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Quantitation of choline glycerophospholipids that contain carboxylate residues by fluorometric high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic method was developed for the quantitation of oxidized choline glycerophospholipids (CGPs) that contain carboxylate residues, after the derivatization of these CGPs with the fluorescent probe 9-anthryldiazomethane (ADAM). The probe reacted efficiently with the carboxylate residues of the CGPs at room temperature. Fluorescent derivatives of carboxylic CGPs were well separated into glutaryl and azelaoyl residues, which are the predominant carboxylic CGP products of the oxidation of naturally occurring CGP. The relationship between the amount of each derivative and the signal was linear over a wide range and amounts as low as a few picomoles of carboxylic glycerophospholipids could be detected.

Keywords: Derivatization, LC; Choline glycerophospholipids; Glycerophospholipids; Phospholipids; 9-Anthryldiazomethane; Lipids

1. Introduction

Previous studies have shown that the peroxidation of glycerophospholipids in biological membranes contributes significantly to processes associated with aging and cellular damage [1–4]. Active oxygen species preferentially attack the double bonds of glycerophospholipids to generate glycerophospholipid hydroperoxides in cell membranes. The breakdown of glycerophospholipid hydroperoxides is accompanied by the generation of a complex variety of oxidized glycerophospholipids that contain short-chain carbonyl or carboxyl moieties, as secondary products, which might be involved in the pathogenesis of various types of injury to tissues [5,6]. Oxidized glycerophospholipids containing carbonyl or carboxyl moieties, derived from oxidatively fragmented polyunsaturated fatty acids in glycerophospholipids, have attracted attention because of their effects on the activation of neutrophils [7], the aggregation of platelets [8], and the lysis of red blood cells, as well as their cytotoxic activity [9]. It is obvious that a specific method for the identification and quantitation of trace amounts of secondary products that originate from peroxidized glycerophospholipids is required if we are to gain new insights into the biological functions of such

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oxidative lipids and the mechanisms responsible for the dysfunction of oxidized biomembranes.

We have already developed a sensitive fluorometric HPLC procedure for analysis of aldehydic choline glycerophospholipids [10]. The present report describes the derivatization of carboxylic CGPs with a fluorescent reagent, as well as the conditions for fractionation and quantitation by HPLC of the fluorescent derivatives of the oxidized CGPs.

2. Experimental

2.1. Chemicals and reagents

1-Palmitoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (16:0–20:4 GPC) were purchased from Sigma (St. Louis, MO, USA). 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0–18:2 GPC) was obtained from Avanti Polar-Lipids (Alabaster, AL, USA). Azelaoyl chloride was obtained from Tokyo Kasei (Tokyo, Japan) and 9-anthryldiazomethane (ADAM) was from Funakoshi (Tokyo, Japan). 2,2′-Azobis (2,4-dimethylvaleronitrile) (AMVN), glutaric anhydride, 4-dimethylaminopyridine and HPLC-grade solvents (methanol, acetonitrile, tetrahydrofuran and distilled water) were obtained from Wako Pure Chemicals (Osaka, Japan).

2.2. Synthesis of carboxylic CGPs and their identification

1-Palmitoyl-2-glutaryl GPC (CGP-5COOH) was prepared by incubation of 1-palmitoyl GPC (30 mg) with glutaric anhydride (90 mg) and 4-dimethylaminopyridine (3 mg) in 1 ml of pyridine at 50°C overnight. The reaction was stopped by addition of 0.2 ml of water and the mixture was evaporated to dryness under a stream of nitrogen gas. Three ml of water were added to the residue and the solution was adjusted to pH 3.0 with 0.1 *M* HCl. The resultant carboxylic CGP was extracted by the method of Bligh and Dyer [11] and purified by TLC (silica gel G; Merck, Darmstadt, Germany) with a mixture of chloroform, methanol and 25% ammonia (65:35:8, v/v) as the mobile phase.

1-Palmitoyl-2-azelaoyl GPC (CGP-9COOH) was prepared by incubation of 1-palmitoyl GPC (10 mg) with azelaoyl chloride (100 μl) and 4-dimethylaminopyridine (50 mg) in 1 ml of chloroform at room temperature, overnight. The reaction mixture was evaporated to dryness under a stream of nitrogen gas. A 3-ml volume of water was added to the residue and the solution was adjusted to pH 3.0 with 2 *M* NaOH. The carboxylic CGP was extracted by the method of Bligh and Dyer and purified by TLC (silica gel G; Merck) with chloroform, methanol, acetic acid and water (100:75:7:4, v/v) as the mobile phase. The carboxylic CGP was further purified by TLC (silica gel G; Merck) with a different mobile phase, namely, a mixture of chloroform, methanol and 25% ammonia (65:35:8, v/v).

2.3. Derivatization of carboxylic CGPs with the fluorescent probe and fractionation by HPLC

Carboxylic CGPs (5 pmol) were dissolved in 200 μl of methanol and the reaction was started by the addition of 200 μl of a 1% solution of ADAM (dissolved in small amount of acetone and then diluted with methanol). The reaction mixture was kept at room temperature for 60 min in darkness. Then the solvent was evaporated under a stream of nitrogen and fluorescent derivatives of carboxylic CGPs were purified by TLC (silica gel G; Merck) with a mixture of chloroform, methanol and 25% ammonia (65:35:8, v/v) as the mobile phase.

Purified fluorescent derivatives were fractionated and quantitated by reversed-phase HPLC. The carboxylic CGP derivatives were monitored with a fluorescence detector (exitation, 384 nm; emission, 415 nm). Fluorescence derivatives were eluted with a mixture of methanol, water, acetonitrile and tetrahydrofuran (90.5:7:4.9:10, v/v) that contained 20 m*M* choline chloride from a reversed-phase column (LiChrosob RP-18; Merck) at the flow-rate of 1 ml/min. The HPLC system consisted of a Model LC-6A liquid chromatograph equipped with a Model RF-550 spectrofluorometric detector (Shimadzu, Tokyo, Japan).

Synthetic carboxylic CGP derivatives were dissolved in a small volume of chloroform for identification by fast atom bombardment-mass spectrometry (FAB-MS). Aliquots of 1 or 2 μl of the solution were mixed with 1 drop of *m*-nitrobenzyl alcohol or

thioglycerol on the probe of a mass spectrometer (JMS-AX 505 HA; JEOL, Japan). The gun high voltage and accelerating voltage were both 3 kV. The filament current and emission current were 2 A and 20 mA, respectively. The m/z values of the positive molecular ions confirmed that the two synthetic carboxylic CGPs were CGP-5COOH (Fig. 1, top) and CGP-9COOH (Fig. 1, bottom).

2.4. Peroxidation of CGP with an azo-initiator

Aliquots of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (4 mg) or 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (4 mg) were incubated at 37°C for 1 h in chloroform (final volume, 1 ml) with or without 20 m*M* AMVN. After the reaction, excess AMVN was removed from the reaction mixture by passage through a silica gel column (2.5 × 0.5 cm I.D., Wako gel C-200, 100–200 mesh, Wako Pure Chemicals) with chloroform as the eluate, the oxidized glycerophospholipids were recovered with methanol. The carboxylic CGPs were then reacted with 1% solution of ADAM and the fluorescent derivatives were quantitated with the HPLC system after purification by TLC, as described above.

3. Results and discussion

Optimal conditions for derivatization were determined with 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (CGP-5COOH) and 1-palmitoyl-2-azelayol-*sn*-glycero-3-phosphocholine (CGP-9COOH), synthesized from 1-palmitoyl glycerophosphocholine. CGP-5COOH and CGP-9COOH would be predominantly produced from the fragmentation of CGP that contained 20:4 and 18:2, respectively, by peroxidation. Several kinds of fluorescence label for short-chain carboxylates have been reported, such as 2-naphthacyl [12], 4-bromophenacyl [13], and 4-bromomethyl-7-methoxycoumarin (Br-Mmc) [14] esters. 9-Anthryldiazomethane (ADAM) [15], which is commonly used as a fluorescent reagent for labeling free fatty acids, was chosen as the reagent for the derivatization of carboxylate residues of glycerophospholipids. ADAM has several advantages over other similar reagents. These advantages include faster derivatization; the absence of any requirement for a catalyst and heating; high reactivity; and high fluorescence intensity. The high concentration of the solution of ADAM (1% instead of the conventional 0.05% or 0.1% solution) was important for derivatization of carboxylic CGPs. The extent of derivatization was 89±2% (n=3), as determined from the recovery of phosphorus in the ADAM derivative of carboxylic CGPs. The relationship between the amount of CGP-9COOH injected onto the column for HPLC and the signal was linear over a wide range of values (2–100 pmol; r=0.99).

A typical chromatogram after reversed-phase HPLC of ADAM derivatives of the synthetic CGP-5COOH and CGP-9COOH standards is shown in Fig. 2. ADAM derivatives of carboxylic CGPs with carboxylic chains of different lengths were well separated within 20 min by reversed-phase HPLC upon isocratic elution with a mixture of methanol, water, acetonitrile and tetrahydrofuran (90.5:7:4.9:10, v/v) that contained 20 m*M* choline chloride (Fig. 2 C). There were no interfering peaks that originated from degradation products of ADAM near the peaks of the derivatives of carboxylic CGPs.

We attempted to detect carboxylic CGPs produced from 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (16:0–20:4 GPC) and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0–18:2 GPC) by treatment with AMVN, a lipophilic radical initiator. Peroxidized CGPs were derivatized with ADAM and the reaction mixture was fractionated by HPLC for detection of carboxylic CGP. A typical chromatogram of ADAM derivatives of the carboxylic CGPs that were produced by AMVN treatment of 16:0–20:4 GPC and 16:0–18:2 GPC is shown in Fig. 3. A small peak was observed in the profile of commercial 16:0–20:4 GPC (Fig. 3 A), but significant amounts of this carboxylic CGP were found in AMVN-treated 16:0–20:4 GPC (Fig. 3 B). The retention time of this carboxylic CGP corresponded to that of synthetic CGP-5COOH. In the case of 16:0–18:2 GPC, a similar phenomenon was observed, but the amount of CGP-9COOH seemed to be lower than that of CGP-5COOH formed from 16:0–20:4 GPC under the same conditions (Fig. 3 B and D). These results suggest that the rate of production of carboxylic CGP from peroxidized CGP

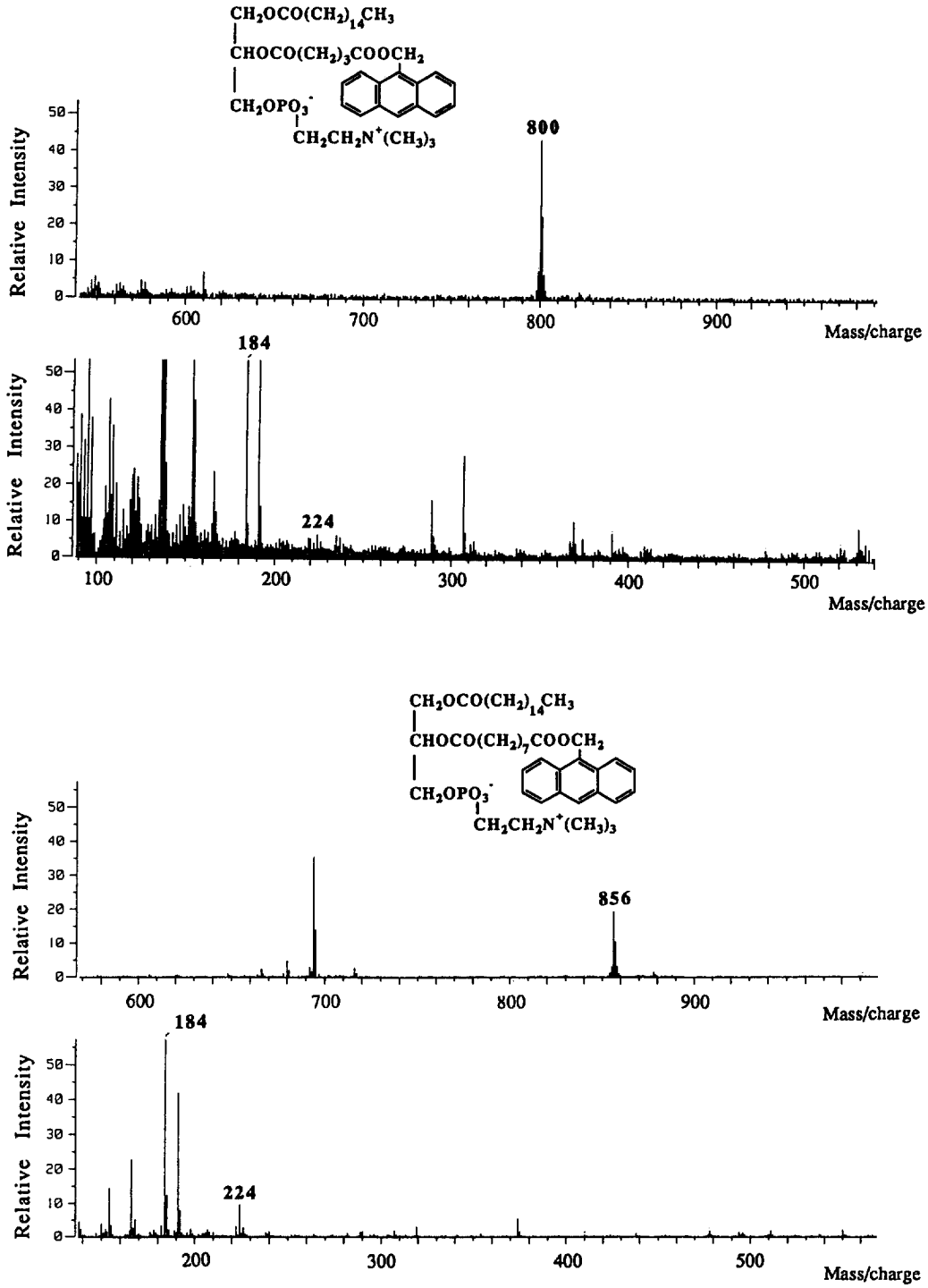

Fig. 1. Positive-ion fast atom bombardment-mass spectrum obtained with a *m*-nitrobenzyl alcohol matrix. (Top) spectrum of ADAM derivatives of 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine. (Bottom) spectrum of ADAM derivatives of 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine.

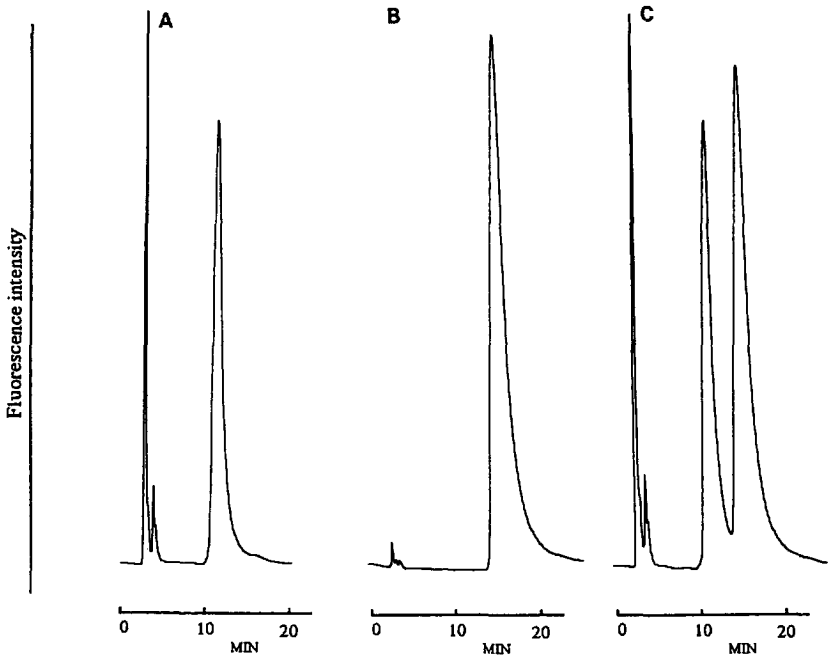

Fig. 2. Chromatograms of ADAM derivatives of the synthetic standards, CGP-5COOH and CGP-9COOH, after reversed-phase HPLC. Each injection contained 3 pmol of the standards. (A) ADAM derivative of CGP-5COOH, (B) ADAM derivative of CGP-9COOH, (C) a mixture of ADAM derivatives of CGP-5COOH and CGP-9COOH.

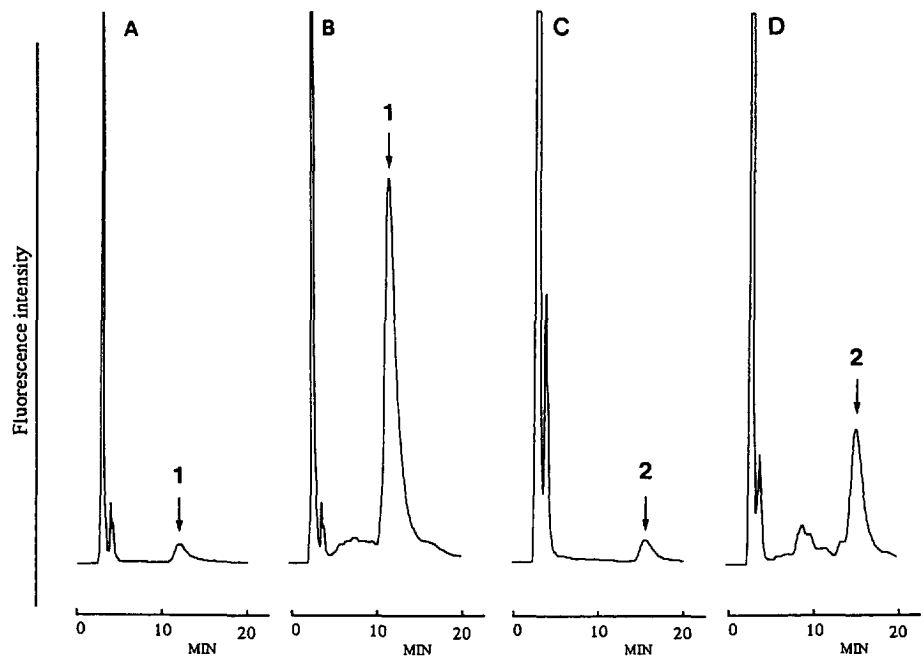

Fig. 3. Typical chromatograms of ADAM derivatives of carboxylic CGPs formed from 16:0–20:4 GPC and 16:0–18:2 GPC after peroxidation with AMVN. The injected amounts corresponded to 50 pmol of each starting CGP. Retention times of peak 1 and peak 2 correspond to those of the synthetic CGP-5COOH and CGP-9COOH standards, respectively. (A) 16:0–20:4 GPC (control), (B) peroxidized 16:0–20:4 GPC, (C) 16:0–18:2 GPC (control), (D) peroxidized 16:0–18:2 GPC.

was closely related to the type of polyunsaturated fatty acid at the 2-position in glycerophospholipids.

Recently, reports have appeared on the biological activities of oxidatively degraded glycerophospholipids. Smiley et al. [16] reported that oxidative glycerophospholipids derived from 1-palmitoyl-2-arachidonoyl GPC strongly stimulated the adhesion of neutrophils to endothelial cells. Itabe et al. [9] detected CGP contained an azelaoyl residue in peroxidized CGP that contained linoleic acid, and this carboxylic CGP had hemolytic activity. 1-Hexadecyl-2-glutaryl GPC which is a major product of the peroxidation of 1-hexadecyl-2-arachidonoyl GPC was found to cause the aggregation of platelets [17]. These published reports suggest that oxidatively fragmented glycerophospholipids that included carboxylic residues are present in tissues after exposure to oxidative stress, where they might affect the biological functions of susceptible cells and organs.

4. Conclusions

The present HPLC method with fluorometric detection, using ADAM, has sufficient sensitivity for the quantitative analysis of trace amounts of carboxylic glycerophospholipids. The methodology described in this report will be useful for investigations of the composition and metabolism of CGPs that contain carboxylate residues, and it will provide new insights into the formation and degradation of carboxylic glycerophospholipids in various tissues that are exposed to oxidative stress.

5. Notation

ADAM	9-anthryldiazomethane
AMVN	2,2′-azobis(2,4-dimethylvaleronitrile)
CGP	choline glycerophospholipid
CGP-9COOH	1-acyl-2-azelaoyl-*sn*-glycerophosphocholine
CGP-5COOH	1-acyl-2-glutaryl-*sn*-glycerophosphocholine
GPC	glycerophosphocholine
HPLC	high-performance liquid chromatography
RBC	red blood cell
TLC	thin-layer chromatography

References

[1] D. Harman, Proc. Natl. Acad. Sci. USA, 78 (1981) 7124.
[2] T.F. Slater, Biochem. J., 222 (1984) 1.
[3] H. Kappush and H. Sies, Experientia, 37 (1981) 1233.
[4] K. Gast, D. Zirwer, A.M. Laddhoff, J. Schreiber, R. Koelsch, K. Kretschme and J. Lasch, Biochim. Biophys. Acta, 686 (1982) 99.
[5] H. Esterbauer, in D.C.H. McBrien and T.F. Slater (Editors), Free Radical Lipid Peroxidation and Cancer, Academic Press, New York, 1982, p.102.
[6] H. Esterbauer and H. Zollner, Free Radicals Biol. Med. 7 (1980) 197
[7] P.L. Smiley, K.E. Stremler, S.M. Prescott, G.A. Zimmerman and T.M. McIntyre, J. Biol. Chem., 266 (1991) 11104.
[8] T. Tanaka, H. Minamino, S. Unezaki, H. Tsukatani and A. Tokumura, Biochim. Biophys. Acta, 1166 (1993) 264.
[9] H. Itabe, Y. Kishi, S. Handa and K. Inoue, Biochim. Biophys. Acta, 962 (1988) 8.
[10] Z.L. Ou, A. Ogamo, L.H. Guo, Y. Konda, Y. Harigaya and Y. Nakagawa, Anal. Biochem., 227 (1995) 289.
[11] E.G. Bligh and W.J. Dyer, Can. J. Biochem. Physiol., 37 (1959) 911.
[12] M.J. Cooper and M.W. Anders, Anal. Chem., 46 (1974) 1849.
[13] P.T.-S. Pei, W.C. Kossa, S. Ramachandran and R.H. Henly, Lipids, 11 (1976) 814.
[14] S. Lam and E. Grushka, J. Chromatogr., 158 (1978) 207.
[15] N. Nimura and T. Kinoshita, Anal. Lett., 13 (1980) 191.
[16] P.L. Smiley, K.E. Stremler, S.M. Prescott, G.A. Zimmerman and T.M. McIntyre, J. Biol. Chem., 266 (1991) 11104.
[17] T. Tanaka, M. Imori, H. Tsukatani and A. Tokumura, Biochim. Biophys. Acta, 1210 (1994) 202.