LITERATURE CITED

Bergmeyer, H. M. *Methods of Enzymatic Analysis*; Verlag Chemie, Academic: New York, 1965.
Blanchard, J. *J. Chromatogr.* **1981**, *226*, 455–460.
Cheynier, V.; Trousdale, E.; Singleton, V. L.; M. Salgues, M.; Wylde, R. *J. Agric. Food Chem.* **1986**, *34*, 217–221.
Colonna, P.; Buleon, A.; Mercier, C. *J. Food Sci.* **1981**, *46*, 88–93.
Delaporte, N.; Macheix, J. J. *Anal. Chim. Acta* **1972**, *59*, 273–277.
Dubernet, M. Thèse 3e cycle, Univeristy of Bordeaux II, 1974.
Esterbauer, H.; Schwarzl, E.; Hayn, M. *Anal. Biochem.* **1977**, *77*, 486–494.
Gunata, Y. Z.; Salgues, M.; Moutounet, M. *Sci. Aliments* **1986**, in press.
Harel, E.; Mayer, A. M.; Shain, Y. *J. Sci. Food Agric.* **1966**, *17*, 389–392.
Myers, T. E.; Singleton, V. L. *Am. J. Enol. Vitic.* **1979**, *30*, 98–102.
Nagel, C. W.; Baranowski, J. D.; Wulf, L. W.; Powers, J. R. *Am. J. Enol. Vitic.* **1979**, *30*, 198–201.
Nickenig, R. Dissertation zur Erlangung des Grades Doktor der Trophologie, Hohen Landwirtschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität, Bonn, 1980.
Ong, B. Y.; Nagel, C. W. *Am. J. Enol. Vitic.* **1978**, *29*, 277–281.
Pierpoint, W. S. *Biochem. J.* **1966**, *98*, 567–579.
Pierpoint, W. S. Rothamsted Experimental Station Report, 1970, Part 2, pp 199–218.
Romeyer, F. M.; Sapis, J. C.; Macheix, J. J. *J. Sci. Food Agric.* **1985**, *36*, 728–732.
Salgues, M.; Cheynier, V.; Gunata, Z.; Wylde, R. *J. Food Sci.* **1986**, *51*, 1191–1194.
Sapis, J. C.; Macheix, J. J.; Cordonnier, R. E. *Am. J. Enol. Vitic.* **1983**, *34*, 157–162.
Schreier, P.; Miller, E. *Food Chem.* **1985**, *18*, 301–317.
Singleton, V. L.; Timberlake, C. F.; Lea, A. G. H. *J. Sci. Food Agric.* **1978**, *29*, 403–410.
Singleton, V. L.; Zaya, J.; Trousdale, E.; Salgues, M. *Vitis* **1984**, *23*, 113–120.
Singleton, V. L.; Salgues, M.; Zaya, J.; Trousdale, E. *Am. J. Enol. Vitic.* **1985**, *36*, 50–56.
Sophianopoulos, J. A.; Durham, S. J.; Sophianopoulos, A. J.; Ragsdale, H. L.; Cropper, W. P. *Arch. Biochem. Biophys.* **1978**, *187*, 132–137.
Vamos-Vigyazo, L. *CRC Crit. Rev. Food Sci. Nutr.* **1981**, 49–109.

Received for review October 8, 1986. Accepted May 18, 1987.

Prostaglandin-like Substances, Precursors of Red Pigment, Formed during Autoxidation of Methyl Arachidonate

Takashi Nakamura* and Yoichiro Hama

Red pigment forming substances (RPS) formed during autoxidation of methyl arachidonate were purified and the chemical structures determined. The production of RPS showed a good relation with lipid peroxidation, until the peroxide value reached a maximum. The RPS were purified successively by gel chromatography on Sephadex LH-20, column chromatography on silica gel 60, and high-performance liquid chromatography on μ-Porasil. Four purified RPS fractions were analyzed by IR spectrometry and by GC–MS after reduction with $NaBH_4$ or $NaBD_4$. Two RPS out of the four predicted by theory were identified as the stereoisomers of 3-(5-hydroxy-3-oxo-2-pentylcyclopentyl)-2-propenal and methyl 4-[2-(2-formylvinyl)-3-hydroxy-5-oxocyclopentyl]butanoate.

The interaction of peroxidized lipids with nitrogenous compounds, such as amino acids and proteins, results in the browning of foods, "rusting" so to speak (Gardner, 1979; Pokorny, 1981), or in the formation of age pigment in vivo (Porta and Hartroft, 1969; Mead, 1976; Hirai et al., 1982). These browning reactions originate from the same Schiff base condensation or carbonyl–amine condensation as do the Maillard-type amine–sugar browning in foods (Reynolds, 1969; Mester et al., 1981). However, there is little documentation on the red coloration induced by autoxidation of lipids. In recent work, we found that autoxidized lipids containing polyunsaturated fatty acids turned reddish brown when reacting with amino acids (Nakamura, 1984) and that development of the reddish color depended on formation of certain conjugated carbonyls in the autoxidized lipids. The carbonyls, red pigment forming substances (RPS), were isolated from autoxidized linolenate and were determined to be 3-(2-ethyl-5-hydroxy-3-oxocyclopentyl)-2-propenal and methyl 8-[2-(2-formylvinyl)-3-hydroxy-5-oxocyclopentyl]octanoate (I and II, respectively) in Figure 1 (Nakamura, 1985, 1986).

Laboratory of Fisheries Technology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Fukuoka 812, Japan.

The RPS (I) had a single intense λ_{max} at 226–227 nm in ethanol (ϵ 14 000), and the red pigment obtained by the reaction with glycine had λ_{max} 515 nm in methanolic solution ($E^{1\%}_{1cm}$ = 400). The coloration occurred rapidly at high temperature but was rather unstable. Although the physiological significance of this prostaglandin-like substance has not been determined, some of these RPS may have biological effects.

According to the mechanism of formation, deduced from findings with methyl linolenate (Nakamura 1985, 1986), at least four RPS (III–VI in Figure 1) could be produced from methyl arachidonate. We now report separation and identification of these compounds, formed during autoxidation of methyl arachidonate.

EXPERIMENTAL SECTION

Coloration and Preparation of Red Pigment Forming Substances (RPS). *Autoxidation.* Methyl arachidonate (99% grade; Sigma Chemical Co., St. Louis, MO) was oxidized at 40 °C in the dark with stirring and with occasional bubbling of air. Aliquots of the autoxidized methyl arachidonate taken at regular intervals were stored at −40 °C until use for assays.

Coloration. Twenty milligrams of the autoxidized methyl arachidonate was dissolved in 2 mL of MeOH, and 1 mL of 0.5 M glycine (pH 7.0) was added to the solution.

0021-8561/88/1436-0015$01.50/0

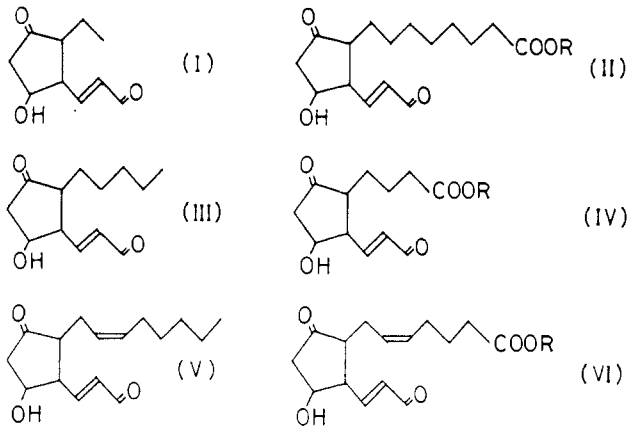

Figure 1. Predicted formation of red pigment forming substances (RPS) by autoxidation of linolenate (I, II) and arachidonate (III–VI).

The mixture was shaken for 1.5 h at 45 °C. To the discolored mixture were added 4 mL of $CHCl_3$ and 0.5 mL of water. After being shaken and centrifuged, the mixture was separated into upper and lower phases. Both were made up to 5 mL with MeOH. Absorbance of the clear solution was measured at 430 nm (brown pigments) and 515 nm (red pigments). Peroxide value was determined by a iodometric method (Standard Method of Japanese Oil Chemists' Society, 2.4.12-17). Unoxidized amounts of the arachidonate during autoxidation were measured by gas–liquid chromatography (GLC), using methyl stearate as the internal standard.

Gel Chromatography. The autoxidized methyl arachidonate was separated on a Sephadex LH-20 column (250 mL, 130 cm long) with $CHCl_3$–MeOH (1:1, v/v) as the eluent. The RPS eluted were monitored by the color reaction with glycine.

Silicic Acid Chromatography. The RPS fraction obtained by gel chromatography was further purified on a silica gel 60 column (24 × 1 cm; E. Merck, Darmstadt, Federal Republic of Germany) with $CHCl_3$–MeOH (97:3, v/v) as the eluent.

High-Performance Liquid Chromatography (HPLC). Crude RPS obtained by silicic acid chromatography were finally purified on a μ-Porasil column (30 × 0.78 cm; Waters Associates Inc., Framingham, Ma) with hexane–2-propanol (9:1, v/v) as the eluent. Purities of RPS were monitored by thin-layer chromatography (TLC) on silica gel 60 plates, with benzene–acetone–ethanol (70:30:2, v/v) as the developing solvent. Reddish spots of RPS were detected after spraying with a glycine solution (0.5 M, pH 7.0) and subsequent heating at 45 °C for 0.5–2 h. Charring spots were detected by spraying 70% H_2SO_4 and heating at 180 °C.

Characterization of RPS. *IR Spectrometry.* IR spectra were measured in $CHCl_3$ with an EPI-G IR spectrometer (Hitachi Ltd., Tokyo).

GLC. Trimethylsilyl (Me_3Si) ethers of RPS were prepared after reduction with $NaBH_4$ or $NaBD_4$ (purity 98%; E. Merck) (Nakamura, 1977; Freedman, 1967). (Trimethylsilyl)dimethylhydrazone derivatives of RPS were prepared as described (Johnson et al., 1970; Nakamura, 1977). Precipitates such as NaCl and NH_4Cl formed during the derivatization were removed by passing through membranes (0.5-μm pore size; Millipore Corp., Bedford, MA). These derivatives were analyzed on a Shimadzu GC-4BPF gas chromatograph equipped with 1.5% silicone GE SE-30 columns (60–80 mesh, 2 m × 3 mm; Shimadzu Co., Kyoto). The flow rate of N_2 carrier gas was 40 mL/min, and the column temperature was 180 °C for

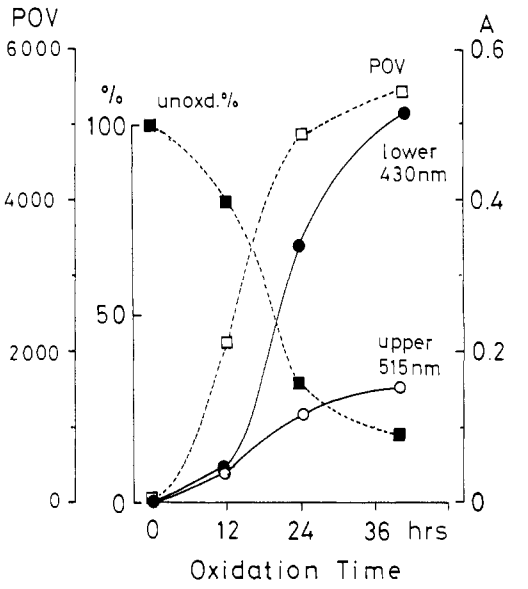

Figure 2. Time course of the oxidation and the coloring ability of methyl arachidonate. Both phases, lower and upper, were prepared as described in the text.

Me_3Si derivatives and an elevating temperature (150–230 °C, 3 °C/min) for (trimethylsilyl)dimethylhydrazone derivatives. The equivalent chain length (ECL) of these derivatives was determined with methyl esters of saturated fatty acids as standards (Mayer, 1978).

Gas Chromatography–Mass Spectrometry (GC–MS). Electron impact mass spectra were obtained on an RM-50 GC mass spectrometer (Hitachi Ltd., Tokyo). The ion accelerating voltage was 1.5 kV, and the ionizing voltage was 20 eV.

RESULTS

Coloration and Preparation of RPS. A colorless solution of the autoxidized arachidonate turned reddish brown when reacted with glycine. The colored mixture was separated with use of the same solvent system ($CHCl_3$–MeOH–water, 8:4:3, v/v) as described for purification of extracted lipids (Folch et al., 1957). Almost all the reddish pigments were distributed into the upper phase, although brown pigments were present in the lower phase. As shown in Figure 2 , the production of RPS showed a good relation with the lipid peroxidation until the peroxide value reached a maximum. Autoxidized methyl arachidonate was separated on the Sephadex column, and RPS eluted were monitored by the coloration with glycine. The pooled RPS fraction (tubes 73–83, Figure 3) was analyzed by TLC. Four RPS spots, R-1–4 were detected, as noted in previous work (Nakamura, 1984). Relative R_f values of the four spots to hydroquinone standard were 1.07 (R-1), 0.98 (R-2), 0.82 (R-3), and 0.70 (R-4), respectively. These four RPS were further purified by silicic acid chromatography and HPLC, until an almost single spot of each RPS appeared on the TLC plate. About 1–2 mg of RPS was recovered from 5 g of methyl arachidonate.

Identification of RPS. *R-1.* The IR spectrum of R-1 measured in $CHCl_3$ was similar to that of 3-(2-ethyl-5-hydroxy-3-oxocyclopentyl)-2-propenal (I) (Nakamura, 1985); absorptions at 1740 (five-membered ring ketone $\nu_{C=O}$, strong), 1690 (conjugated aldehyde $\nu_{C=O}$, strong), 1635 ($\nu_{C=C}$ of conjugated carbonyl, weak), 3500 region (ν_{OH}, medium), and 980 cm^{-1} (trans $\delta_{=CH}$, medium) were found. The relatively stronger absorption bands at 2700–3100 cm^{-1} (ν_{CH}) found in R-1, however, indicated the presence of a longer side chain in R-1 than in the RPS (I) found in the autoxidized linolenate.

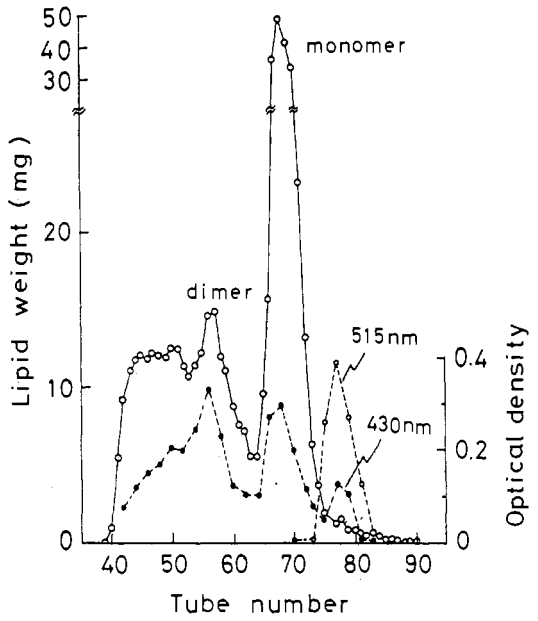

Figure 3. Separation of oxidation products of methyl arachidonate on Sephadex LH-20 and coloration of the separated fractions by the reaction with glycine. Conditions: load, 520 mg; fraction volume, ca. 2 mL/tube. Key: O—O, lipid weight; ●---●, 430 nm of lower phase; O---O, 515 nm of upper phase. Both phases were prepared as described in the text.

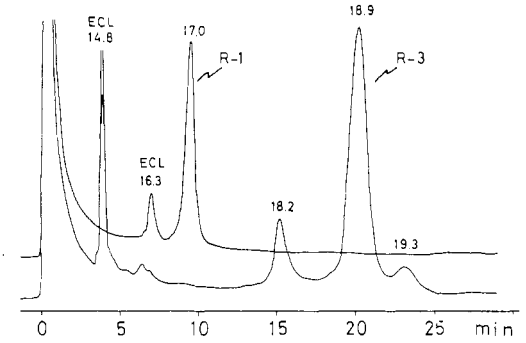

Figure 4. Gas chromatogram of trimethylsilyl ethers of RPS (R-1 and R-3) prepared after $NaBH_4$ reduction.

Trimethylsilyl ethers of R-1 prepared after reduction with $NaBH_4$ were analyzed on a 1.5% SE-30 column. Two peaks, ECL 16.3 and 17.0, were observed (Figure 4). The mass spectrum of the main compound (ECL 17.0) has an apparent molecular ion M^+ 444 [Figure 5 (1)]. The fragment ions m/z 243, 217, and 191 indicate the trimethylsilyl ether of a dihydroxycyclopentane ring in the molecule (Hamberg and Israelsson, 1970). The major peaks M – 206 [loss of Me_3SiOH (90) and $C_2H_3OMe_3Si$ (116)], M – 219 [loss of $C_3H_5(OMe_3Si)_2$], m/z 167 ($C_6H_6OMe_3Si$, M – 116 – 90 – 71), and small peaks m/z 328 (M – 116) and 313 (M – 15 – 116) were common to the trimethylsilyl ethers of RPS (Nakamura, 1985, 1986). The fragment ions m/z 373 (M–71), 283 (M – 90 – 71) and 257 (M – 116 – 71) indicate that one substituent group of the dihydroxycyclopentane ring, other than 2-propenal, was m/z 71 [$CH_3(CH_2)_4$]. From the results described above, the main component of R-1 is assigned as 3-(5-hydroxy-3-oxo-2-pentylcyclopentyl)-2-propenal (III) in Figure 1. The validity of this estimation was confirmed with the mass spectrum of the trimethylsilyl ether, obtained after reduction with $NaBD_4$ [Figure 5 (2)]. The fragment ions containing deuterium such as m/z 360 (M – 116), 270 (M – 116 – 90), and 169 (M – 116 – 90 – 71) suggest the position of the carbonyl groups in the original compound. Namely, close similarity of the fragmentation pattern to that of RPS (I) (Nakamura, 1986) indicates that the oxo group is attached not to C-5 but C-3. The mass spectrum of the (trimethylsilyl)dimethylhydrazone derivative of R-1 was also measured: M^+ 380 (18%), 365 (M – 15, 1), 336 (M – 44, 21), 291 (M – 89, 11), 290 (M – 90, 8), 246 (M – 90–44, 12), 194 (M – 196, 19), 146 (16), 141 (29), 123 (100), 83 (8), 73 (11). This fragmentation pattern was also similar to that of (trimethylsilyl)dimethylhydrazone of the RPS (I) from linolenate (Nakamura, 1985). Thus, the proposed structure of R-1 (ECL 17.0) was reconfirmed.

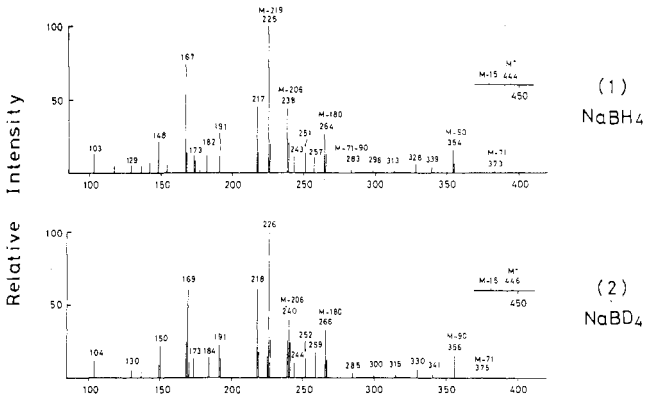

Figure 5. Mass spectra of trimethylsilyl ethers of RPS (R-1) prepared after $NaBH_4$ or $NaBD_4$ reduction.

The mass fragmentation of $NaBH_4$–trimethylsilyl ether of the minor component (ECL 16.3) in Figure 4 was as follows: m/z 354 (M – 90, 2%), 339 (M – 15 – 90, trace), 283 (M – 71 – 90, 5), 282 (11), 264 (M – 180, 6), 256 (11), 254 (14), 238 (M – 206, 23), 225 (M – 219, 100), 217 (30), 191 (16), 185 (35), 167 (30), 166 (27), 149 (12), 145 (20), 136 (12), 130 (15), 119 (10), 110 (13), 103 (13), 95 (13), 75 (18), 73 (22). Although some discrepancies between the spectra of ECL 17.0 and of ECL 16.3 such as absence of molecular ion and presence of m/z 254, 185, 166, and so on in the ECL 16.3 were observed, most of the fragmentation patterns were similar. The discrepancy might relate to a slight contamintion of another compound.

R-2. Four peaks of much the same size (ECL 16.1, 16.3, 16.8, 17.1) were detected by gas chromatography of both $NaBH_4$– and $NaBD_4$–trimethylsilyl derivatives of R-2. The mass spectra of the former two peaks (ECL 16.1, 16.3) closely resembled that of R-1 (ECL 16.3), and the latter two, that of R-1 (ECL 17.0). Thus, R-1 and R-2 may be stereoisomers.

R-3. In addition to similar IR absorption bands observed in R-1, absorptions of the ester group such as ν_{COO} (1170-cm^{-1} region) and ester $\nu_{C=O}$ (overlapping peaks, 1730-cm^{-1} region) were observed in R-3. The trimethylsilyl ether of R-3 prepared after reduction with $NaBH_4$ was analyzed by gas chromatography, under the same conditions as for R-1. As shown in Figure 4, a main peak (ECL 18.9) and three minor peaks (ECL 14.8, 18.2, 19.3) were detected. The mass spectrum of the main peak (ECL 18.9) is shown in Figure 6 (1). Fragment ions such as M – 15, M – 90, M – 180, M – 193, M – 206, M – 219, m/z 243, 216, 191, 167, and so on in the spectrum were in common with the RPS already identified (Nakamura, 1986). The molecular ion was estimated to be M^+ 474. The presence of ions such as M – 31 (loss of CH_3O), m/z 373 (M – 101), and m/z 358 (M – 15 – 101) were also observed. The m/z 101 ion was assigned to the fragment $(CH_2)_3COOCH_3$. The structure of the main compound was assumed to be the derivative of methyl 4-[2-(2-formylvinyl)-3-hydroxy-5-oxocyclopentyl]butanoate (IV). One of the minor compounds (ECL 14.8) in Figure 4 was an impurity because no change in retention time and no significant fragment

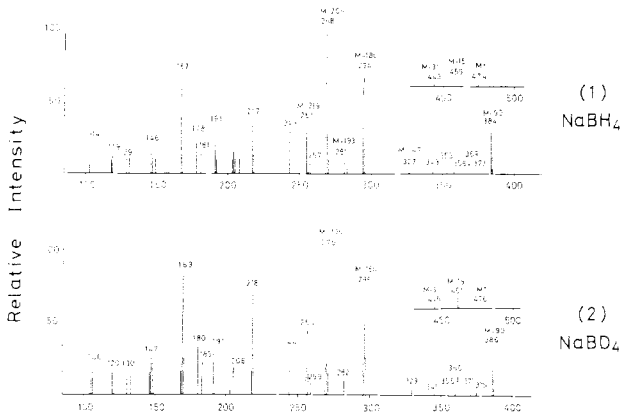

Figure 6. Mass spectra of trimethylsilyl ethers of RPS (R-3) prepared after $NaBH_4$ or $NaBD_4$ reduction.

ions in common with RPS were observed before and after the derivatization. The mass spectra of the other two peaks (ECL 18.2, 19.3) were similar to that of the main peak (ECL 18.9). Thus, these may be stereoisomers.

R-4. Three pairs of peaks (ECL 18.1, 18.3; 18.8, 19.0; 19.3, 19.5), which were like the isomers produced by splitting of three R-3 peaks (ECL 18.2, 18.9, 19.3), were observed on the gas chromatogram. Judging from the close similarity of the mass spectra to those of R-3, it may be concluded that the R-4 and R-3 were stereoisomers.

DISCUSSION

Two RPS out of the four predicted were detected in the present experiment. According to the formation mechanism deduced from the study of linolenate (Nakamura, 1985, 1986) both RPS found in this study originated from abstraction of the hydrogen atom at the C-10 position and the subsequent cyclization of 12-hydroperoxyeicosatetraenoate or 8-hydroperoxyeicosatetraenoate. Namely, RPS (III) was formed from the 12-isomer and RPS (IV) from the 8-isomer. The remaining two RPS (V, VI), which would originate from cyclization of the 9- and 11-isomers, were not detected. A possible explanation may be instability of the side chain containing a double bond or the inferior production of the compounds. However, one of them, 7-[2-(2-formylvinyl)-3-hydroxy-5-oxocyclopentyl]hept-5-enoate, was found to be present in autoxidized icosapentaenoate (unpublished data).

The stereoconfiguration of the RPS found in the present study remains to be elucidated as numerous isomers of the trimethylsilyl derivatives were detected. some may be produced secondarily by reduction of the keto group in the molecule to the hydroxy group. O'Connor et al. (1981, 1984) studied the stereochemistry of prostaglandin-like substances produced by the nonenzymic conversion of methyl 13-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate and methyl 9-hydroperoxy-*cis*-6,*trans*-10,*cis*-12-octadecatrienoate. They concluded that cis ring substituents were predominantly produced, while the isomers with trans ring substituents like that of natural prostaglandins were also slightly produced. This may account for the presence of each two main stereoisomers of III (R-1, R-2) and IV (R-3, R-4). Namely, their distinction may depend on two different stereoconfigurations of the hydroxy group attached to the five-membered ring. However, further experimentation is necessary to explain the stereochemistry. The possible production of positional isomers, depending upon the location of oxo and hydroxy groups on the cyclopentane ring, was also estimated, using the distribution of deuterium in the mass fragment ions. According to the mass fragmentation analysis of the known RPS from linolenate, fragment ions such as M − 116, M − 116 − 90, and m/z 167 (M − 116 − 90 − 71) originated from loss of fragment 116 containing the trimethylsilyl ether of the hydroxy group adjacent to the formylvinyl (or 2-propenal) side chain. Thus, the presence of superior peaks such as M − 117, M − 117 − 90, and m/z 168 instead of M − 116, M − 116 − 90, and m/z 169 in the spectra of $NaBD_4$–trimethylsilyl derivatives [Figures 5 (2) and 6 (2)] may suggest the presence of the positional isomer. However, such a fragmentation pattern was not observed in all of the spectra examined. The RPS we detected probably have the same location of oxo and hydroxy groups as that of linolenate.

ACKNOWLEDGMENT

We thank M. Kitamikado for advice and encouragement and M. Ohara for critical comments on the paper.

Registry No. III, 111006-17-8; IV (R = Me), 111006-18-9; glycine, 56-40-6; methyl arachidonate, 2566-89-4.

LITERATURE CITED

Folch, J.; Lees, M.; Sloane Stanley, G. H. *J. Biol. Chem.* **1957**, *226*, 497–509.
Freedman, B. *J. Am. Oil Chem. Soc.* **1967**, *44*, 113–116.
Gardner, H. W. *J. Agric. Food Chem.* **1979**, *27*, 220–229.
Hamberg, M.; Israelsson, U. *J. Biol. Chem.* **1970**, *245*, 5107–5114.
Hirai, S.; Okamoto, K.; Morimatsu, M. In *Lipid Peroxides in Biology and Medicine*; Yagi, K., Ed.; Academic: New York, London, 1982; pp 305–315.
Johnson, C. B.; Pearson, A. M.; Dugan, L. R., Jr. *Lipids* **1970**, *5*, 958–963.
Mayer, J. J. In *Fatty acids and Glycerides*; Kuksis, A., Ed.; Plenum: New York, London, 1978; p 144.
Mead, J. F. *Free Radicals Biol.* **1976**, *1*, 51–68.
Mester, L.; Szabados, L.; Mester, M.; Yadav, N. *Progr. Food Nutr. Sci.* **1981**, *5*, 295–313.
O'Connor, D. E.; Mihelich, E. D.; Colemann, M. C. *J. Am. Chem. Soc.* **1981**, *103*, 223–224.
O'Connor, D. E.; Mihelich, E. D.; Coleman, M. C. *J. Am. Chem. Soc.* **1984**, *106*, 3577–3584.
Nakamura, T. *Bull. Jpn. Soc. Sci. Fish.* **1977**, *43*, 1097–1104.
Nakamura, T. *Bull. Jpn. Soc. Sci. Fish.* **1984**, *50*, 477–479.
Nakamura, T. *Lipids* **1985**, *20*, 180–186.
Nakamura, T. *Lipids* **1986**, *21*, 553–557.
Pokorny, J. *Prog. Food Nutr. Sci.* **1981**, *5*, 421–427.
Porta, E. A.; Hartroft, W. S. In *Pigments in Pathology*; Wolman, M., Ed.; Academic: New York, London, 1969; pp 192–230.
Reynolds, T. M. *Adv. Food Res.* **1969**, *14*, 168–283.

Received for review December 29, 1986. Revised manuscript received July 9, 1987. Accepted August 27, 1987.