CHAPTER IV

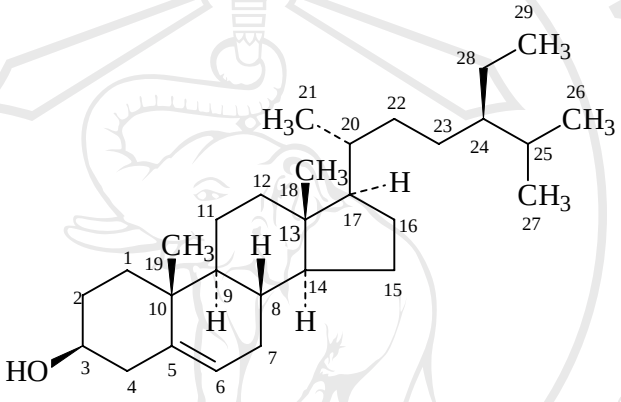
RESULTS AND DISCUSSION

A. Isolation and antimalarial activity of *P. javanica*

The eight crude extracts of *P. javanica*, four extracts from the stembark and four extracts from wood, were tested for in vitro antimalarial activities against *P. falciparum* K1 by using the [3H]hypoxanthine incorporation method reported by Desjardins *et al*. (1978) and modified by Kamchowongpaisan *et al*. (1995). The hexane extract of stembark was shown to be the most effective against *P. falciparum* K1 with IC_{50} of 3.3 μg/mL (Table 4.1), while the chloroform extract of stembark was effective with IC_{50} of 20.0 μg/mL. The chloroform extract of wood showed the activity against *P. falciparum* K1 with IC_{50} of 21.6 μg/mL, which was comparable to that of the chloroform extract of stembark.

The scale up of extraction provided about 13 g of hexane extract. Further isolation of 12.152 g of hexane extract obtained ten fractions. The *in vitro* antimalarial activity test of these fractions showed that Fraction V was the most active with IC_{50} of 4.4 μg/ml (Table 4.2). Purification of Fraction V (0.7593 g) provided six fractions and their antimalarial activities were shown in Table 4.3. It was found that the white crystal from Fraction V-3 was not dissolved in DMSO, therefore its antimalarial activity was not determined. Fraction V-2 and V-4 showed antimalarial activities against *P. falciparum* K1 with IC_{50} of 2.8 and 3.4 μg/ml, respectively. The major compound of Fraction V-3 could be assigned as a known steroid, β-sitosterol through analysis of its ^{1}H- and ^{13}C-NMR spectra. The ^{1}H-NMR spectrum showed the signals at δ 0.66-0.98 ppm which were the signals of methyl protons of steroid compound. The signals at 1.00-2.29 ppm were the signals of methylene and methine protons of steroid compound. The multiplet signal at δ 3.50 ppm was the signal of the proton at C-3. The olefinic signal at δ 5.33 ppm (m) could be assigned as H-6 which was trisubstituted vinylic proton. The ^{13}C-NMR spectrum showed the signals of 3 quaternary, 9-methine, 11 methylene and 6 methyl carbons. The assigned carbons are C-1 (37.22 ppm), C-2 (31.61 ppm), C-3 (71.78 ppm), C-4 (42.25 ppm), C-5 (140.71 ppm), C-6 (121.69 ppm), C-7 (31.87 ppm), C-8 (31.87 ppm), C-9 (50.10 ppm), C-10 (36.48 ppm), C-11

(21.06 ppm), C-12 (39.75 ppm), C-13 (42.25 ppm), C-14 (56.73 ppm), C-15 (24.29 ppm), C-16 (28.22 ppm), C-17 (56.02 ppm), C-18 (11.83 ppm), C-19 (19.37 ppm), C-20 (36.11 ppm), C-21 (18.76 ppm), C-22 (33.91 ppm), C-23 (26.04 ppm), C-24 (45. 80 ppm), C-25 (29.11 ppm), C-26 (19.79 ppm), C-27 (19.01 ppm), C-28 (23.04 ppm) and C-29 (11.96 ppm). Both the ^{1}H- and ^{13}C-NMR spectra were in accordance with those published previously of β-sitosterol (Wright *et al*., 1978 and Kosin, 1996). Therefore, it could be concluded that the major compound of fraction V-3 was β-sitosterol.



β-sitosterol

In order to isolate 4-methoxy-1-vinyl-β-carboline, the chloroform extract of stembark was prepared again using the same process as described. Further isolation of 21.901g of chloroform crude extract using acid-base extraction method provided alkaloidal portion 17.5 mg and non-alkaloidal portion of about 19 g. The in vitro antimalarial activity test of these portion showed that alkaloidal portion was effective against P. falciparum K1 with IC_{50} of 15.0 μg/ml, while non-alkaloidal portion showed IC_{50} of 22.0 μg/ml (Table 4.4). The GC chromatogram of alkaloidal portion from the first run showed the major peak at the retention time of 13.14 min with some small peaks. The EI mass spectrum of the major GC peak showed the important peaks at *m/z* 224 (100 %), 209 (14 %), 181 (39 %), 154 (19 %) and 126 (15 %). The GC chromatogram from the second run showed the major peak at the retention time of 13.03 min with some small peaks. The CI mass spectrum of the major GC peak showed the main two peaks at *m/z* 225 (100%) and 253 (29 %). According to the mass spectra, the major compound of alkaloidal portion could be assigned as a known 1-substituted-4-oxygenated-β-carboline; 4-methoxy-1-

vinyl-β-carboline (M). The EI mass spectrum showed the molecular ion peak ($M^{\bullet+}$) at m/z 224. The peaks at m/z 209, 181 and 154 represented fragment ions $M^{\bullet+}$-$CH_3^{\bullet}$, $M^{\bullet+}$- $CH_3^{\bullet}$- C_2H_4 and $M^{\bullet+}$- $CH_3^{\bullet}$- C_2H_4 – CHN respectively. The CI mass spectrum showed the M + H^+ ion peak at m/z 225. The peak at *m/z* 253 represented for M + $C_2H_5^+$ ion. In addition, the EI mass spectrum was in accordance with that of 4-methoxy-1-vinyl-β-carboline reported by Pavanand *et al*. (1988). Further purification of alkaloidal portion using preparative TLC provided only small amount of degradation product, 1-ethyl-4-methoxy-β-carboline (3). This compound showed molecular ion peak at *m/z* 226.

Table 4.1 Crude extracts obtained from *Picrasma javanica* Bl. stembark and woot, and their antimalarial activities against *Plasmodiun falciparum* K1

Crude extracts	**Weight (g)**	**% Yield**	**IC_{50} against *P. falciparum* K1 (μg/ml)**
Extract from stembark			
methanol crude extract	3.674	2.84	22.1
chloroform crude extract	1.674	1.61	20.0
n-hexane crude extract	0.453	0.38	3.3
water crude extract	10.487	9.03	inactive at 50.0
Extract from wood			
methanol crude extract	1.634	1.35	inactive at 50.0
chloroform crude extract	0.254	0.24	21.6
n-hexane crude extract	0.157	0.15	33.2
water crude extract	3.819	3.42	inactive at 50.0

Table 4.2 Isolated fractions obtained from hexane crude extract of *Picrasma javanica* Bl. stembark and their antimalarial activities against *Plasmodium falciparum* K1

Isolated fractions	**Weight (g)**	**% Yield**	**IC_{50} against *P. falciparum* K1 (μg/ml)**
I	1.202	9.89	Inactive
II	0.340	2.80	Inactive
III	0.521	4.29	Inactive
IV	1.246	10.25	30.0
V	1.840	15.14	4.4
VI	0.723	5.95	20.0
VII	1.005	8.27	18.0
VIII	1.044	8.59	13.0
IX	0.292	2.40	21.0
X	0.188	1.55	12.0

Table 4.3 Isolated fractions obtained from Fraction V and their antimalarial activities against *Plasmodium falciparum* K1

Isolated fractions	**Weight (mg)**	**% Yield**	**IC_{50} against *P.* falciparum K1 (μg/ml)**
V-1	9.6	1.26	Inactive
V-2	70.6	9.30	2.8
V-3	102.1	13.45	Not dissolved in DMSO
V-4	25.5	3.36	3.4
V-5	17.5	2.30	11.8
V-6	4.7	0.62	12.5

Table 4.4 Isolated portions obtained from chloroform extract of *Picrasma javanica* Bl. stembark and their antimalarial activities against *Plasmodium falciparum* K1

Isolated portions	**Weight (g)**	**% Yield**	**IC_{50} against *P. falciparum* K1 (μg/ml)**
Alkaloid portion	0.0175	0.08	15.0
Non-alkaloid portion	19	86.75	22.0

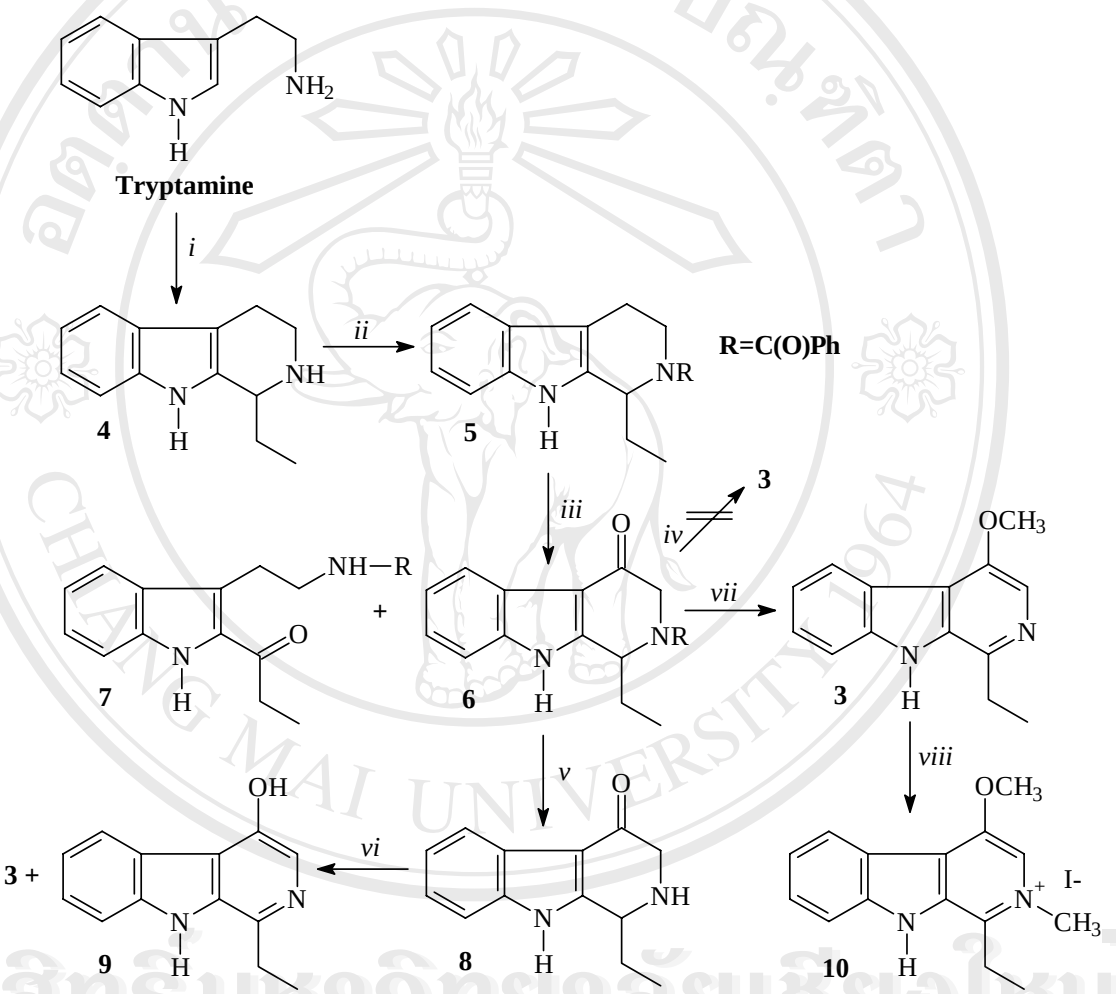
B. Synthesis, cytotoxicity and antimalarial activity of 1-substituted-4-oxygenated-β-carbolines

Chemistry

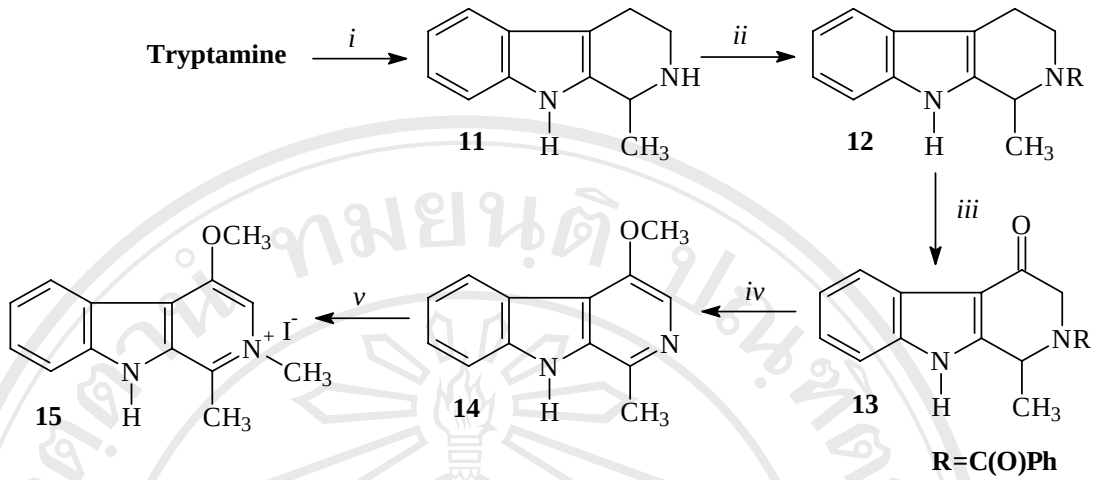
The convenient methods for synthesis 1-substituted-4-oxygenated-β-carbolines were developed by using **3** as the model compound (Scheme1). Compound **3** was synthesized by modification of Cook's method (Cain *et al*., 1982; Hagen *et al*., 1989; see in appendix B) and combined with modification of Suzuki's method (Suzuki *et al*., 1999). First, 1-Ethyl-1,2,3,4-tetrahydro-β-carboline (**4)** was synthesized in the high yield by a reaction of tryptamine with propionaldehyde. Benzolation of 4 with benzoyl chloride gave N-1 protected compound, 1-Ethyl-2-benzoyl-1,2,3,4-tetrahydro-β-carboline (**5**). Treatment of **5** with DDQ provided a mixture of 1-Ethyl-2-benzoyl-4-oxo-1,2,3,4-tetrahydro-β-carboline (**6**) and side product **7**. When compound **6** was refluxed with methanol in the present of trimethyl orthoformate and *p*-toluenesulfonic acid, the desired product **3** did not occur. Thus, compound **6** was hydrolized with sodium hydroxide to yield 1-Ethyl-4-oxo-1,2,3,4-tetrahydro-β-carboline (**8**). Compound **8** then methylated with methanol in the present of trimethyl orthoformate and *p*-toluensulfonic acid, the result provided the target compound **3** and 1-Ethyl-4-hydroxy-β-carboline (**9**) in low yield. The high yield of **3** was achieved when modified Suzuki's method that used to synthesized 4-methoxy-8-tosyloxy-β-carboline from 2-formyl-4-oxo-8-tosyloxy-1,2,3,4-tetrahydro-β-carboline (Suzuki *et al*., 1999). The reaction was carry in the refluxed mixture of benzene and *p*-toluenesulfonic acid monohydrate in the Dean-Stark apparatus. Compound **6** was methylated with dimethoxypropane, then oxidized with *p*-chloranil and finally lost the benzoyl moielty to provide compound **3**.

The four-step method for the synthesis of 1-substituted-4-oxygenated-β-carbolines using tryptamine as a starting material was applied to 1-methyl-4-methoxy-β-carboline (14) as show in scheme 2. First, 1-Methyl-1,2,3,4-tetrahydro-β-carboline (**11**) was synthesized in a high yield by a reaction of tryptamine with acetadehyde. Benzolation of **11** with benzoyl chloride gave N-1 protected compound, 1-Methyl-2-benzoyl-1,2,3,4-tetrahydro-β-carboline (**12**). Treatment of **12** with DDQ provided a mixture of 1-Mthyl-2-benzoyl-4-oxo-1,2,3,4-tetrahydro-β-carboline (**13**) and an unknown side product. In the refluxed mixture of benzene and *p*-toluenesulfonic acid monohydrate in the Dean-Stark apparatus, compound **13** was methylated with dimethoxypropane,

then oxidized with p-chloranil and finally lost the benzoyl moiety to achieve the high yield of compound **14**. In addition, compound **3** and **14** were methylated with iodomethane in tetramethylenesulfone (Wright *et al*., 2001) to give the coresponding analogues **10** and **15**.



Scheme1. Reagents and conditions: (*i*) C_2H_5CHO, 10% MeOH, conc. H_2SO_4, reflux (6.5 h); (*ii*) PhC(O)Cl, DMAP, Et_3N, CH_2Cl_2 60°C (0.5 h); (*iii*) DDQ, THF/H_2O (9:1), -78°C (3 h), at (18 h); (*iv*) MeOH, *p*-TSA, $(CH_3O)_3CH$, reflux (24 h); (*v*) 6 *N* NaOH, reflux (3 h); (*vi*) MeOH, *p*-TSA, $(CH_3O)CH$, reflux (24 h); (*vii*) $(MeO)_2CMe_2$, *p*-TSA, *p*-chloranil, benzene, at (20h); (*viii*) CH_3I, tetramethylene sulfone, 50°C (17h)



Scheme 2. Reagents and conditions: (*i*) CH_3CHO, 2 *N* H_2SO_4, H_2O, heat (0.75 h); (*ii*) PhC(O)Cl), DMAP, Et_2N, CH_2Cl_2, 60°C (2 h); (*iii*) DDQ, THF/H_2O (9:1), -78°C, at; (*iv*) *p*-TSA, $(MeO)_2CMe_2$, chloranil, benzene, at (20 h); (*v*) CH_3I, tetramethylene sulfone, at (15 h)

***In vitro* antimalarial activity and cytotoxicity**

The in vitro antimalarial activities against *P. falciparum* (chloroquine sensitive FCR-3 strain) of the synthesized compounds and their cytotoxicities against mouse mammary tumor FM3A were evaluated (Table 4.5). Selective toxicities, defined by the ratio of EC_{50}(FM3A/EC_{50}(*P. falciparum*), were determined. The tetrahydro-β-carboline compound **4** showed low activity againt *P. falciparum* chloroquine sensitive strain FCR-3 and low cytotoxicity. Structure modification to 4-oxo-tetrahydro-β-carboline compound **6** dramatically decreased not only antimalarial activity but also toxicity. The 1-substituted-4-oxygenated-β-carbolines compound **3** and **14** were 2-fold more toxic than compound **4** and a little less active against *P. falciparum*. However, their N-methylated compound **10** and **15** were 15-fold and 38-fold more active respectively, while their cytotoxicities were eqivalent to tetrahydro-β-carboline compound **4**. Thus their selective toxicities were increased particularly in compound **15**. It is indicated that the methyl group at N-2 position is necessary for antimalarial activity. This is in good agreement with report of Wright *et al*. (2001) that the methyl group at N-5 of cryptolepine (5-methyl, 10H-indolo[3,2-b]quinoline) is a prerequisite for antiplasmodial activity . In addition, the accidental synthesized compound **9** was noted to increased antimalarial activity and decrease

toxicity. Therefore, further structure modification of 1-substituted-4-oxygenated-β-carbolines could lead to high potential candidates for antimalarial agent.

Table 4.5 *In vitro* antimalarial activities and cytotoxicity of 1-substituent-4-oxygenated-β-carbolines

Compound	EC_{50} (M)		Selective toxicity [c]
	P. falciparum [a]	FM3A [b]	
4	1.4×10^{-5}	2.9×10^{-5}	2
6	$> 4.9 \times 10^{-5}$ [d]	$> 4.9 \times 10^{-5}$ [e]	-
9	1.2×10^{-6}	$>3.1 \times 10^{-5}$ [f]	>25
3	1.6×10^{-5}	1.8×10^{-5}	11
10	9.5×10^{-7}	$> 3 \times 10^{-5}$ [g]	>31
14	2.2×10^{-5}	1.8×10^{-5}	0.8
15	3.7×10^{-7}	3.0×10^{-5}	81

[a]Chloroquine sensitive strain (FCR-3). [b]Mouse mammary tumor FM3A cells representing a model of host. [c]Selective toxicity = EC_{50} for (FM3A)/EC_{50} for (P. falciparum). [d]EC_9 value (91 % growth of for P. falciparum was observed). [e]EC_6 value (94 % growth of FM3A cells was observed). [f]EC_{20} value (80% growth of FM3A cells was observed). [g]EC_{37} value (63 % growth of FM3A cells was observed).