



# An Improved Preparation of Methylpheophorbide A and Chlorin-E<sub>6</sub> Trimethylester from Spirulina and Skillworm Waste

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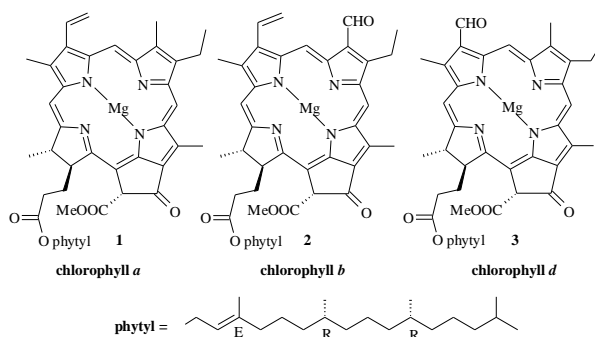
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**Abstract** Chlorophyll and its derivatives are using as food additives, cosmetic and photodynamic therapy of cancer such as pheophytin a, methylpheophorbide a, chlorin-e<sub>6</sub> trimethylester, copper chlorophyllin. Herein, we report an improved extraction chlorophyll from spirulina by soxhlet extraction method and an improved directly transformation chlorophyll to methyl pheophorbide a without purification. The transformation methylpheophorbide a to chlorin-e<sub>6</sub> trimethylester was performed by opening carbocyclic ring catalyzed by potassium methoxide. Pure obtained methylpheophorbide a and chlorin-e<sub>6</sub> trimethylester are important materials for the production of chlorin-e<sub>6</sub> and its derivatives. The structures of these compounds were determined by IR, UV, NMR and MS spectroscopy.

**Keywords** Chlorophyll, pheophorbide, pheophytin, chlorin-e<sub>6</sub>, 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*

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-*e*<sub>6</sub> **4**, mono L-aspartyl chlorin-*e*<sub>6</sub> **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, esophagus, stomach, urinary bladder and rectum [1-3,7].

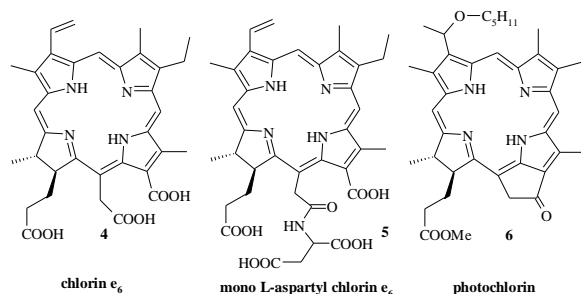


Figure 2: Chlorophyll derivatives as possible sensitizer for photodynamic therapy

In our synthetic studies in the field of chlorophyll chemistry, we were interested to develop and improve a partial synthetic pathway methylpheophorbide **8** and chlorin-*e*<sub>6</sub> 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 demethoxycarboxylation 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 ( $\epsilon=0,8579.10^5$ ) 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

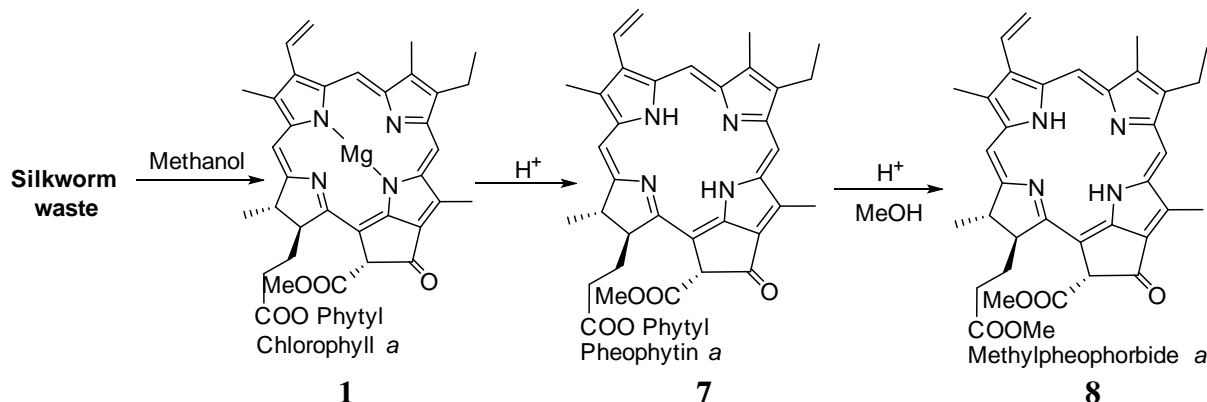
No	Samples	Chlorophyll (% mass)
1	Erythrina variegata L (at Bac Ninh, Vietnam)	$0.028 \pm 0.002$
2	Bamboo leaves (at Bac Ninh, Vietnam)	$0.046 \pm 0.005$
3	Erythrina orientalis leaves (at Bac Ninh, Vietnam)	$0.034 \pm 0.001$
4	Mulberry leaves (at Bac Ninh, Vietnam)	$0.023 \pm 0.002$
5	Spinat (at Ba Vi,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

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**

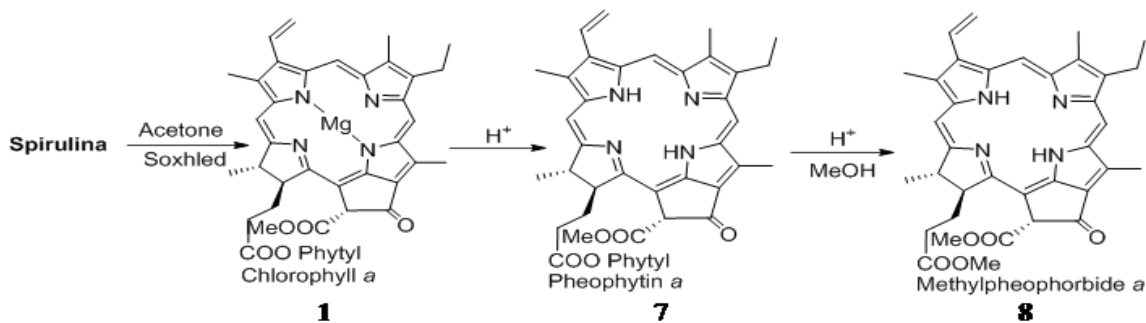
The extraction of **1** from silkworm waste and transformation to methylpheophorbide *a* were performed by one-pot manner (**Scheme 1**). The mixture of silkworm waste and methanol was stirred with methanol for 2 hours at ambient temperature. The chlorophyll *a* **1** solution was obtained by removal 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  $\pi$ -stacking interaction of 18 electrons  $\pi$  conjugated and can be solved by recrystallization. Pure **8** is very important intermediate for the preparation of copper chlorophyllin and chlorin-*e*<sub>6</sub> 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 methylpheophorbide *a* **8** was received by chromatography and recrystallization by CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH. 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 diastereomers at position C<sub>13</sub> in carbocyclic ring. The ratio between <sup>13</sup>C<sup>2</sup>*R* and <sup>13</sup>C<sup>2</sup>*S* diastereomers is 89/11 which was determined by <sup>1</sup>H-MNR. Hyperchem calculation shows that the energy of *R*-diastereomer is less than 3.15 kcal/mol compare to <sup>13</sup>C<sup>2</sup>*S*-diastereomer.



*Scheme 2: Preparation of methylpheophorbide *a* **8** from spirulina*





of 25 ml of concentrated H<sub>2</sub>SO<sub>4</sub> and 500 ml CH<sub>3</sub>OH 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<sub>f</sub>**: 0.57 (Alox, CH<sub>2</sub>Cl<sub>2</sub>/ pet/ acetone 10:10:1).

**Mp**: 226-227°C (CH<sub>2</sub>Cl<sub>2</sub>/ MeOH, Lit.: 220-225°C, Lit.: 228°C).

**IR** (KBr):  $\tilde{\nu}$  = 3387 cm<sup>-1</sup> (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):  $\lambda_{\max}$  (ex10<sup>-3</sup>) = 320 (20.63), 410 (106.05), 505 (11.31), 535 (9.95), 610 (7.87), 670 (48.86).

**<sup>1</sup>H-NMR** (200 MHz, CDCl<sub>3</sub>):  $\delta$  13<sup>2</sup>R [13<sup>2</sup>S] = - 1.61 ppm [- 1.43] (s, br, 1H, NH), 0.54 [0.89] (s, br, 1H, NH), 1.71 (t, <sup>3</sup>J = 7.82 Hz, 3H, CH<sub>3</sub>, 8<sup>2</sup>), 1.83 (d, <sup>3</sup>J = 7.34 Hz, 3H, CH<sub>3</sub>, 18<sup>1</sup>), 2.10 - 2.70 (m, 4H, 2CH<sub>2</sub>, 17<sup>1</sup> and 17<sup>2</sup>), [3.21] 3.25 (s, 3H, CH<sub>3</sub>, 7<sup>1</sup>), [3.39] 3.42 (s, 3H, CH<sub>3</sub>, 2<sup>1</sup>), [3.54] 3.59 (s, 3H, CH<sub>3</sub>, 12<sup>1</sup>), 3.70 (q, <sup>3</sup>J = 7.82 Hz, 2H, CH<sub>2</sub>, 8<sup>1</sup>), 3.71 (s, 3H, CH<sub>3</sub>, 17<sup>4</sup>), [3.84] 3.89 (s, 3H, CH<sub>3</sub>, 13<sup>3</sup>), 4.05 - 4.25 (m, 1H, CH, 17), 4.48 (q, <sup>3</sup>J = 7.34 Hz, 1H, CH, 18), 6.20 (d, <sup>3</sup>J = 11.24 Hz, *cis* - vinyl, <sup>3</sup>J = 11.24 Hz, =CH<sub>2</sub>, 3<sup>2</sup>), 6.28 (s, 1H, CH, 13<sup>2</sup>), 6.30 (d, <sup>3</sup>J = 17.66 Hz, *trans* - vinyl, 1H, =CH<sub>2</sub>, 3<sup>2</sup>), 8.01 (dd, <sup>3</sup>J = 11.24 Hz, *cis* - vinyl, <sup>3</sup>J = 17.66 Hz, *trans* - vinyl, 1H, =CH, 3<sup>1</sup>), [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<sup>2</sup>R/ 13<sup>2</sup>S = 89:11.

**MS**: (EI, 70 eV, direct, T = 200 °C): m/z (% rel. intensity) = 606 (100) [M<sup>+</sup>], 548 (29), 459 (17), 236 (10), 44 (40), 28 (100) [C<sub>2</sub>H<sub>4</sub>]<sup>+</sup>.

**MS**: (ESI, positive, CH<sub>2</sub>Cl<sub>2</sub>/MeOH 1:10): 607 [M + H]<sup>+</sup>, 629 [M + Na]<sup>+</sup>, 645 [M + K]<sup>+</sup>.

**MS**: (ESI, negative, CH<sub>2</sub>Cl<sub>2</sub>/ MeOH 1:10): 605 [M - H]<sup>-</sup>.

**Precision mass**: 606.2842 (Cal.).

**BRN**: 604709.

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

### Synthesis of chlorin e<sub>6</sub>-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<sub>3</sub>OH, 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<sub>2</sub>Cl<sub>2</sub>/ EtOAc 7:1). At the end of the reaction, 500 ml of H<sub>2</sub>O, 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<sub>2</sub>O and dried by filtration through cotton wool. The solvent was removed by rotary. The crude product was purified by column chromatography (silicagel, CH<sub>2</sub>Cl<sub>2</sub>/ 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<sub>f</sub>**: 0.59 (silicagel, CH<sub>2</sub>Cl<sub>2</sub>/ EtOAc 7:1).

**IR** (KBr):  $\tilde{\nu}$  = 3296 cm<sup>-1</sup> (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):  $\lambda_{\max}$  (ex10<sup>-3</sup>) = 285 (12.59), 305 (10.72), 400 (123.51), 500 (10.86), 530 (4.46), 610 (4.45), 665 (42.44).

**<sup>1</sup>H-NMR** (500 MHz, CDCl<sub>3</sub>):  $\delta$  = - 1.47 ppm (s, br, 1H, NH), - 1.30 (s, br, 1H, NH), 1.73 (t, <sup>3</sup>J = 7.34 Hz, 3H, CH<sub>3</sub>, 8<sup>2</sup>), 1.77 (d, <sup>3</sup>J = 7.30 Hz, 3H, CH<sub>3</sub>, 18<sup>1</sup>), 2.18 - 2.22 (m, 4H, 2 CH<sub>2</sub>, 17<sup>1</sup> and 17<sup>2</sup>), 3.31 (s, 3H, CH<sub>3</sub>, 7<sup>1</sup>), 3.49 (s, 3H, CH<sub>3</sub>, 2<sup>1</sup>), 3.59 (s, 3H, CH<sub>3</sub>, 12<sup>1</sup>), 3.78 (s, 3H, CH<sub>3</sub>, 17<sup>4</sup>), 3.80 (q, <sup>3</sup>J = 7.34 Hz, 2H, CH<sub>2</sub>, 8<sup>1</sup>), 4.27 (s, 3H, CH<sub>3</sub>, 15<sup>3</sup>), 4.44 (q, <sup>3</sup>J = 7.30 Hz, 1H, CH, 18), 5.23 (d, 1H, <sup>2</sup>J = 18.58 Hz, CH<sub>2</sub>, 15<sup>1</sup>), 5.37 (d, 1H, <sup>2</sup>J = 18.58 Hz, CH<sub>2</sub>, 15<sup>1</sup>), 6.16



(d,  $^3J = 11.72$  Hz, *cis* - vinyl, 1H, =CH<sub>2</sub>, 3<sup>2</sup>), 6.37 (d,  $^3J = 17.60$  Hz, *trans* - vinyl, 1H, =CH<sub>2</sub>, 3<sup>2</sup>), 8.08 (dd,  $^3J = 11.72$  Hz, *cis* - vinyl,  $^3J = 17.60$  Hz, *trans* - vinyl, 1H, =CH, 3<sup>1</sup>), 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<sup>+</sup>], 579 (13) [M - COOCH<sub>3</sub>]<sup>+</sup>, 479 (20), 289 (7), 236 (7), 28 (19) [C<sub>2</sub>H<sub>4</sub>]<sup>+</sup>.

**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*<sub>6</sub> 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*<sub>6</sub> trimethylester **10** can be used for food additive or transformed to chlorin-*e*<sub>6</sub> and its derivatives using for photodynamic therapy.

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