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IDENTIFICATION OF *sn*-2-ω-HYDROXYCARBOXYLATE-CONTAINING PHOSPHOLIPIDS IN A LIPID EXTRACT FROM BOVINE BRAIN

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Phospholipids having both a long-chain acyl(palmitoyl or stearoyl) and a short-chain hydroxycarboxylyl(C_3-C_9) residue were identified by GC-MS in a fraction with PAF-like activity from a bovine brain lipid extract. The hydroxyl group in the hydroxycarboxylate residue was determined to be at the ω-position by comparison of the mass spectra of the *tert*-butyldimethylsilyl derivatives of these compounds with those of synthetic hydroxybutyrate-containing phosphatidylcholines. The co-existence of short-chain hydroxycarboxylate-, monocarboxylate- and dicarboxylate-containing phospholipids in the bovine brain lipid extract suggested that these compounds were formed by peroxidation of membrane phospholipids, especially phosphatidylcholines.

Platelet-activating factor(PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a phospholipid mediator with diverse biological activities, is synthesized in a variety of animal tissues including brain (1).

Previously, we found a PAF-like vasodepressor phospholipid(2) in a bovine brain lipid extract, and characterized it as a mixture of PAF homologs and various 1-long-chain acyl analogs(3,4). The molecular composition of the 1-acyl analogs of PAF was complex: these compounds included species with an *sn*-2-short-chain acyl group such as a propionyl and butyryl group in addition to those with an *sn*-2-acetyl group(3,4). This finding prompted us to study the PAF-like compounds in the lipid extract by GC-MS, leading to the identification of a novel series of phospholipids with an

Abbreviations used: PAF, platelet-activating factor; PC, phosphatidylcholine; SPM, sphingomyelin; lysoPC, lysophosphatidylcholine; GPC, *sn*-glycero-3-phosphocholine; tBDMS, *tert*-butyldimethylsilyl.

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sn-2-short-chain dicarboxylyl residue(5). Here, we report the existence in the lipid extract of a third series of PAF-like phospholipid containing an *sn*-2-short-chain ω-hydroxycarboxylyl residue. The profiles of the PAF-like phospholipids in the bovine brain lipid extract suggest that all these phospholipids except those with an *sn*-2-acetyl group were formed by peroxidation of membrane phosphatidylcholines.

Methods

GC-MS of hydroxycarboxylate-containing phospholipids in a bovine brain lipid extract

Lipids were extracted from bovine cerebrum, and fractionated by silicic acid column chromatography, as reported previously(3). The fraction with PAF-like activity was purified further by reverse-phase TLC on a Merck Silica gel plate 60 in a solvent system of acetone-methanol-water(1:2:1, by vol.). In this solvent system, phosphatidylcholine(PC) and sphingomyelin(SPM) remained at the origin, and lysophosphatidylcholine(lysoPC) moved with an Rf value of 0.6(3). To separate PAF and its analogs from PC, SPM and lysoPC, we scraped off silica gel in the zone of 0.05-0.55, and extracted the phospholipids from it by the method of Bligh and Dyer(6). We then hydrolyzed these phospholipids with phospholipase C(*Bacillus cereus*, Sigma), and converted the resultant glycerides to *tert*-butyldimethylsilyl (*t*BDMS) derivatives(3). The reaction products were fractionated by TLC, eluted from the silica gel with 4 ml chloroform, and analyzed by GC-MS, as described previously(3,4), except that a Hewlett Packard capillary column(50 m x 0.32 mm I.D.) coated with cross-linked methylsilicone(0.52 μm thickness) was used.

Synthesis of hydroxybutyrate-containing PCs

2-Hydroxybutyrate-containing-PC The ethyl ester of 2-hydroxybutyrate (5 g) was converted to the corresponding benzyl ether by treatment with 11 g of benzylbromide in 30 ml of dry ethyl ether in the presence of 40 g of silver oxide, by a reported method(7). The benzyl ether of the 2-hydroxybutyrate ethyl ester was hydrolyzed to the benzyl ether of 2-hydroxybutyrate by hydrolysis in methanolic 1*N* NaOH at room temperature for 2 hours. The product was then converted to the benzyl ether of 2-hydroxybutyric chloride by treatment with five equivalents of oxalyl chloride at 25°C for 2-3 hours.

1-Palmitoyl-2-lyso-GPC(10 mg) was mixed with 50 mg of *N,N'*-dimethylaminopyridine in 1 ml of dry chloroform. The reaction was initiated by adding the benzyl ether of 2-hydroxybutyric chloride(80 mg) at room temperature. After incubation for 2 hours with stirring, the phospholipid was recovered from the reaction mixture by the method of Bligh and Dyer(6). The phospholipid containing a benzyl ether of the 2-hydroxybutyrate residue was dissolved in 3 ml of chloroform. The solution was mixed with a trace amount of 10 % palladium carbon and one equivalent of acetate under a stream of hydrogen gas. The debenzylated phospholipid was purified by TLC on a Merck Silica gel 60 plate in a solvent system of chloroform-methanol-28 % ammonium hydroxide(65:35:8, v/v/v).

4-Hydroxybutyrate-containing-PC 4-Bromobutyric acid(3 g) was converted to 4-bromobutyric chloride by treatment with 2 g of oxalyl chloride at 4°C for 12 hours. 1-Palmitoyl-2-lyso-GPC(10 mg) was mixed with 4-bromobutyric chloride(50 mg) in 1 ml of dry chloroform in the presence of 50 mg *N,N'*-dimethylaminopyridine at room temperature for 1 hour with stirring. The PC with a 4-bromobutyrate residue was dissolved in 1 ml of chloroform, and the solution was stirred with 55 µl of water and 40 mg of silver carbonate in the dark at 4°C for 60 hours, resulting in replacement of the bromine by a hydroxyl group. The 4-hydroxybutyrate-containing PC was purified by silica gel TLC in a solvent system of chloroform-methanol-28 % ammonium hydroxide (65:35:8, v/v/v).

The purities of synthetic PCs having a hydroxybutyrate residue were checked by fast atom bombardment mass spectrometry in a JEOL JMS-D300. Both PCs gave an intense signal of $[M+H]^+$ at m/z 582 together with characteristic fragment ions which were assignable to $[M-85]^+$(m/z 496), $[phosphocholine]^+$(m/z 184) and $[choline]^+$(m/z 104).

Results and Discussion

The phospholipid fraction with PAF activity purified from a bovine brain lipid extract was hydrolyzed with phospholipase C, and the resultant

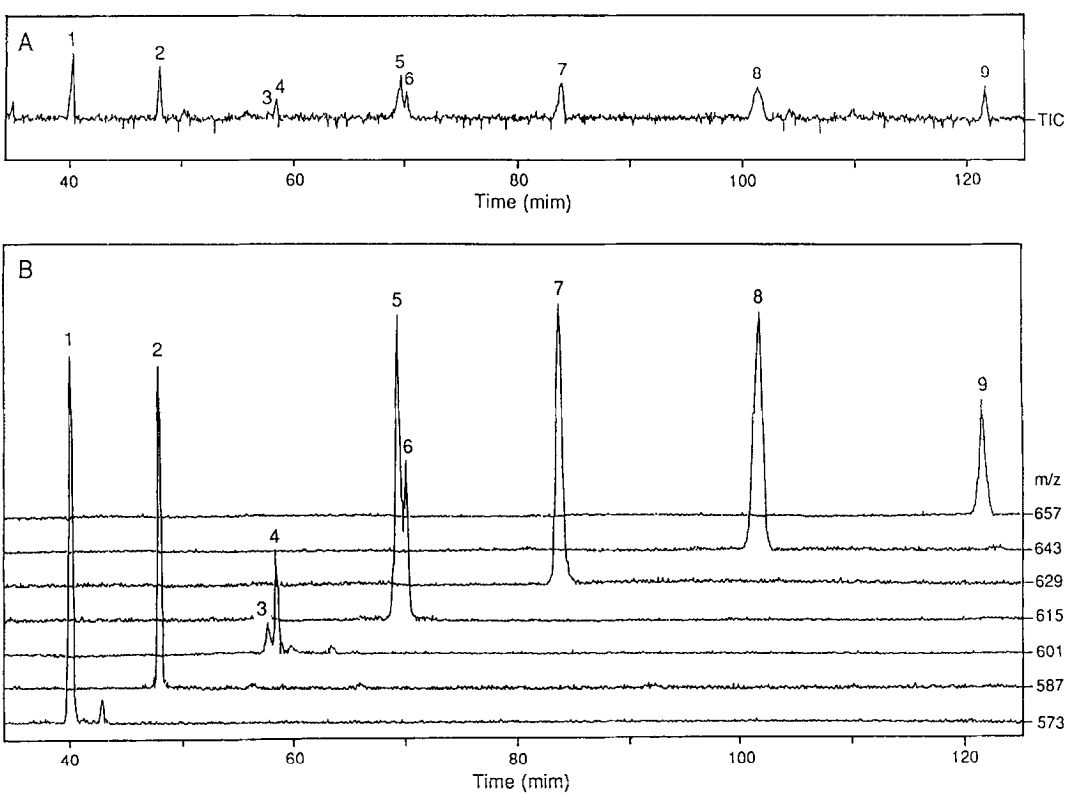

Figure 1. Total ion monitoring(A) and ion-current profiles(B) on GC-MS of tBDMS derivatives of glycerides of the phospholipid fraction with PAF-like activity from a bovine brain lipid extract.

glycerides were fractionated by TLC after their conversion to *t*BDMS derivatives, as described in Methods. Figure 1-A shows the total ion chromatogram of the *t*BDMS derivative recovered from a zone of Rf 0.4-0.6 on the TLC plate. The 9 major ion peaks(compounds 1-9) were expected to be due to a homologous series of *t*BDMS derivatives of glycerides on the basis of their mass spectra. These compounds gave intense ion signals assignable to the corresponding [M-*tert*-butyl]$^+$ at 14 mass unit intervals(m/z 573, 587, 601, 615, 629, 643 and 657, Fig. 1-B). It should be mentioned that the *t*BDMS derivatives derived from PAF and 1-long-chain acyl-2-short-chain acyl-GPCs, which we detected previously in the active fraction, were eluted within 30 min(data not shown).

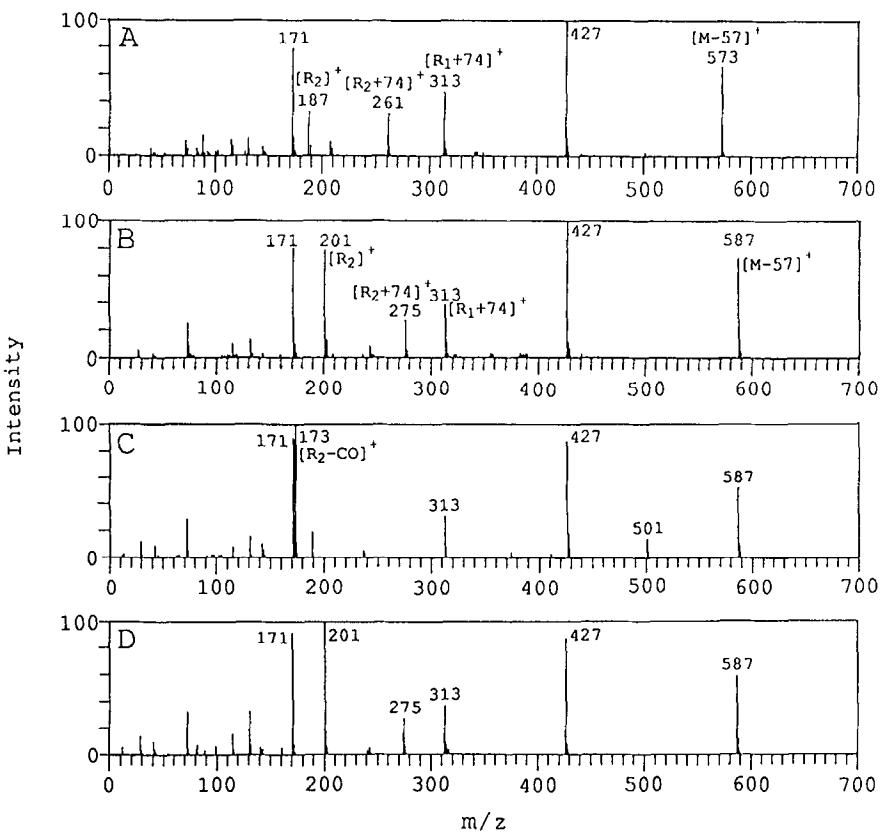

Figure 2. Mass spectra of components in the gas chromatogram of tBDMS derivatives of hydroxycarboxylate-containing phospholipids.
A and B: Mass spectra measured at the top of peak 1 and 2, respectively(Fig. 1). C and D: Mass spectra of tBDMS derivatives of glycerides derived from synthetic 2-hydroxybutyrate or 4-hydroxybutyrate-containing-PC, respectively.

Figure 2-A and B show the mass spectra of compound 1 and compound 2, respectively. High resolution mass spectrometric analysis indicated that the elemental composition of the intense ion at m/z 313 in the mass spectrum of compound 1 was $C_{18}H_{37}O_2Si$(observed mass 313.2609, calculated mass 313.2562), which can be assigned to $[CH_3(CH_2)_{14}CO + OSi(CH_3)_2]^+$, as shown in Fig. 3. An intense ion with the same elemental composition was also observed in the mass spectra of compounds 2(Fig.2-B),3,5,7,8 and 9(data not shown), indicating that these are homologs of glycerides having with a palmitoyl residue. The intense ions at m/z 187 and m/z 261 in the mass spectra of compound 1 were found to have the elemental composition of $C_9H_{19}O_2Si$(observed mass 187.1126, calculated mass 187.1154) and $C_{11}H_{25}O_3Si_2$(observed mass 261.1328, calculated mass 261.1342), respectively. These ions would be due to a hydroxypropionyl-tBDMS residue(R_2), probably attached to the *sn*-2-position of glycerides, and a rearrangement ion of R_2 and dimethylsilanol(Fig. 3). Similarly, a diagnostic ion pair(m/z 201 and 275) in the mass spectrum of compound 2 was found to be due to $[R_2]^+$ and $[R_2+OSi(CH_3)_2]^+$. High resolution mass spectral data for $[M-57]^+$ ($C_{30}H_{61}O_6Si_2$; observed mass 573.3983, calculated mass 573.4006, and $C_{31}H_{63}O_6Si_2$; observed mass 587.4208, calculated mass 587.4163) supported the conclusion that compounds 1 and 2 were *t*BDMS derivatives of glycerides having both a palmitoyl and a hydroxypropionyl or hydroxybutyryl group. The other diagnostic ions were found to be $[M-R_2O]^+$(m/z 427) and $[M-R_1O-R_2O-H]^+$(m/z 171), respectively.

De Jong et al.(8) reported that on mass spectrometry, *t*BDMS derivatives of lactate and 2-hydroxyvaleric acid(α-hydroxycarboxylate) and 3-hy-

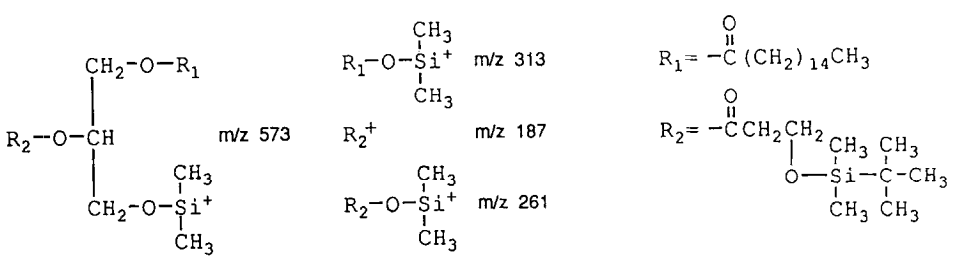

Figure 3. Structure of tBDMS derivative of hydroxypropionate-containing phospholipid.

droxybutyric acid(β-hydroxycarboxylate) gave a characteristic ion assignable to $[M\text{-}tert\text{-butyl-CO}]^+$ for α-hydroxyacids and $[M\text{-}tert\text{-butyl-CH}_2CO]^+$ for β-hydroxyacids. We confirmed this finding using *t*BDMS derivatives of α-hydroxycarboxylates with C_3-C_9 and β-hydroxycarboxylates(C_4), and found that ω-hydroxycarboxylate(C_3 and C_4) gave no significant ion peak that corresponded to $[M\text{-}tert\text{-butyl-CO}]^+$ and $[M\text{-}tert\text{-butyl-CH}_2CO]^+$. We presumed that the diagnostic ions described above could be detected in the mass spectra of *t*BDMS derivatives of glycerides having a short-chain hydroxycarboxylate residue. To verify this assumption, we synthesized 1-palmitoyl-2-(2-hydroxy)butyryl-GPC and 1-palmitoyl-2-(4-hydroxy)butyryl-GPC, and analyzed the corresponding *t*BDMS derivatives by GC-MS after the hydrolysis with phospholipase C. As expected, a significant ion assignable to $[R_2\text{-CO}]^+$ was seen at m/z 173 in the mass spectrum of the *t*BDMS derivative derived from 2-hydroxybutyrate-containing PC(Fig. 2-C). On the other hand, diagnostic ions at m/z 201($[R_2]^+$) and 275($[R_2+74]^+$) were observed in the mass spectrum of the *t*BDMS derivative of glyceride derived from 4-hydroxybutyrate-containing PC(Fig. 2-D), which was identical to that of compound 2. Judging from the similarity in the fragmentation patterns in their mass spectra, compounds 1-9 seemed to be ω-hydroxycarboyxlate-containing phospholipids. Table I lists the relative abundances of the major ions in mass spectra of compounds 1-9, and their possible assignments together with their retention times on GC. A plot of the logarithms of the relative retention times of compounds having a palmitoyl group against the chain lengths of their hydroxycarboxylate residues, was linear(r=1.00).

Previously, we reported the presence of monocarboxylate(C_2-C_7)-containing PCs, and dicarboxylate(C_4-C_9)-containing phospholipids in a bovine brain lipid extract. Considering the co-existence of ω-hydroxycarboxylate-containing-phospholipids(C_3-C_9) with these unique phospholipids and the similar patterns of molecular heterogeneity of these three series of phospholipids, it seems likely that all these compounds,except the species having an acetyl group were formed by lipid peroxidation of various molecu-

Table I. Major ions in mass spectra measured at the top of peak 1-9(Fig. 1) and their possible assignments

Compound	Retention time	Major ions in EI-MS				Possible group	
		$[M-57]^+$	$[R_1+74]^+$	$[R_2]^+$	$[R_2+74]^+$	*sn*-1	*sn*-2
	min	m/z (Relative intensity, %)					
1	40.1	573 (68)	313 (48)	187 (32)	261 (32)	palmitoyl	3-hydroxy-propionyl
2	47.9	587 (72)	313 (40)	201 (80)	275 (28)	palmitoyl	4-hydroxy-butyryl
3	57.6	601 (100)	313 (15)	215 (70)	289 (14)	palmitoyl	5-hydroxy-valeroyl
4	58.3	601 (100)	341 (41)	187 (33)	261 (36)	stearoyl	3-hydroxy-propionyl
5	69.5	615 (100)	313 (62)	229 (67)	303 (15)	palmitoyl	6-hydroxy-caproyl
6	70.0	615 (100)	341 (23)	201 (70)	275 (16)	stearoyl	4-hydroxy-butyryl
7	83.9	629 (100)	313 (59)	243 (49)	317 (12)	palmitoyl	7-hydroxy-enanthoyl
8	101.5	643 (100)	313 (56)	257 (27)	331 (17)	palmitoyl	8-hydroxy-capryloyl
9	121.7	657 (100)	313 (85)	271 (28)	345 (5)	palmitoyl	9-hydroxy-pelargonoyl

lar species of PC. In this connection, it should be mentioned that Itabe et al.(9) identified an *sn*-2-azelaoyl-containing PC as a cytotoxic phospholipid toward erythrocytes which was formed during peroxidation of liposomes of linoleate-containing PC with oxyhemoglobin, and that Stremler et al.(10) reported the peroxidative formation of 5-oxovalerate-containing PC from PC having an arachidonate residue.

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References

1. Braquet, P., Touqui,L., Shen, T. Y. and Vargraftig, B. B.(1987) Pharmacol. Rev. 39. 97-145.
2. Tsukatani, H., Yamada, S., A. Tokumura, Miyamoto, T. and Takauchi, K.(1976) Chem. Pharm. Bull. 24, 2294-2300.
3. Tokumura, A., Kamiyasu, K., Takauchi, K., Tsukatani, H.(1987) Biochem. Biophys. Res. Commun. 145, 415-425.
4. Tokumura, A., Takauchi, K., Asai. T., Kamiyasu, K., Ogawa, T. and Tsukatani, H. (1989) J. Lipid Res. 30, 219-224.

5. Tokumura, A., Asai, T., Takauchi, K., Kamiyasu, K., Ogawa, T. and Tsukatani, H.(1988) Biochem. Biophys. Res. Commun. 155, 863-869.
6. Bligh, E.G. and Dyer, W.J.(1959) Can. J. Biochem. Physiol. 37, 911-917.
7. Mislow, K., O'Brien, R.E. and Schaefer, H. (1962) J. Am. Chem. Soc. 84, 1940-1944.
8. de Jong, A.P.J.M., Elema, J., van de Berg, B.J.T.(1980) Biomed. Mass spectrom. 7, 359-364.
9. Itabe, H., Kushi, Y., Handa, S. and Inoue, K.(1988) Biochim. Biophys. Acta 962, 8-15.
10. Stremler, K.E., Stafforini, D.M., Prescott, S.M., Zimmerman, G.A.and McIntyre, T.M.(1989) J. Biol. Chem. 264, 5331-5334.