ELSEVIER

Journal of Chromatography A, 839 (1999) 85–91

JOURNAL OF CHROMATOGRAPHY A

Determination of chemical oxidation products of chlorophyll and porphyrin by high-performance liquid chromatography[1]

Yasuyo Suzuki[a], Kimiko Tanabe[b], Yuzo Shioi[a,*]

[a]*Department of Biology and Geoscience, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan*
[b]*Materials Research Center, Miyazaki University, Miyazaki 889-2192, Japan*

Received 10 November 1998; received in revised form 15 January 1999; accepted 2 February 1999

Abstract

Chemical oxidation products of chlorophyll and porphyrin were analyzed by LC with UV_{280} detection using a wide-pore, octadecyl polyvinyl alcohol polymer column and isocratic elution with acetonitrile–water (25:75, v/v) containing 100 m*M* ammonium acetate. Next to hematinic acid and C-E-ring derivative of chlorophyll, methyl ethyl maleimide and methyl vinyl maleimide which usually coelute on a monomeric ODS column, were also separated. The technique was used to analyze degradation products, especially monopyrrole derivatives, which emerged during chlorophyll breakdown in biological systems.

Keywords: Chlorophyll; Porphyrin

1. Introduction

The rapid breakdown of chlorophylls (Chls) occurs periodically in the stages of senescence or fruit ripening, in addition to the normal turnover of Chls in green leaves. The degradation process includes loss of phytol, magnesium and carbomethoxy groups from the macro and isocyclic rings of Chl in reactions catalyzed by several enzymes [1–6]. Subsequently, the oxidative cleavage of the tetrapyrrole macrocyclic ring system occurs with the involvement of molecular oxygen, probably by monooxygenase [7,8]. The main catabolites of degraded Chls after the cleavage of macrocyclic ring systems were identified as derivatives of bilin [9–13].

The accumulation of these derivatives has been reported in senescent leaves of several higher plants [9,11,12] and algae [10,13]. However, little is known about the subsequent reactions involved in the breakdown of these derivatives into low-molecular-mass compounds, except for the studies on the products of photodegradation of bilirubin and Chls. In the photodegradation of bilirubin, three groups of more than 10 species of products have been reported [14]. These include dipyrroles, monopyrroles and their hydrolytic products. With respect to Chls, methyl ethyl maleimide has previously been found in the photo-bleached endproduct of Chl dissolved in solvents [15]. The formation of several hydrophilic colorless products, low-molecular-mass organic acids, were measured in Chl *a* adsorbed on lipophilic particles during photodegradation [16,17].

*Corresponding author. Fax: +81-54-238-0986.
E-mail address: yshioi@sci.shizuoka.ac.jp (Y. Shioi)
[1]This article is dedicated to Prof. Dr. Wolfhart Rüdiger on the occasion of his 65th birthday.

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00189-2

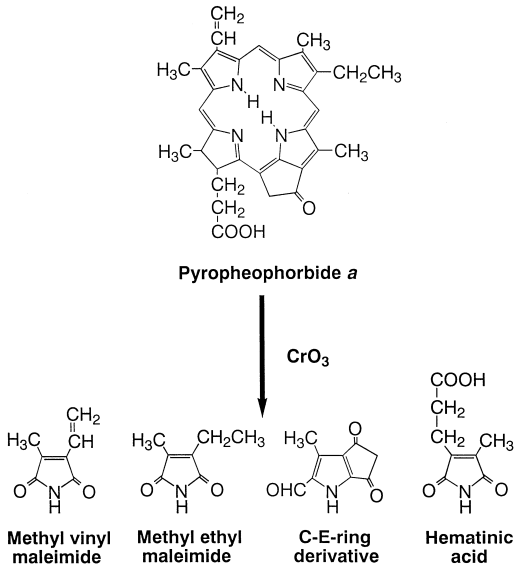

Fig. 1. Structures of degradation products of pyropheophorbide *a* by oxidation with chromic acid. Four compounds, methyl vinyl maleimide, methyl ethyl maleimide, C-E-ring derivative and hematinic acid are primarily formed.

To elucidate the intermediary degradation step of Chls, we required a simple and rapid method for the separation and identification of degradation products of bilin derivatives, especially for monopyrrole derivatives. We, therefore, prepared putative catabolites of Chl, monopyrrole derivatives from Chl, and porphyrin, by oxidation with chromic acid according to the established method which has been used to determine the structure of bile pigments [18,19] (Fig. 1).

In this report, we present a chromatographic method using a wide pore octadecyl polyvinyl alcohol polymer (ODP) column which enables the separation of ethyl and vinyl species of maleimide in addition to hematinic acid and C-E-ring derivative of Chl by a rather simple isocratic elution system with a buffered eluent.

2. Experimental

2.1. Chlorophylls and porphyrins

Pheophorbide *a* and pyropheophorbide *a* were obtained from Wako Pure Chemical (Osaka, Japan). Protoporphyrin IX was purchased from Porphyrin Products (Logan, UT, USA). Chls *a* and *b* were extracted from spinach leaves with acetone and were partially purified by precipitation with dioxane [20]. The dioxane-precipitated Chls were separated and further purified by sugar-column chromatography [21]. Pheophorbide *b* was prepared by acid treatment of Chl *b* according to the method of Hynninen [22]. Pyropheophorbide *b* was prepared from pheophorbide *b* by dissolving pheophorbide *b* in pyridine and heating it in an oven at 100±1°C for 48 h in a sealed tube [23]. The resultant pyropheophorbide *b* was then identified by high-performance liquid chromatography (HPLC) analysis. Mesoporphyrin IX was prepared from protoporphyrin IX by reduction with hydrogen according to the method of Shemin [24].

2.2. Oxidation of chlorophylls and porphyrin with chromic acid

Oxidation with chromic acid was carried out essentially as described by Rüdiger [18,19]. Oxidation at pH 1.2 was done at 25°C with 1% (w/v) CrO_3 solution containing 1% $KHSO_4$ for 15 min to 1 h for Chl *a* and for 1 to 15 h for Chl *b*. Oxidation of Chl *a* in 1 *M* H_2SO_4 was done with 1% CrO_3 in 1 *M* H_2SO_4 at 25°C for 1 h. In the oxidation of Chls, an equal volume of acetone was added to dissolve Chls. After the reaction, acetone was evaporated under reduced pressure. The oxidation products were then extracted with ethyl ether and concentrated for chromatography.

Methyl esterification of the samples was carried out with a diazomethane generator (Wheaton, Millville, NJ, USA), if necessary, according to the manufacturer's instructions.

2.3. Identification of chromic acid oxidation products

The concentrated oxidation products were analyzed by thin-layer chromatography (TLC) with two solvent systems, solvent A: dichloroethane–ethyl acetate (10:1, v/v) and solvent B: dichloroethane–ethyl acetate–ethanol–acetic acid (200:10:5:0.5, v/v) using Kieselgel 60 plates (20×5 cm) (Merck, Darmstadt, Germany). Maleimides were detected by color reaction with chlorin-tetramethyl benzidine, and

pyrrole aldehydes were detected with 2,4-dinitrophenylhydrazine, both basically according to the method described by Rüdiger [19]. Major bands were scraped off and extracted with ether. The ether solution was concentrated and analyzed by absorption and mass spectrometry to confirm the identification. Major compounds thus identified were separately analyzed by HPLC to determine the elution properties such as retention time. Conversely, peak fractions collected from LC were also analyzed by spectrophotometry and with TLC under the conditions described above.

Absorption measurements were carried out with a Hitachi spectrophotometer, Model U-3210 (Tokyo, Japan). Mass spectra were measured with a Hitachi mass spectrometer, M-2000AM (70 eV) connected with gas chromatography (GC) or LC apparatus using an electron impact (EI) or atmospheric pressure chemical ionization (APCI) methods.

2.4. Chromatography

LC was carried out with a Shimadzu LC-10A chromatograph system (Kyoto, Japan). Separations were performed using an Asahipak ODP-50 (Showa Denko, Tokyo, Japan) (250×4.6 mm I.D.) packed with ODP. Degradation products were eluted with acetonitrile–water (25:75, v/v) containing 100 m*M* ammonium acetate at a flow-rate of 1.0 ml per min at room temperature (ca. 22–25°C). Separated products were detected with a Waters LC spectrophotometer, Lambda-Max, Model 481 (Milford, MA, USA) measuring at 280 nm and quantified by a Shimadzu Chromatopac C-R6A.

2.5. Enzymatic degradation of pyropheophorbide a by peroxidase

Pyropheophorbide a was enzymatically degraded by peroxidase (EC 1.11.1.7) to ensure the applicability of this technique. The reaction was performed basically as described by Kato and Shimizu [25]. The reaction mixture contained 50 m*M* acetate buffer (pH 5.6), 40 μM 2,4-dichlorophenol, 8 units horseradish peroxidase (Type II) (Sigma, St. Louis, MO, USA), 20 μM pyropheophorbide *a* dissolved in 0.17% Triton X-100, and 15 μM H_2O_2 in a total volume of 12 ml. The reaction was started by adding H_2O_2 and measured absorbance at 665 nm. At the appropriate time periods, an aliquot was taken and degradation products were extracted with ether and the resulting ether solution was concentrated under reduced pressure to use for LC analyses.

3. Results and discussion

3.1. Identification of authentic oxidation products

Oxidation of mesoporphyrin IX with chromic acid is known to yield two monopyrroles, hematinic acid and methyl ethyl maleimide [24]. These two compounds were separated by TLC and determined as described in Experimental. The R_F values obtained from TLC using solvent B were 0.16 for hematinic acid and 0.61 for methyl ethyl maleimide. The molecular masses of these compounds were determined by mass analyses: 139 (M^+) for methyl ethyl maleimide by GC–MS with EI; 183 (M^+) for hematinic acid by LC–MS with APCI.

Oxidation products of pyropheophorbide *a* with chromic acid gave additional components, methyl vinyl maleimide and C-E-ring derivative of Chl in addition to the above two compounds, as demonstrated by Shimomura [26]. These compounds were tentatively identified by analyses by TLC in solvent system B; the R_F values of methyl vinyl maleimide and C-E-ring derivative were 0.64 and 0.28, respectively. Besides these compounds, one more aldehyde compound (R_F=0.63 in solvent B) was detected by color reaction with dinitrophenylhydrazine as described by Shimomura [26]. This compound was tentatively identified as degraded C-E-ring derivative from the presence of aldehyde. The appearance of this peak in oxidation products from Chl *a* and in enzymatic degradation will be described below.

3.2. Separations of degradation products

The effect of acetonitrile concentration in the presence of 100 m*M* ammonium acetate on the log k' of monopyrroles and C-E-ring derivative is shown in Fig. 2. The values of the log k' of four oxidation products increased with decreasing acetonitrile concentration and the individual plots gave parallel lines. This indicates that the ratio of the log k' (α) is

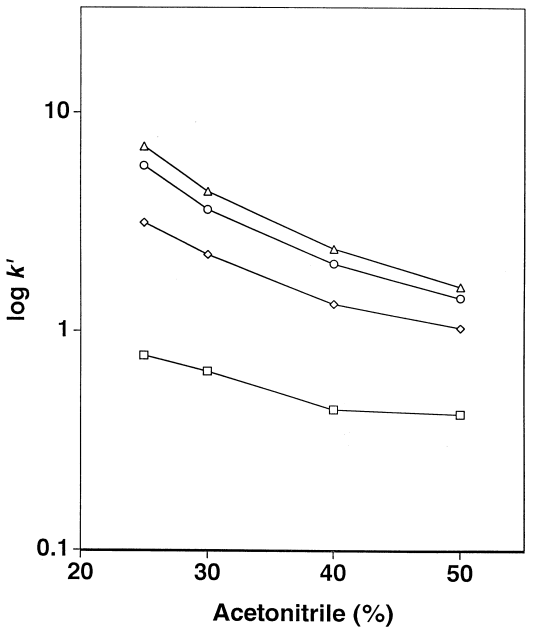

Fig. 2. Log k' versus percent acetonitrile of the oxidation products. LC conditions: ODP column, acetonitrile–water (25:75, v/v) containing 100 m*M* ammonium acetate (1.0 ml/min); detection at 280 nm. For details, see text. □, Hematinic acid; ◇, C-E-ring derivative; ○, methyl ethyl maleimide; △, methyl vinyl maleimide.

not changed with increasing eluent polarity. Omission of ammonium acetate resulted in the slight decrease of peak resolution due to weak retention of ionic compounds, but the elution order remained unchanged.

The separation of esterified and non-esterified samples in the presence of ammonium acetate was compared. There were, however, no significant differences between their separation parameters such as log k' and resolution. The separation of methyl ethyl and methyl vinyl maleimides can be repeatedly performed using an ODP column, but not the usual ODS column (see below and Fig. 4). Thus, the use of an ODP column with acetonitrile–water (25:75, v/v) containing 100 m*M* ammonium acetate is suitable for the separation of oxidation products, in particular monopyrrole derivatives of Chls and porphyrins.

The repeatability of the separation in this system strongly depends on several factors. The coefficient of variation of the retention times approached 2.8% as calculated from 13 measurements of methyl ethyl maleimide at ambient temperature. This slightly poor repeatability is due mainly to the fact that the retention strongly depends on the eluent polarity, as can be seen in Fig. 2, and also column temperature which changes the viscosity of the eluent. To attain good repeatability of the retention times for identification, a fixed column temperature is recommended.

Fig. 3 shows the separation of extracts of oxidation products of mesoporphyrin IX under the suitable elution conditions noted above. The oxidation products were separated in less than 12 min. Peak 1 (t_R=2.9 min) was hematinic acid and peak 2 (t_R= 10.1 min) was methyl ethyl maleimide by the comparison with authentic samples. A peak at 2.4 min and peaks that appeared around 6 to 7 min were unknown degradation products. Peaks emerged that around 17 to 19 min are not determined yet, but are probably dipyrrole derivatives from comparison with photodegradation products of bilirubin. The presence of dipyrrole derivatives in the oxidation products was supported by mass analysis.

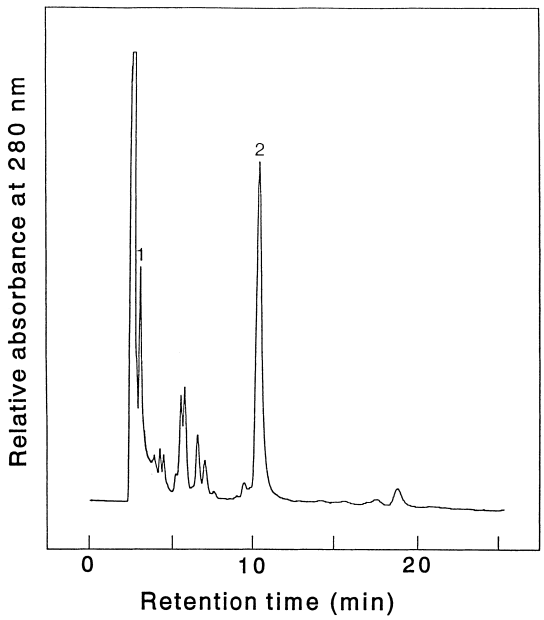

Fig. 3. LC elution profile of extract of chemical oxidation products of mesoporphyrin IX with chromic acid. Chemical oxidation and extraction of the products are described in Experimental. LC conditions as in Fig. 2. Peaks: 1=hematinic acid; 2=methyl ethyl maleimide; non-numbered=unknown degradation products.

The extract of oxidation products of pyropheophorbide *a* was analyzed on an ODP column with acetonitrile–water (25:75, v/v) containing 100 m*M* ammonium acetate (Fig. 4). The oxidation products were separated in less than 14 min. Peak 1 (t_R=2.9 min) was hematinic acid and peak 2 (t_R=6.5 min) was determined to be C-E-ring derivative of Chl by comparison with TLC isolated samples. Peaks 3 (t_R=10.1 min) and 4 (t_R=12.1 min) were methyl ethyl maleimide and methyl vinyl maleimide, respectively. The structure of these compounds is very similar, and differentiation could not be resolved completely by the usual ODS column [27]. However, the ODP column that was used here was capable of the separation of these ethyl and vinyl species as reported previously [27].

In the case of oxidation in more acid conditions, a new peak (t_R=11.2 min) appeared between peaks 3 (methyl ethyl maleimide) and 4 (methyl vinyl maleimide). This peak was tentatively identified as degraded C-E-ring derivative from TLC analyses. This compound is found commonly in the enzymatic degradation products (see Fig. 5A, peak 2) and in the senescent cotyledons of higher plants (in preparation).

Faint peaks eluted around 17 to 19 min were also found in this extract of short-term oxidation with chromic acid and these are tentatively identified as dipyrrole derivatives as described above. Peaks that

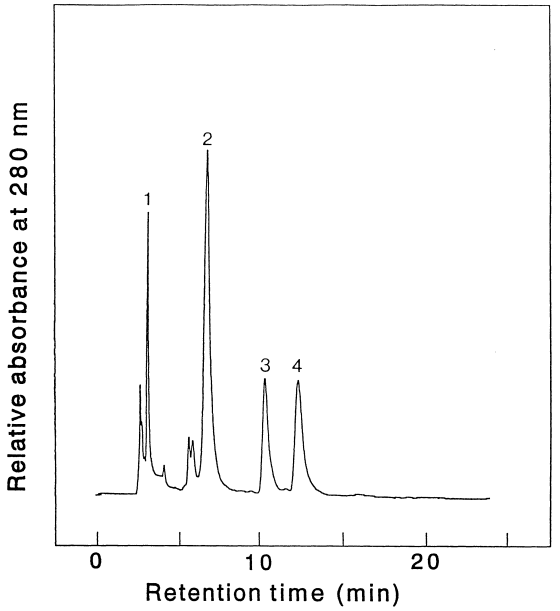

Fig. 4. LC elution profile of extract of chemical oxidation products of pyropheophorbide *a* with chromic acid. Chemical oxidation and extraction of the products are described in Experimental. LC conditions as in Fig. 2. Peaks: 1=hematinic acid; 2=C-E-ring derivative; 3=methyl ethyl maleimide; 4=methyl vinyl maleimide. Non-numbered=unknown degradation products.

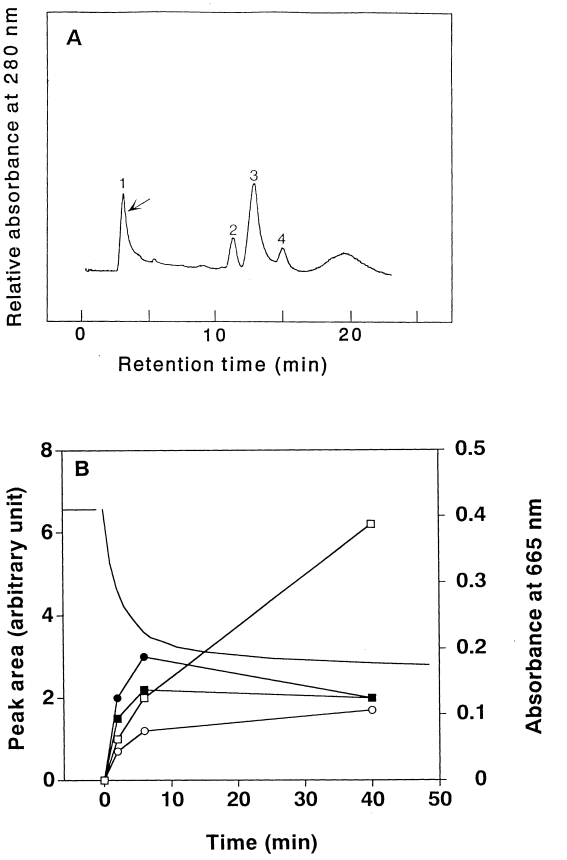

Fig. 5. (A) LC elution profile of extract of enzymatic degradation products of pyropheophorbide *a* after 6 min of peroxidase reaction. Enzymatic degradation, extraction and LC conditions of the products are described in Experimental. Peaks: 1=unknown compounds; 2=degraded C-E-ring derivative; 3=methyl vinyl maleimide; 4=2,4-dichlorophenol; shoulder (arrow), hematinic acid. Non-numbered peaks were not determined. (B) Time-dependent change of degradation products in enzymatically degraded pyropheophorbide *a*. Contents of oxidation products were estimated quantitatively by LC as in (A). Pyropheophorbide *a* was determined by measuring at 665 nm. □, Unknown product(s); ■, hematinic acid; ○, degraded C-E-ring derivative; ●, methyl vinyl maleimide. Solid line without symbol=pyropheophorbide *a* (right scale).

appeared at 2.4 min and around 6 to 7 min were unknown degradation products. Interestingly, these degradation products can be seen in the extracts obtained from both mesoporphyrin IX and pyropheophorbide *a*. It is likely that these may be derived by further degradation of main compounds and they appear to have a similar structure from their elution properties.

With respect to the separation of pyropheophorbide *b*, hematinic acid and C-E-ring derivative were clearly separated. Two other primary products, methyl vinyl and methyl formyl maleimides were eluted as a mixture with similar retention times as methyl ethyl and methyl vinyl maleimides. However, their identities could not be resolved completely with the samples that had been prepared with oxidation times from 1 to 15 h.

3.2. Application

The present method was applied to analyze oxidation products of pyropheophorbide *a* by the enzymatic reaction with peroxidase. This experiment was constructed as a model of Chl degradation by biological system using a simple enzymatic reaction. As shown in Fig. 5A, four peaks and one shoulder were clearly observed. These were compared to the standard sample to identify peak compounds. Peak 1 was hydrophilic compounds probably derived from further degradation of pyrrole derivatives. The shoulder around 2.9 min contained hematinic acid. Peak 2 (t_R=11.2 min) was tentatively identified as degraded C-E-ring derivative as described above. Peak 3 (t_R= 12.1 min) is assigned as methyl vinyl maleimide from the comparison of the standard sample. Peak 4 (t_R=ca. 15 min) was 2,4-dichlorophenol used as a cofactor for the enzymatic reaction, from coelution and the comparison with an authentic sample.

The time course of formation of enzymatic breakdown products of pyropheophorbide *a* is shown as well as the decrease of pyropheophorbide *a* (Fig. 5B). Three degradation products, hematinic acid, methyl vinyl maleimide, and degraded C-E-ring derivative increased sharply up to 5 min with a concomitant decrease of pyropheophorbide *a*. Unknown hydrophilic compound(s), which appeared with a slight lag time from the first three compounds, may be due to further degradation of main monopyrroles into more hydrophilic low molecular mass compounds, although further quantitative study is necessary to obtain the stoichiometric answer. Similar to the photodegradation of bilirubin [14], degradation of pyropheophorbide *a* through enzymatic reaction is first initiated by a break of the bridge linkage and subsequent hydrolytic reactions leading to the formation of hydrophilic compounds such as simple organic acids.

4. Conclusions

The experimental system described here allows the rapid separation of breakdown products of Chl, especially monopyrrole derivatives by a rather simple isocratic elution system with a buffered eluent. The use of an ODP column and the addition of ammonium acetate to the eluent is the key to the successful resolution of ethyl and vinyl derivatives of maleimide and also for ionic degradation products of Chls. This procedure is a useful tool for the analyses of degradation products of Chls in biological system, in particular monopyrrole derivatives.

Acknowledgements

This work was supported by grants from the Ministry of Education, Science, Sport and Culture of Japan and Yamada Science Foundation.

References

[1] S.B. Brown, J.D. Houghton, G.A.F. Hendry, in: H. Scheer (Ed.), Chlorophylls, CRC Press, Boca Raton, FL, 1991, p. 465.
[2] P. Matile, S. Hörtensteiner, H. Thomas, B. Kräutler, Plant Physiol. 112 (1996) 1403.
[3] A. Gossauer, N. Engel, J. Photobiochem. Photobiol. B 32 (1966) 141.
[4] T. Tsuchiya, H. Ohta, T. Masuda, B. Mikami, N. Kita, Y. Shioi, K. Takamiya, Plant Cell Physiol. 38 (1997) 1026.
[5] Y. Shioi, N. Tomita, T. Tsuchiya, K. Takamiya, Plant Physiol. Biochem. 34 (1996) 41.
[6] Y. Shioi, K. Watanabe, K. Takamiya, Plant Cell Physiol. 37 (1996) 1143.
[7] C. Curty, N. Engel, A. Gossauer, FEBS Lett. 364 (1995) 41.

[8] S. Hörtensteiner, K.L. Wührich, P. Matile, K.-H. Ongania, B. Kräutler, J. Biol. Chem. 273 (1998) 15335.
[9] B. Kräutler, B. Jaun, K. Bortlik, M. Schellenberg, P. Matile, Angew. Chem. Int. Ed. 30 (1991) 1315.
[10] N. Engel, T.A. Jenny, V. Mooser, A. Gossauer, FEBS Lett. 293 (1991) 131.
[11] W. Mühlecker, B. Kräutler, S. Ginsburg, P. Matile, Helv. Chim. Acta 76 (1993) 2976.
[12] J. Iturraspe, N. Moyano, J. Org. Chem. 60 (1995) 6664.
[13] M. Doi, S. Shima, T. Egashira, K. Nakamura, S. Okayama, J. Plant Physiol. 150 (1997) 504.
[14] D.A. Lightner, Photochem. Photobiol. 26 (1977) 427.
[15] J.J. Jen, G. Mackinney, Photochem. Photobiol. 11 (1970) 303.
[16] C.A. Llewellyn, R.F.C. Mantoura, R.G. Brereton, Photochem. Photobiol. 52 (1990) 1037.
[17] C.A. Llewellyn, R.F.C. Mantoura, R.G. Brereton, Photochem. Photobiol. 52 (1990) 1043.
[18] W. Rüdiger, in: T.W. Goodwin (Ed.), Porphyrins and Related Compounds, Academic Press, London, 1968, p. 121.
[19] W. Rüdiger, Hoppe-Seylers Z. Physiol. Chem. 350 (1969) 1291.
[20] K. Iriyama, N. Ogura, A. Takamiya, J. Biochem. 76 (1974) 901.
[21] H.J. Perkins, D.W.A. Roberts, Biochim. Biophys. Acta 58 (1962) 486.
[22] P.H. Hyninnen, Acta Chem. Scand. 27 (1973) 1771.
[23] W.A. Svec, in: D. Dolphin (Ed.), The Porphyrins, Academic Press, New York, 1978, p. 341.
[24] D. Shemin, in: S.P. Colowick, N.O. Kaplan (Eds.), Methods in Enzymology, Vol. IV, Academic Press, New York, 1957, p. 643.
[25] M. Kato, S. Shimizu, Plant Cell Physiol. 26 (1985) 1291.
[26] O. Shimomura, FEBS Lett. 116 (1980) 203.
[27] Y. Shioi, K. Wantanabe, K. Takamiya, J.L. Garrido, M. Zapata, Anal. Biochem. 231 (1996) 225.