Chemistry Research Journal, 2018, 3(6):82-87

Available online www.chemrj.org



Research Article

ISSN: 2455-8990 CODEN(USA): CRJHA5

An Improved Preparation of Methylpheophorbide A and Chlorin- \mathbf{E}_6 Trimethylester from Spirulina and Skillworm Waste

Duy Tien Doan*¹, Truong Giang Le¹, Quang Trung Nguyen², Van Tuyen Nguyen¹, Franz-Peter Montforts³

Abstract Chlorophyll and its derivatives are using as food additives, cosmetic and photodynamic therapy of cancer such as pheophytin a, methylphephobide a, chlorin-e₆ trimethylester, copper chlorophyllin. Herein, we report an improved extraction chlorophyll from spirulina by soxhlet extraction method and an improved directly transformation chlorophyll to methyl pheophorbide a without purification. The transformation methylphephobide a to chlorin-e₆ trimethylester was performed by opening carbocylic ring catalyzed by potassium methoxide. Pure obtained methylphephobide a and chlorin-e₆ trimethylester are important materials for the production of chlorin-e₆ and its derivatives. The structures of these compounds were determined by IR, UV, NMR and MS spectroscopy.

Keywords Chlorophyll, pheophorbide, pheophytin, chlorin-e₆, food additive, photodynamic therapy

Introduction

Photosynthesis is the energetic basic of life on the earth. Light harvesting and conversion of light into chemical energy which occur in higher plants, marine algae, aquatic algae and photosynthetic bacteria are the processes accomplished by the photosystems [1]. Chlorophyll, a member of the most important class of pigments involved in photosynthesis, the process by which light energy is converted to chemical energy through the synthesis of organic compounds. Examples of chlorophyll family are shown in **Fig. 1** including chlorophyll a **1**, chlorophyll b **2** and chlorophyll d **3**.

Figure 1: Main members of the chlorophyll family



¹Institute of Chemistry, Vietnam Academy of Science and Technology

²Center for research and technology transfer, Vietnam Academy of Science and Technology

³Faculty of chemistry and biology, University of Bremen, Germany

Compound 1 was isolated in 1913 and the complete structure was determined in 1940 by Hans Fischer [1-2]. Isolation of chlorophylls from natural sources has been known to be difficult as a result of its various possible decomposition reactions such as demetallation, dephytylation, photooxidation, epimerization, allomerization, solvolysis and demethoxycarbonylation [1]. Chlorophyll *a* 1 and its derivatives are very important model compounds in theoretical chemistry to test the concepts of electron structure, aromaticity, electron transfer, quantum chemistry, coordination and photochemistry. Application of chlorophylls derivatives such as chlorin-e₆ 4, mono L-aspartyl chlorin-e₆ 5 and photochlorin 6 (Fig. 2) concerns food chemistry, cosmetic, nanotechnology and treatment of cancer [3-6]. Photodynamic therapy was employed to treat malignant tumors of the skin, mammary gland, mucous membrane of the oral cavity, tongue, lower lip, larynx, lung, esophugus, stomach, urinary bladder and rectum [1-3,7].

Figure 2: Chlorophyll derivatives as possible sensitizer for photodynamic therapy

In our synthetic studies in the field of chlorophyll chemistry, we were interested to develop and improve a partial synthetic pathway methylphephobide a 8 and chlorin- e_6 trimethylester 10 leading from extracted 1 [1,4,7-8].

Results and Discussion Semi-quantitative of 1

HPLC method must be implemented in the special conditions because chlorophylls are very easy decomposed by demetalization, dephytylation, oxidation, allomerization and demetoxycarboxylation but UV-VIS is more convenient method for semi-quantitative of 1. UV-VIS of 1, 5.10^{-6} M in acetone, is showed including two main peaks of Soret band at 410nm and Q-band at 667nm. The peak at 667nm (ε =0,8579.10⁵) was selected to determine the content of 1 by the calibration curve method. The chlorophyll a 1 content of bamboo leaves, mulberry leaves, erythrina variegata L, spinat, silkworm waste and spirulina were shown in the **Table 1**.

Table 1: The content of **1** in some sources collected in Vietnam

No	Samples	Chlorophyll
		(% mass)
1	Erythrina variegata L (at Bac Ninh, Vietnam)	0.028 ± 0.002
2	Bamboo leaves (at Bac Ninh, Vietnam)	0.046 ± 0.005
3	Erythrina orientalis leaves (at Bac Ninh, Vietnam)	0.034 ± 0.001
4	Mulberry leaves (at Bac Ninh, Vietnam)	0.023 ± 0.002
5	Spinat (at Ba Vi, Vietnam)	0.109 ± 0.008
6	Pennisetum purpureum (at Bac Ninh, Vietnam)	0.162 ± 0.002
7	Silkworm waste (at Bac Ninh, Vietnam)	0.265 ± 0.009
8	Spirulina (at Vinh Hao, Vietnam)	0.682 ± 0.013

Chlorophyll contents in some sources are arranged from 0.023-0.682% by mass. Spirulina (0.682%) and silkworm waste (0.265%) are potent of chlorophyll sources.



Extraction of chlorophyll a 1 from silkworm waste and directed transformation to methylpheophorbide a 8

The extraction of 1 from silkworm waste and transformation to methylpheophorbide a were performed by one pot manner (**Scheme 1**). The mixture of silkworm waste and methanol was stirred with methanol for 2 hours at ambient temperature. The chlorophyll a 1 solution was obtained by removal biomass. The transformation 1 to 8 was performed directly without purification by addition of concentrated sulfuric acid with the removal magnesium in the chromophore forming pheophytin a intermediate and methyl transesterification. Chlorophyll b 2 derivatives impurity was removed by column chromatography on alox. Purification of compound 8 has trouble due to π -stacking interaction of 18 electrons π conjugated and can be solved by recrystalization. Pure 8 is very important intermediate for the preparation of copper chlorophyllin and chlorin- e_6 trimethylester. The yield of recovered 8 is higher than 80% from 1 content in the silkworm waste with the mass of 3.85 g from 2 kg silkworm waste.

Scheme 1: Preparation of methylpheophorbide a 8 from silkworm waste

An improved soxhlet extraction of chlorophyll a 1 from spirulina and transformation to methylpheophorbide a 8.

Spirulina, one kind of cyanobacterium, have only compound 1 and can be considered as the best chlorophyll source. The procedure was performed by soxhlet extraction method with acetone (**Scheme 2**). The transformation crude compound 1 to 8 was performed by a solution of 5% concentrated sulfuric acid in methanol and stirred 24 hours at room temperature, darkness and inert atmosphere. Analytical methylpheophobide a 8 was received by chromatogaraphy and recrystallization by CH_2Cl_2/CH_3OH . The yield of recovered pheophorbide a is higher than 85% from chlorophyll contents in the spirulina with the mass of 2.96 g (0.592%) from 500 g spirulina biomass. Methylpheophorbide a has two diasteriomers at position of carbon 13^2 in carbocyclic ring. The ratio between 13^2R and 13^2S diasteriomers is 89/11 which was determined by 1H -MNR. Hyperchem calculation shows that the energy of R-diasteriomer is less than 3.15 kcal/mol compare to 13^2S -diasteriomer.

Scheme 2: Preparation of methylpheophorbide a 8 from spirulina



Preparation of chlorin e₆-trimethylester 10

Chlorin- e_6 trimethylester **10** is an intermediate for the synthesis of chlorin- e_6 and its derivatives. Compound **10** was conveniently prepared by methanolysis of methylpheophorbide a **8** by treatment with 0.5% potassium methanolate in methanol [9] (**Scheme 3**).

Scheme 3: Methanolysis of methyl pheophorbide a 8 to form chlorin e₆-trimethylester 10

Methylpheophorbide a **8** and chlorin- e_6 trimethylester **10** are poorly soluble in methanol at low temperature. This prevents the homogeneity of the reaction so that a second solvent is necessary to keep the reaction homogenously. For these purposes, a second solvent is added for instance THF, CH_2Cl_2 or pyridine. During the methanolysis reaction to prevent allomerization, oxygen should be avoided although methanolysis occurs more rapidly than allomerization [7]. Theoretically and practically, the methanolysis reaction cannot proceed completely due to the reverse reaction, therefore the reaction mixture always contains **10** and small amount of **8**. Retention times of methyl pheophorbide a and chlorin- e_6 trimethylester **10** on silicagel or aluminium oxide are quite similar so that they cannot be separated clearly by flash chromatography or HPLC. Isothermic crystallization of compound **10** represents a useful purification procedure. Purity of **10** is more than 99% by HPLC analysis.

Experimental Section

All the chemicals and solvents used for the synthesis work acquired from commercial sources, were of analytical grade, and used without further purification. IR spectra were measured on GX-Perkin Elmer FT-IR spectrophotometer. ¹H-NMR spectra were recorded at 500 MHz (Bruker XL-500) with CDCl₃ as solvent and tetramethylsilane as internal standard. Electrospray ionization mass spectrometry (ESI-MS) were obtained with an Agilent 1100 Series MS mass spectrometer. High resolution electron spray (ES) were obtained with an Agilent technologies 6210 series time of flight. Melting points of crystalline compounds were measured with a Gallenkamp.

Preparation of 8 from silkworm waste

The mixture of 2 kg of silkworm waste and 3 liters of methanol was stirred for 2 hours at ambient temperature. At the end of the extraction the biomass was removed by filtration and washed twice with portions of 200 ml of MeOH. 25 ml of concentrated sulfuric acid was added to the filtrate and the mixture was stirred in the dark under an argon atmosphere for 24 hours. The mixture was added 500 ml of CH₂Cl₂, 700 ml of H₂O and 300 ml of 10% NaHCO₃ aq. solution. The organic phase was separated, and the water phase was extracted twice with portions of 200 ml of CH₂Cl₂. The extract was washed with 200ml of water, dried though cotton wood and removed solvent by rotary following by high vacuum to get crude methylpheophobide *a* in red colour. Analytical methylpheophobide was received by column chromatogaraphy (Alox; CH₂Cl₂/acetone 15:1) and recrystallization from acetone/ MeOH. The yield of 8 is higher than 80% from chlorophyll content in the silkworm waste with the mass of 3.85 g.

Preparation of 8 from spirulina

The soxhlet extractor containing 500g of dry spirulina and 5 liters of acetone was heated to reflux in the dark under an argon atmosphere for 2 hours. The extract was removed solvent by rotary and then by high vacuum. The solution



of 25 ml of concentrated H_2SO_4 and 500 ml CH_3OH was added to the dried residual and the mixture was stirred for 24 hours at room temperature, darkness and inert atmosphere. At the end of the reaction, the treatment and purification were performed similar to the silkworm waste procedure. The yield of recovered 8 is higher than 85% from chlorophyll content in the spirulina with the mass of 2.96 g (0.592% of biomass).

 R_f : 0.57 (Alox, CH₂Cl₂/ pet/ acetone 10:10:1).

Mp: 226-227°C (CH₂Cl₂/ MeOH, Lit.: 220-225°C, Lit.: 228°C).

IR (KBr): $\tilde{V} = 3387 \text{ cm}^{-1}$ (w, N-H), 2957 (w), 2865 (w), 1732 (s, C=O, ester), 1700 (s, C=O, ester), 1621 (m, C=C, aromatic), 1557 (w), 1498 (w), 1434 (w), 1366 (w), 1347 (w), 1298 (w), 1203 (s, C-O-C, ester), 1164 (s, br, C-O-C, ester), 1123 (w), 1036 (w), 991 (w), 910 (w), 894 (w), 830 (w), 753 (w), 672 (w).

UV/VIS (THF): $\lambda_{\text{max}} (\epsilon x 10^{-3}) = 320 (20.63), 410 (106.05), 505 (11.31), 535 (9.95), 610 (7.87), 670 (48.86).$

¹**H-NMR** (200 MHz, CDCl₃): δ 13^2 R [13^2 S] = - 1.61 ppm [- 1.43] (s, br, 1H, NH), 0.54 [0.89] (s, br, 1H, NH), 1.71 (t, 3 J = 7.82 Hz, 3H, CH₃, 8^2), 1.83 (d, 3 J = 7.34 Hz, 3H, CH₃, 18^1), 2.10 - 2.70 (m, 4H, 2CH₂, 17^1 and 17^2), [3.21] 3.25 (s, 3H, CH₃, 7^1), [3.39] 3.42 (s, 3H, CH₃, 2^1), [3.54] 3.59 (s, 3H, CH₃, 12^1), 3.70 (q, 3 J = 7.82 Hz, 2H, CH₂, 8^1), 3.71 (s, 3H, CH₃, 17^4), [3.84] 3.89 (s, 3H, CH₃, 13^3), 4.05 - 4.25 (m, 1H, CH, 17), 4.48 (q, 3 J = 7.34 Hz, 1H, CH, 18), 6.20 (d, 3 J = 11.24 Hz, *cis* - vinyl, 3 J = 11.24 Hz, *cis* - vinyl, 3 J = 17.66 Hz, *trans* - vinyl, 1H, =CH₂, 3), 8.01 (dd, 3 J = 11.24 Hz, *cis* - vinyl, 3 J = 17.66 Hz, *trans* - vinyl, 1H, =CH, 3 J, [8.52] 8.59 (s, 1H, meso proton), [9.37] 9.41 (s, 1H, meso proton), [9.51] 9.54 (s, 1H, meso proton). Ratio 13^2 R/ 13^2 S = 89:11.

MS: (EI, 70 eV, direct, T = 200 °C): m/z (% rel. intensity) = 606 (100) [M⁺], 548 (29), 459 (17), 236 (10), 44 (40), 28 (100) [C₂H₄]⁺.

MS: (ESI, positive, $CH_2Cl_2/MeOH\ 1:10$): 607 $[M + H]^+$, 629 $[M + Na]^+$, 645 $[M + K]^+$.

MS: (ESI, negative, CH₂Cl₂/ MeOH 1:10): 605 [M - H]⁻.

Precision mass: 606.2842 (Cal.).

BRN: 604709.

CAS: 5594-30-9, 64070-09-3, 138812-88-1.

Synthesis of chlorin e₆-trimethylester 10 [9]

1516 mg (2.5 mmol) of **8** was dissolved in 50 ml of dry THF under an argon atmosphere. Thereafter, 200 ml of CH_3OH , 5 ml of a 30-35% solution of potassium methoxide in methanol were added. The mixture was stirred in the dark under an argon atmosphere for 30 minutes. The progress of the reaction was monitored by TLC (silicagel, CH_2Cl_2 / EtOAc 7:1). At the end of the reaction, 500 ml of H_2O , 10 ml of 2N aq. HCl and 100 ml aq. sat. NaCl were added. The mixture was extracted twice with portions of 100 ml of diethylether. The combined organic phases were washed twice with portions of 200 ml of H_2O and dried by filtration through cotton wool. The solvent was removed by rotary. The crude product was purified by column chromatography (silicagel, CH_2Cl_2 / EtOAc 7:1).

Analytically pure 10 was obtained after recrystallization from acetone/ MeOH as lustrous dark blue needles.

Yield: 1350 mg (yield 85 %).

Mp: 208-209°C (acetone/ MeOH, Lit. [9]: 207-208 °C).

R_f: 0.59 (silicagel, CH₂Cl₂/ EtOAc 7:1).

IR (KBr): $\tilde{V} = 3296 \text{ cm}^{-1}$ (w, N-H), 2954 (m, C=CH), 2914 (w), 2868 (w), 1731 (s, br, C=O, ester), 1601 (m, C=C, aromatic), 1496 (w), 1439 (m), 1370 (w), 1306 (w), 1240 (m, br, C-O-C), 1165 (m), 1118 (w), 1064 (m), 1028 (w), 986 (w), 907 (w), 894 (w), 845 (w), 796 (w), 730 (w), 670 (w).

UV/ VIS (THF): $\lambda_{\text{max}} (\epsilon x 10^{-3}) = 285 (12.59)$, 305 (10.72), 400 (123.51), 500 (10.86), 530 (4.46), 610 (4.45), 665 (42.44).

¹**H-NMR** (500 MHz, CDCl₃): δ = -1.47 ppm (s, br, 1H, NH), -1.30 (s, br, 1H, NH), 1.73 (t, 3 J = 7.34 Hz, 3H, CH₃, 8²), 1.77 (d, 3 J = 7.30 Hz, 3H, CH₃, 18¹), 2.18 - 2.22 (m, 4H, 2 CH₂, 17¹ and 17²), 3.31 (s, 3H, CH₃, 7¹), 3.49 (s, 3H, CH₃, 2¹), 3.59 (s, 3H, CH₃, 12¹), 3.78 (s, 3H, CH₃, 17⁴), 3.80 (q, 3 J = 7.34 Hz, 2H, CH₂, 8¹), 4.27 (s, 3H, CH₃, 15³), 4.44 (q, 3 J = 7.30 Hz, 1H, CH, 18), 5.23 (d, 1H, 2 J = 18.58 Hz, CH₂, 15¹), 5.37 (d, 1H, 2 J = 18.58 Hz, CH₂, 15¹), 6.16



(d, ${}^{3}J = 11.72$ Hz, cis - vinyl, 1H, =CH₂, 3^{2}), 6.37 (d, ${}^{3}J = 17.60$ Hz, trans - vinyl, 1H, =CH₂, 3^{2}), 8.08 (dd, ${}^{3}J = 11.72$ Hz, cis - vinyl, ${}^{3}J = 17.60$ Hz, trans - vinyl, 1H, =CH, 3^{1}), 8.75 (s, 1H, meso proton), 9.58 (s, 1H, meso proton), 9.87 (s, 1H, meso proton), 9.70 (s, 1H, meso proton).

MS: (EI, 70 eV, direct, T = 200 °C): m/z (% rel. intensity) = 638 (100) $[M^+]$, 579 (13) $[M - COOCH_3]^+$, 479 (20), 289 (7), 28 (19) $[C_2H_4]^+$.

Precision mass: 638.3104 (Cal.).

BRN: 76355. **CAS**: 15592-17-3.

Conclusion

An improved isolation and transformation chlorophyll a 1 to methylpheophorbide a and chlorin- e_6 trimethylester were performed in the safety conditions and can be scaled up to the pilot plant. The yield of recovered pheophorbide a 8 is higher than 80% from chlorophyll content. Pure methylpheophorbide a and chlorin- e_6 trimethylester 10 can be used for food additive or transformed to chlorin- e_6 and its derivaties using for photodynamic therapy.

Acknowledgements

The authors are indebted to the Vietnam Academy of Science and Technology for financially supported in part by scientific research and technological development project (code: VAST.ĐT.TP.04/16-18).

References

- 1. P. H. Hynninen, Chemistry of Chlorophyllls: Modification in Chlorophyllls, *Chlorophyllls*, H. Scheer, Eds., Boca Raton Ann Arbor Boston London , *1*, 145-209 (1991).
- 2. F. P. Montforts, M. G. Breiling, Naturally Occurring Tetrapyroles: Progress in the Chemistry of Organic Natural Products, Ed, *Springer Wien New York*, 84, 1-51 (2004).
- 3. W. Schmidt, F.-P. Montforts, Synthesis of a novel enantiomerically pure chlorin as a potential pubunit for an artificial, *Synlett*, 903-904, (1997).
- 4. Webber, B. Leeson, D. Fromm, D. Kessel, Effects of photodynamic therapy using a fractionated dosing of mono-l-aspartyl chlorin e₆ in a murine tumor, *J. Photochem. Photobiol. B: Biol.* 78, 135-140, (2005).
- 5. Y. Amao, T. Komori, Biosensors and Bioelectronics, 19, 843-847 (2004).
- 6. C. L. Bergstrom, I. Vucenik, I. K. Hagen, S. A. Chernomorsky, R. D. Poretz, *J. Photochem. and Photobiol. B: Biol.*, 24, 17-23 (1994).
- 7. D. Kessel, K. Woodburn, C. J. Gomer, N. Jagerovic, K. M. Smith, Photosensitization with derivatives of chlorin, *J. Photochem. Photobiol. B: Biol*, 28, 13-18, (1995).
- 8. W. Kühlbrandt, D. N. Wang, Y. Fujiyoshi, Atomic model of plant light-harvesting complex by electron crystallography, *Nature*, *367*, 614, (1994).
- 9. M. Stapelbroek-Möllmann, Dissertation, Helsinki/Bremen 1997

