RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2006.32.017

Localization of Plaunotol in the Leaf of *Croton stellatopilosus* Ohba

Worapan Sitthithaworn,[a] Buppachart Potduang[b] and Wanchai De-Eknamkul[c*]

[a] Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Srinakharinwirot University, Nakornnayok 26120, Thailand.
[b] Department of Pharmaceuticals and Natural Products, Thailand Institute of Scientific and Technological Research, Bangkok 10900, Thailand.
[c] Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand.

* Corresponding author, E-mail: Wanchai.D@Chula.ac.th

Received 27 Sep 2005
Accepted 1 Nov 2005

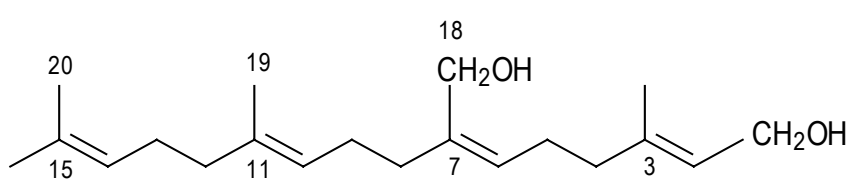
ABSTRACT: Plaunotol, a well-known protective factor-enhancing agent isolated from the leaves of *Croton stellatopilosus* Ohba (Thai name : Plau-Noi, known previously as *Croton sublyratus* Kurz), was shown to be accumulated in the chloroplasts of palisade mesophyll cells. This was confirmed by both detection of plaunotol in specific leaf tissues and examination of electron microscopic pictures of subcellular organelles of the plaunotol-containing tissue. The detection of plaunotol by a TLC-densitometric technique showed the presence of 95% of the plaunotol in the palisade cells and only about 5% in the spongy mesophyll cells. Inside the palisade mesophyll cells, the electron micrographs showed the presence of numerous oil globules in the chloroplasts. Since plaunotol is an acylic diterpenoid (C-20) and is nonpolar, it was suggested that the observed oil globules are sites of plaunotol accumulation.

KEYWORDS: *Croton stellatopilosus* Ohba, Euphorbiaceae, plaunotol, subcellular localization, chloroplasts, palisade mesophyll cells.

INTRODUCTION

Plaunotol **1**, the active ingredient of a commercial drug named Kelnac®, is a mucosal protective factor-enhancing antiulcer agent[1]. It was originally found in the stem and leaves of *Croton sublyratus* Kurz (Thai name : Plau-Noi)[2]. This plant was subsequently identified as *Croton stellatopilosus* Ohba[3]. Plaunotol is an acyclic diterpene alcohol with two hydroxyl groups attached to C-1 and C-18 of the molecule. Biosynthetically, we have recently reported that the four isoprene units of plaunotol are originated exclusively from the deoxyxylulose phosphate pathway[4]. No isoprene units derived from the mevalonate pathway are involved in the formation of this acyclic diterpenoid. This is contrast with the biosynthesis of β-sitosterol and stigmasterol which we have found to proceed via a mixed origin of isoprene units[5]. For subsequent biosynthetic steps, we have reported that plaunotol is formed from geranylgeraniol diphosphate (GGPP) by the action of two consecutive enzymes, namely GGPP phosphatase and geranylgeraniol-18-hydroxylase[6]. The discovery of the latter has led to a patent in the United States and suggests the possiblility of using the enzyme for plaunotol production[7].

Although the biosynthesis of plaunotol has been clarified at the enzyme level, the subcellular localization of these enzymes and the final product plaunotol has not yet been investigated. This information is important since it can lead to a better understanding of the whole picture of plaunotol biosynthesis in *C. stellatopilosus*. For this aspect, we have developed a rapid TLC-densitometric method for plaunotol detection and analysis[8]. This technique appears to be as sensitive as gas chromatographic method and allows plaunotol of less than 0.1 μg to be detected. This study aimed to



1

detect plaunotol in different tissue types of *C. stellatopilosus* leaf using the TLC-densitometric analytical technique. The tissue containing plaunotol was then observed by electron microscopy to examine characteristics of the organelles in the tissue.

Materials and Methods

Plant Material

The leaves of *C. stellatopilosus* Ohba (Euphorbiaceae) used in this study were obtained from plants growing in an open field at the Faculty of Pharmaceutical Sciences, Chulalongkorn University. A voucher specimen is deposited in the Herbarium, Royal Forest Department in Bangkok, Thailand under no. 21867.

Transmission Electron Microscopy

Leaf samples for electron microscopic observation were prepared according to the method described previously[9]. Young fresh leaves of *C. stellatopilosus* were cut into small pieces (1.0 x 1.0 mm) and fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4°C for 1 h. The specimens were post-fixed in 1% osmium tetroxide at 4°C for 2 h. They were then dehydrated in a graded series of acetone solutions (30%, 50%, 70%, and 90% 15 min each, and 100% 10 min 3 times) and infiltrated using a mixture of acetone and Spurr's medium at the ratios of 2:1 for 3 h, 1:2 for 3 h, and in pure Spurr's medium for another 3 h. The resulting samples were embedded in liquid paraffin wax for at least 8 h at 70°C. Blocks of specimens were sectioned at 60-90 nm thickness with an ultramicrotome. The sections were viewed and photographed under a JEOL JEM-200 CX TEM at 80 kV.

Separation of Leaf Tissues Containing Palisade and Spongy Mesophyll Cells

From a young fresh leaf of *C. stellatopilosus*, the upper layer of the leaf was removed using a pair of sharp forceps to separate the leaf into 2 parts, dorsal and ventral sides. The dorsal (upper) side of the leaf was obtained as small pieces of approximately 1 x 1 cm in size whereas the ventral side was obtained as the whole under-portion of the leaf. Observation under a light microscope indicated the presence of mainly palisade mesophyll cells in the dorsal part and mainly spongy mesophyll cells in the ventral part. The two leaf tissue parts were separated from five fresh leaves to give sufficient tissue for plaunotol analysis.

Detection of Plaunotol in Leaf Tissues

The presence of plaunotol in the leaf tissues was analyzed by the TLC-densitometric method described previously[8]. Leaf tissues obtained from dorsal sides, ventral sides, and from whole fresh leaves, were each extracted with 10 ml of boiling 95% ethanol. After filtering through Whatman no. 1 filter, 0.5 µl of each aliquot was spotted onto a TLC plate (silica gel 60 F_{254}, 0.2 mm thickness). The plate was developed three times using the same solvent system of chloroform : *n* propanol, 96:4 with 10 cm height of the solvent front. The TLC plate was then scanned using a TLC densitometer (Shimadzu Model CS-930) at a wavelength of 220 nm.

Results

Electron Microscopic Study of Leaf Tissues of *C. stellatopilosus*

Figure 1A illustrates a transverse section of a *C. stellatopilosus* leaf. It clearly shows that the cell arrangement of the leaf is dorsiventral (the upper and lower parts of the leaf have a different cell structures). It can be seen that beneath the upper epidermis is a layer of vertically elongated palisade mesophyll cells. The lower half of the leaf contains the spongy mesophyll, which is a loosely arranged network of cells of irregular shape and large air spaces. Below the spongy mesophyll

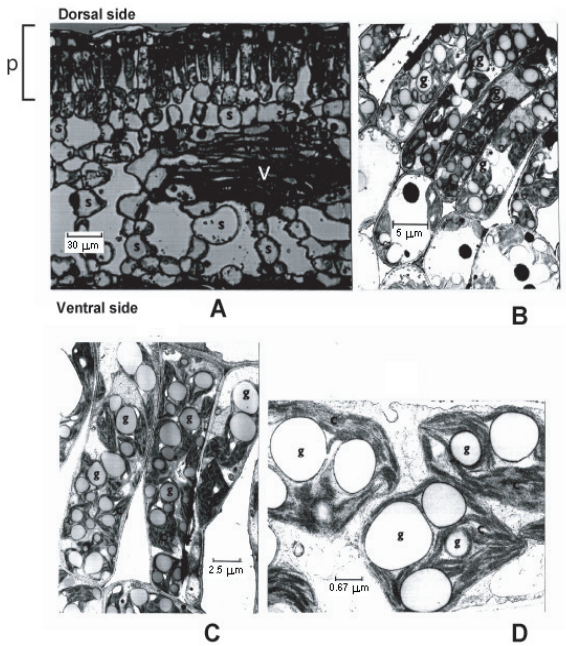


Fig 1. (A) A transverse section of a *C. stellatopilosus* leaf (x 400) showing palisade mesophyll cells (p), spongy mesophyll cells (s), a vein (v). (B) An electron micrograph (x 2,300) of palisade mesophyll cells showing number of oil-like globules (g). (C) Electron micrograph of palisade mesophyll cells with 3,800 times magnification. (D) Electron micrograph (x 15,200) showing oil globules (g) in the chloroplasts (c).

cells is the lower epidermis. The palisade mesophyll cells are packed with chloroplasts containing a number of oil globule-like structures (Fig. 1B). Similar subcellular structures were also found in the spongy cells connected with the palisade cells, although to a much lesser extent. At higher magnification, it was clear that the globule-like structures were located in the chloroplasts which are present densely inside the palisade cells (Fig. 1C). Each chloroplast appears to be able to accommodate 2-4 globules of various sizes (Fig. 1D).

Detection of Plaunotol in Leaf Tissues

Extraction and analysis of plaunotol in the whole leaf of *C. stellatopilosus* showed the presence of a plaunotol peak in the TLC-densitometric chromatogram with *Rf* value of 0.35 (Fig. 2A). With separate tissue parts of the upper epidermis and the lower half of spongy mesophyll tissues, it was found that most of plaunotol content present in the whole leaf was detected in the upper palisade mesophyll part (Fig. 2B). Only a small amount of the acyclic diterpenoid was detected in the spongy mesophyll tissues (Fig. 2C). Quantitatively, the content of plaunotol found in the palisade mesophyll cells appeared to be almost 95% of that found in the whole leaves (Table 1). This strongly suggested plaunotol is accumulated almost exclusively in the palisade cells of the leaves of *C. stellatopilosus*.

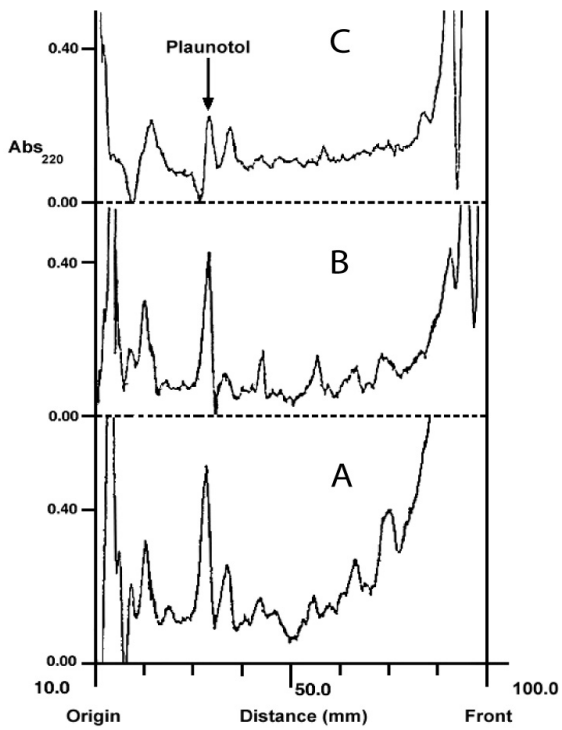


Fig 2. TLC-densitometric chromatograms of the ethanolic extracts of the whole leaf (A) palisade mesophyll tissue (B) and of spongy mesophyll tissue (C).

Table 1. Plaunotol content in the dorsal and ventral sides of *C. stellatopilosus* leaves compared with the content in whole leaves.

Leaf part	Plaunotol content* (mg per 5 leaves)	% Distribution
Whole leaf	1.60 ± 0.16	100.0
Dorsal side containing palisade mesophyll tissue	1.50 ± 0.20	94.5
Ventral side containing spongy mesophyll tissue	0.07 ± 0.03	4.5

* Each value represents mean ± SD of triplicate analysis

This result of chemical analysis was consistent with the EM observation described in Fig. 1. Since plaunotol itself is a hydrophobic diterpenoid which is well dissolved in non-polar solvents, one would expect that the accumulation of plaunotol in the leaves should be in the form of oil globules. Figure 1B clearly shows that the oil globules, presumably containing plaunotol, are present in the palisade cells. At the subcellular level, it was also clear that the oil globules were located in the chloroplasts (Fig. 1C - D).

Discussion

We have shown that the leaf of *C. stellatopilosus* has a characteristic cell arrangement belonging to the dorsiventral type with plaunotol accumulation mainly in chloroplasts of the palisade mesophyll cells. Under electron microscopic observation, the vertically elongated palisade mesophyll cells appear to be filled with chloroplasts. The chloroplasts are present in various sizes and each contains a few round-shaped structures similar to oil globules. The results obtained from this study suggest that these oil globules are very likely to be sites of plaunotol accumulation. This is due to the oily properties of plaunotol, which, structurally, has twenty carbons and two hydroxyl groups in an acyclic linear form. The presence of a high number of oil globules in the palisade mesophyll cells is also consistant with the high content of plaunotol (0.8%) found in the leaf as reported previously[8].

The biosynthesis of diterpenoids in higher plants has recently been proposed to occur with their isoprene units originating via a new mevalonate-independent route, namely the deoxyxylose phosphate (DXP) pathway[10]. This DXP pathway has been reported to operate in the chloroplasts for the formation of terpenoids required for the photosynthetic machinery (eg. phytol, plastoquinone etc.) and the groups of mono- and diterpenoids[11]. Our finding that plaunotol which is an acyclic diterpenoid accumulated in the chloroplasts, therefore, supports this proposed

concept.

It should be noted that the last two enzymes involved in the biosynthesis of plaunotol, namely GGPP phosphatase[12] and geranylgeraniol-18-hydroxylase, have been found in the 20,000 g pellet fraction of *C. stellatopilosus* leaves[7]. The two membrane-bound enzymes have been shown to convert geranylgeranyl diphosphate effectively to plaunotol. Based on these results and our present study, it is very likely that the 20,000 g pellet fraction containing the two enzymes might be a part of the membrane system of the chloroplast. This aspect of the subcellular localization of the enzymes is being investigated in our laboratory.

Acknowledgements

This work was supported by the Thailand Research Fund and The National Center for Genetic Engineering and Biotechnology (BIOTEC).

References

1. Ogiso A, Kitazawa E, Kobayashi S, Komai T, Matsunuma N, Kataumi S (1985) Plaunotol (CS-684), a new antipeptic ulcer agent. *Sankyo Kenkyusho Nempo,* **37**, 1-39.
2. Ogiso A, Kitazawa E, Mikuriya I, Promdej C (1978) Isolation and structure of antipeptic ulcer diterpene from Thai medicinal plant. *Chem Pharm Bull*, **26**, 3117-23.
3. Esser J, Chayamarit K (2001) Two new species and a new name in Thai *Croton* (Euphorbiaceae). *Thai Forest Bulletin (Bot),* **29**, 51-7.
4. Wungsintaweekul J, De-Eknamkul W (2005) Biosynthesis of plaunotol in *Croton stellatopilosus* proceeds via the deoxyxylulose phosphate pathway. *Tetrahedron Lett,* **46**, 2125-8.
5. De-Eknamkul W and Potduang B (2003) Biosynthesis of bð-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene units. *Phytochemistry*. **62**, 389-98.
6. Tansakul P, De-Eknamkul W (1998) Geranylgeraniol-18-hydroxylase : The last enzyme on the plaunotol biosynthesis in *Croton sublyratus*. *Phytochemistry*, **47**, 1241-6.
7. De-Eknamkul W, Tansakul P (1999) Geranylgeraniol-18-hydroxylase from *Croton sublyratus*. *United States Patent*, no. 5, 879, 916, Mar 9, 1999.
8. Vongchareonsathit A, De-Eknamkul W (1998) Rapid TLC-densitometric analysis of plaunotol from *Croton sublyratus* leaves. *Planta Medica,* **64**, 279-80.
9. Glauert A M (1984) *Fixation, Dehydration and Embedding of Biological Specimens (Practical Methods in Electron Microscopy)*, 208 pp. North-Holland Publishing Company, Amsterdam, the Netherlands.
10. Arigoni D. and Schwarz M (1999) In: *Comprehensive Natural Product Chemistry* Vol. 2 (Edited by Cane D), pp 45-68. Pergamon, Oxford, UK.
11. Schwender J, Zeidler J, Groner R, Muller C, Focke M, Braun S, Lichtenhaler F W, Lichtenhaler K (1997) Incorporation of 1-deoxy-D-xylulose into isoprene and phytol by higher plants and algae. *FEBS Lett.* **414**, 129-34.
12. Nualkaew N, De-Eknamkul W, Kutchan T, Zenk M H (2005) Geranylgeraniol formation in *Croton stellatopilosus* proceeds via successive monodephosphorylations of geranylgeranyl diphosphate. *Tetrahedrol, Lett*, **46**, 8727-8731.