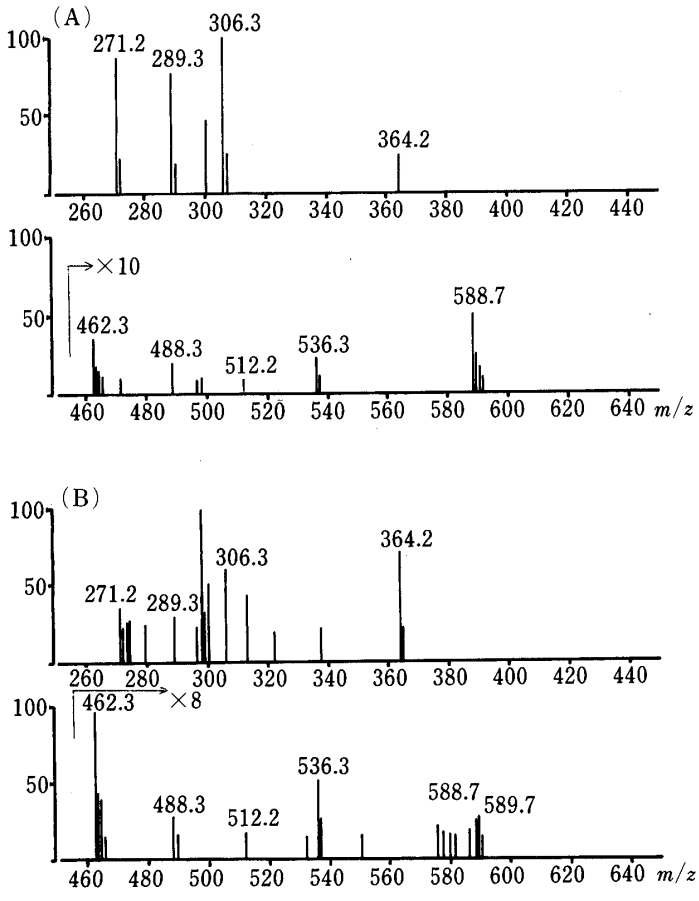

Fig. 7. GC-MS of Authentic (A) and *in Vitro* Formed (B) Plaunotol-1-oleate

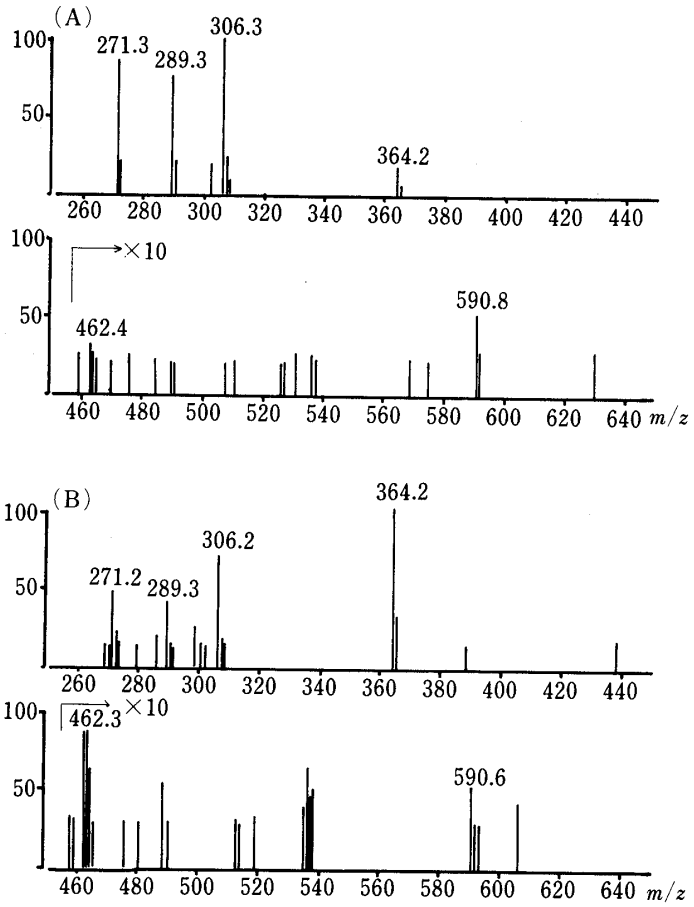

Fig. 8. GC-MS of Authentic (A) and *in Vitro* Formed (B) Plaunotol-1-stearate

(palmitic, oleic and stearic acids, at least) in the rat liver homogenate system.

Discussion

Generally speaking, drug metabolism increases the polarity or hydrophilicity of xenobiotics, promoting the excretion of the xenobiotics from the body. Although reported cases are limited, some compounds gain lipophilicity rather than hydrophilicity after metabolism, consequently remaining for a relatively long time in the body.[4] These highly lipophilic metabolites can be practically classified into three groups: 1) carboxylic metabolites incorporated into hybrid triglycerides (cycloprate,[5-8] dodecylcyclohexane,[9] ethyl-4-benzyloxybenzoate,[10,11] methoprene,[12] 3-phenoxybenzoate[13]); 2) carboxylic metabolites incorporated into cholesterol ester (BRL24139,[14] cycloprate, fenvalerate,[15,16] methoprene, prednimustine[17]) and 3) hydroxylic metabolites conjugated with fatty acids (tetrahydrocannabinol and its metabolites,[18-20] etofenamate,[21,22] the metabolite of dichlorodiphenyltrichloroethane (DDT),[23] dipyridamol and mopidamol[24] and phytol[25]). All the lipophilic metabolites in groups 1 and 2 possess a carboxylic group and were produced through the endogenous metabolic pathway for lipids functioning as pseudo-fatty acids.[26] In contrast, however, there is apparently no structural relationship among the compounds in group 3. Plaunotol, the xenobiotic newly classified into the third group in this paper, is a naturally occurring isoprenoid, as are tetrahydrocannabinol, its metabolites and phytol. Thus lipophilic isoprenoids that possess a hydroxyl group may be conjugated with fatty acid *in vivo*.

As described in this paper, fatty acid conjugates of plaunotol served as substrates for cholesterol esterase and were hydrolyzed into plaunotol and fatty acids. The substrate specificity of cholesterol esterase seems not to be strict because fatty acid conjugates of

tetrahydrocannabinol are also hydrolyzed by this enzyme.[27] These results suggest that all or part of the structures of plaunotol and tetrahydrocannabinol are recognized as quite similar to cholesterol by cholesterol esterase. The enzymatic systems producing endogenous cholesterol ester may contribute to the formation of fatty acid conjugates of plaunotol and other isoprenoids, *i.e.*, acyl-CoA: cholesterol *O*-acyltransferase (ACAT), lecithin: cholesterol *O*-acyltransferase (LCAT) and cholesterol esterase. In fact, the participation of ACAT was suggested in the production of fatty acid conjugates of tetrahydrocannabinol.[28,29] As described in the present paper, however, the addition of palmitoyl-CoA did not enhance the formation of fatty acid conjugates of plaunotol in the liver homogenate system, so it is unlikely that ACAT participates in the case of plaunotol. The transesterification between endogenous cholesterol esters and plaunotol may be catalyzed by cholesterol esterase as another possibility. The enzymatic mechanisms that produce fatty acid conjugates of plaunotol are now under detailed investigation and the results will be reported elsewhere.

Lipophilic metabolites remain in the body for a rather long period of time because of their nature and may cause chronic toxicity. The fatty acid conjugates of plaunotol, however, were only observed as such in the gastric mucosa and as glucuronide in the bile, and were not detected in any other tissue including adipose tissue. This is presumably because the fatty acid conjugates of plaunotol formed in the liver are further conjugated with glucuronic acid and effectively excreted into the bile. The fatty acid conjugates of plaunotol in gastric mucosa rather contribute to anti-ulcer activity since the production of anti-ulcer prostaglandins (PGE_2 and PGI_2) *in vitro* is enhanced by the addition of these conjugates (Ushiyama and Matsuda, unpublished data), as is also the case with plaunotol and its carboxylic metabolites.[30]

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