

A bioactive diterpene; Nasimalun A from *Croton oblongifolius* Roxb.

Kanchana Wijesekera

BPharm Degree Program, Faculty of Medicine, University of Ruhuna, Sri Lanka

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## Abstract

A clerodane type diterpene, nasimalun A was isolated from a dichloromethane extract of the leaves of *Croton oblongifolius* Roxb. Nasimalun A showed moderate cytotoxicity toward MOLT-3 cell line with  $IC_{50}$  value of 26.44  $\mu\text{g/mL}$ . Furthermore, nasimalun A showed minimum inhibitory concentration (MIC) of 50, 12.5, and 100  $\mu\text{g/mL}$  for *Bacillus cereus* and both *Staphylococcus aureus*, and *Staphylococcus epidermidis* respectively.

**Keywords:** Clerodane, nasimalun A, cytotoxicity, MIC

## 1. Introduction

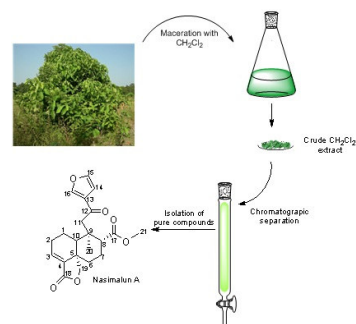
*Croton* is a genus of the family Euphorbiaceae and known to have around 1300 species. The species of *Croton* are widely distributed in tropical regions. Several species have a long history in the traditional medicine systems in South America, Asia, and Africa.<sup>1,2</sup> *Croton oblongifolius*, *C. kongensis*, *C. tigilium*, and *C. sublyratus* are few of the species belong to the genus *Croton* which are used in the traditional medicine systems in Asia. The plant *C. oblongifolius*, in Thailand known as "Plau Yai" has a long history in the traditional Thai medicine.<sup>3</sup>

Different parts of this plant have been used in several medical purposes including leaves as a tonic, flowers for the treatment of flat worms, fruits to alleviate dysmenorrhea, seeds as a purgative, bark for the treatment of dyspepsia, and roots as an ailment for dysentery. Moreover, in the traditional Thai medicine, *C. oblongifolius* in combination with *C. sublyratus* has been used to treat gastric ulcers and gastric cancers.<sup>4,5</sup> Chemical constituents of *C. oblongifolius* have been extensively studied by Seshadri *et al*, and a number of metabolites have been isolated. These diterpenoids belong to cembrane, halimane, labdane, cleistanthane, and isopimarane types and showed cytotoxicity towards several cancer cell lines including HepG2, SW620, CHAGO, KATO3, and BT474.<sup>6</sup> Herein reports the isolation, characterization and biological activities of a diterpene type compound nasimalun A which was isolated from the leaves of *C. oblongifolius* Roxb.

## 2. Materials and Methods

## 2.1 Plant material

Leaves of *C. oblongifolius* were collected from Nakhonsawan province, Thailand, in October 2014. The plant, *C. oblongifolius*, was previously authenticated by



Panarat Charoenchai, and the specimen (no. CRI 285) was deposited at the Laboratory of Natural Products, Chulabhorn Research Institute, Bangkok, Thailand.<sup>3</sup>

## 2.2 Extraction and isolation

Powdered, air-dried leaves (0.8 kg) of *C. oblongifolius* were macerated with dichloromethane to yield a crude extract of 37.6 g. The crude extract was subjected to silica gel column chromatography (CC) (10×56 cm), eluted with hexane/CH<sub>2</sub>Cl<sub>2</sub> and CH<sub>2</sub>Cl<sub>2</sub>/MeOH stepwise gradients, and yielded 19 fractions (A-1 to A-19). Fraction A19 (3.18 g) was separated by SepCC (4×46 cm), eluted with MeOH, to yield 20 fractions (B1-B20). Fractions B16-B19 which had similar TLC patterns and <sup>1</sup>H NMR spectra were combined (487.6 mg) and further separated by SepCC (2×122 cm), eluted with MeOH:CH<sub>2</sub>Cl<sub>2</sub> (1:3), to yield nasimalun A (76.4 mg)

## 2.3 Biological activity

## 2.3.1 Measurement of oxygen radical absorbance capacity (ORAC)

Peroxy or hydroxyl radical absorbance capacity of test sample was tested by a modified ORAC assay, following the method previously described by Gerhauser *et al*.<sup>7</sup> Results were expressed as ORAC units, where 1 ORAC unit equals the net protection of  $\beta$ -phycoerythrin ( $\beta$ -PE) produced by 1  $\mu\text{M}$  trolox (6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid), a water soluble vitamin E analog. Only scavenging capacities of more than 1 ORAC unit were considered as positive.

## 2.3.2 Aromatase inhibitory assay (AIA)

The inhibition of aromatase was performed according to the method previously described by Stresser *et al*. using a CYP19/methoxy-4-trifluoromethyl-coumarin (MFC) high throughput inhibition screening kit. Ketoconazole, which

typically has IC<sub>50</sub> value of 2.4 μM, was used as the reference compound.<sup>8</sup>

### 2.3.3 Cytotoxic activity

Cytotoxic activities against adhesive cell lines; HepG2 (human hepatocellular carcinoma cell line), HUCA-1 (cholangiocarcinoma cell line), and A549 (human alveolar epithelial cell line) were evaluated using the MTT assay. XTT assay was used for the assessment of cytotoxicity of non-adhesive cell line MOLT-3 (human acute lymphoblastic leukemia-T cell type). Etoposide and doxorubicin were used as the reference drugs.<sup>9,10</sup>

### 2.3.4 Antibacterial activity

Minimum inhibitory concentration (MIC) of the compound was tested on gram positive (*Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*), and gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Shigella flexneri*) bacteria by broth microdilution method described by Andrews *et al.*<sup>11</sup> The compound was dissolved in DMSO, and a two-fold serial dilution in 96-well plates was carried out. Bacterial suspensions were prepared in normal saline solution and adjusted to a turbidity of the 0.5 McFarland standard. Final concentration of the DMSO did not exceed 0.5% (v/v). The plates were incubated at 36 °C (±1) for 20 h, and the absorbance was measured at 600 nm to determine the MICs of the tested compounds. Chloramphenicol, tetracycline, and vancomycin were used as the standard drugs. The MIC is defined as the lowest concentration of the compound that inhibits the growth of microorganism.

## 3. Results

### 3.1 Structure elucidation and characterization of the compound

The compound was obtained as white crystals. The HRESI-MS showed a pseudomolecular ion peak at *m/z* of 395.1479 (M+H)<sup>+</sup>, calcd. *m/z* 395.1473 for (C<sub>21</sub>H<sub>24</sub>NaO<sub>6</sub>), suggesting the molecular formula of the compound as C<sub>21</sub>H<sub>24</sub>O<sub>6</sub>. The IR spectrum of the compound exhibited a strong absorption peak for C-H stretching at 2951 cm<sup>-1</sup>, and strong absorption peaks for carbonyl groups at 1767, 1728, and 1674 cm<sup>-1</sup>. The UV spectrum of compound the showed absorption peaks at 665.5, 606.5, 409.5, and 206 nm.

The <sup>1</sup>H NMR spectrum (300 MHz, CDCl<sub>3</sub>) of the compound indicated the presence of two methyl protons at δ 0.83 (s, H<sub>3</sub>-20) and 3.61 (s, H<sub>3</sub>-21), six methylene protons which resonate between δ 1.10 – 4.35, two *sp*<sup>3</sup> methine protons that resonate at δ 2.74 (d, *J* = 12.1, H-10) and 3.25 (dd, *J* = 12.4, 4.3, H-8), four *sp*<sup>2</sup> methine protons that resonate at δ 6.74 (dd, *J* = 8.1, 2.5, H-3), 6.76 (d, *J* = 2.0, H-14), 7.44 (d, *J* = 2.0, H-15), and 8.03 (s, H-16) ppm.

The <sup>13</sup>C NMR spectrum (75 MHz) of compound displayed signals for 21 carbons. DEPT spectral data classified these carbons as six methine carbons, six methylene carbons, two methyl carbons, and seven non-protonated carbons. Analysis of the chemical shifts of <sup>13</sup>C NMR spectrum indicated the presence of four *sp*<sup>2</sup> methine carbons, two *sp*<sup>3</sup> methine carbons, six *sp*<sup>2</sup> methylene carbons, two methyl carbons, five *sp*<sup>2</sup> non-protonated carbons, and two *sp*<sup>3</sup> non-protonated carbons. The carbon resonances at δ 108.5 (C-14), 136.2 (C-3), 144.3 (C-15), and 147.1 (C-1) were *sp*<sup>2</sup> methine carbons. The carbon resonances at δ 128.6 (C-13) and 137.6 (C-4) were *sp*<sup>2</sup> non-protonated carbons. Moreover, carbon resonances at δ 169.0 (C-18), 174.0 (C-17), and 193.7 (C-12) were carbonyl carbons. The HSQC spectral data were used to assign the protons being attached to carbon (Table 1). The core structure of the compound was established by analyzing the <sup>1</sup>H-<sup>1</sup>H COSY HMBC, and NOESY spectral data.

**Table 1:** <sup>1</sup>H and <sup>13</sup>C NMR data of the compound in acetone-*d*<sub>6</sub>

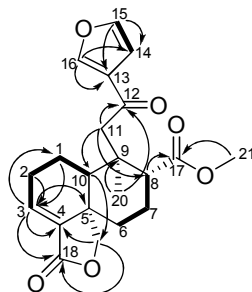
Nasimalun A		
Position	δ <sub>c</sub> (ppm)	δ <sub>H</sub> (ppm), multiplicity ( <i>J</i> in Hz)
1	20.0	1.10, (ddd, <i>J</i> = 11.7, 6.2, 4.2) 1.68 (m)
2	27.2	2.26 (m)
3	136.2	6.76 (dd, <i>J</i> = 8.1, 2.5)
4	137.6	-
5	45.0	-
6	33.1	1.38 (m) 2.00 (m)
7	22.0	1.90 (m) 2.05 (m)
8	48.6	3.25 (dd, <i>J</i> = 12.4, 4.3)
9	39.5	-
10	46.5	2.74 (d, <i>J</i> = 12.1) 2.85 (d, <i>J</i> = 17.8)
11	46.5	3.05 (d, <i>J</i> = 17.8)
12	193.7	-
13	128.6	-
14	108.5	7.74 (d, <i>J</i> = 2.0)
15	144.3	7.44 (d, <i>J</i> = 2.0)
16	147.1	8.03 (s)
17	174.0	-
18	169.0	-
19	71.4	3.95 (dd, <i>J</i> = 8.0, 1.4) 4.35 (d, <i>J</i> = 8.1)
20	19.1	0.83 (s)
21	51.4	3.61 (s)

300 MHz for <sup>1</sup>H and 75 MHz for <sup>13</sup>C, chemical shift (δ) in ppm

#### Establishment of a core structure of the compound

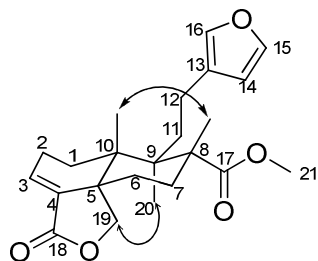
The <sup>1</sup>H-<sup>1</sup>H COSY spectrum of the compound established the fragments of H<sub>2</sub>-1/H<sub>2</sub>-2/H-3; H<sub>2</sub>-6/H<sub>2</sub>-7/H-8;

and H-14/H-15. The cyclic core structure of this compound was established by analyzing the HMBC correlations including H<sub>2</sub>-2 to C-3; H<sub>2</sub>-2 to C-4; H-3 to C-1, C-4, and C-18; H<sub>2</sub>-6 to C-8; H-8 to C-7, C-9, and C-20; H-10 to C-1, C-5, C-9, and C-11; H<sub>2</sub>-11 to C-8, C-9, and C-12; H-14 to C-12, C-13 and C-16; and H<sub>2</sub>-19 to C-4, C-5, C-6 and C-18 (Figure 1).



**Figure 1:** <sup>1</sup>H-<sup>1</sup>H COSY and HMBC correlations of a core structure of compound

The relative configuration of the compound was established by analysis of NOESY spectrum. The NOESY correlations between H-8 and H-10 which denoted that H-8 and H-10 were in the same plane. Furthermore, NOESY correlations between H<sub>2</sub>-19 and H<sub>3</sub>-20 suggested that H<sub>2</sub>-19 and H<sub>3</sub>-20 were co-planer (Figure 2).



**Figure 2:** NOESY correlations of a core structure of compound

On the basis of these spectral data and literature data comparison, this compound was identified as Nasimalun A, which was previously isolated from the roots of *Barringtonia racemosa*.<sup>12</sup>

**Table 2** Antibacterial activities of nasimalun A

Compound	MIC (µg/mL)		
	BC	SA	SE
Nasimalun A	50	12.5	100
Chloramphenicol	0.78	0.78	0.78
Tetracycline	6.25	0.78	1.56
Vancomycine	3.12	1.56	3.12

### 3.2 Biological activities of the compound

Nasimalun A showed oxygen radical absorbance capacity (ORAC) value of 0.2 and aromatase inhibitory activity (AIA) with an IC<sub>50</sub> value of 12.0 µM. Moreover, this compound showed moderate cytotoxicity toward only MOLT-

3 cell line with IC<sub>50</sub> value of 26.44 µg/mL, but it was not active against other cell lines. Furthermore, nasimalun A showed MIC of 50, 12.5, and 100 µg/mL for *B. cereus* (BC) and both *S. aureus* (SA), and *S. epidermidis* (SE) respectively (Table 2).

### 4. Conclusions

Natural products, especially the universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine.<sup>13</sup> Even though most of the diterpene compounds isolated from *Croton oblongifolius* Roxb. exhibited potent cytotoxic activities, nasimalun A showed moderate cytotoxicity. Compared to the standard drugs used in the determination of MIC, nasimalun A is not a potent antibacterial agent against the organisms tested.

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## Author Information

Kanchana Wijesekera is a Lecturer at the BPharm Degree Program, Faculty of Medicine, University of Ruhuna, Sri Lanka. She received her B.Sc. Honours in Chemistry in the year 2010 from University of Sri Jayewardenepura, Sri Lanka. She received the Post-Graduate Scholarship from Chulabhorn Graduate Institute, Thailand for a Master Degree in Chemical Biology. She completed her Master Degree research under the supervision of Dr. Prasat Kittakoop, Chulabhorn Graduate Institute, Thailand on the topic "Bioactive Compounds from the Endophytic Fungus *Dothideomycete* sp. CRI7 and a Plant *Croton oblongifolius* Roxb." in the year 2016.

