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Biosynthesis of β-sitosterol and stigmasterol proceeds exclusively via the mevalonate pathway in cell suspension cultures of *Croton stellatopilosus*

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Abstract

Cell suspension cultures of *Croton stellatopilosus* were fed with [1-^{13}C]glucose and [2-^{13}C]sodium acetate and cultured under control conditions. β-Sitosterol and stigmasterol were isolated and their ^{13}C-labeling patterns examined using quantitative NMR spectroscopy. Analysis of the patterns of ^{13}C-enrichment revealed that all the isoprene units in the molecules of both phytosterols originated exclusively from the mevalonate pathway. These results were in contrast with our previous study using callus cultures of *C. stellatopilosus*, which showed that the isoprene units of β-sitosterol and stigmasterol were supplied equally from both the deoxyxylulose phosphate (DXP) pathway and the mevalonate pathway. Observation by transmission electron microscopy of sub-cellular structures of both cell types revealed that the callus cells contained partially differentiated chloroplasts, whereas the suspension cultured cells did not. Since the DXP pathway is known to be located in the chloroplasts, it was suggested that the presence of chloroplasts is essential for expression of the DXP pathway. Therefore, the sole operation of the phytosterol biosynthesis by the mevalonate pathway observed in this study was likely to be the result of non-expression of the DXP pathway in the chloroplast-free cell suspension cultures of *C. stellatopilosus*.

Keywords: *Croton stellatopilosus*; Cell suspension culture; β-Sitosterol; Stigmasterol; Feeding experiments; The mevalonate pathway

β-Sitosterol is a phytosterol distributed widely throughout the plant kingdom and is known to be involved in the stabilization of cell membranes.[1] Closely related to β-sitosterol is stigmasterol which possesses an additional unsaturation in the side-chain of the molecule, a feature seen in many plant sterols.[2] The biosynthesis of these phytosterols in plant cells has demonstrated that their isoprene units are supplied exclusively from the mevalonate (MVA) pathway which is located in the cytoplasm.[3] On the other hand, isoprene units of the terpenoid groups of monoterpenes, diterpenes and tetraterpenes are biosynthesized predominantly from the deoxyxylulose phosphate (DXP) pathway located in the plastid.[4] The first study reporting the co-existence of both pathways in higher plants was the incorporation experiments of various labeled glucoses in *Ginkgo biloba* seedlings.[4] Ginkgolides appeared to be formed preferentially from the DXP pathway, whereas phytosterols were formed from the MVA pathway. Thereafter, a number of studies on the origin of isoprene units of plant terpenoids and steroids have been reported, for example, *Catharanthus roseus*,[5] *Daucus carota*, *Hordeum vulgare*, *Lemna gibba*,[6] *Mentha piperita*[7], and *Nicotiana tabacum*.[8]

In our laboratory, the Thai medicinal plant, *Croton stellatopilosus* (formerly known as *C. sublyratus*[9]), has been used as a model for biosynthetic studies on isoprene origin. This plant has been reported to contain a variety of

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terpenoid compounds, especially the anti-peptic acyclic diterpene, plaunotol which appears to be a major constituent of the leaves.[10] In vitro cultures of *C. stellatopilosus* including callus[11] and cell suspension[12] cultures have been established in order to observe their potential for terpenoid biosynthesis in comparison with the whole plant system. However, we found that our callus culture accumulates considerable amounts of β-sitosterol and stigmasterol, with no detectable amount of plaunotol.[11] Feedings of [1-^{13}C]glucose into the callus culture have shown that the labeling patterns of the two phytosterols are the result of a mixed origin of isoprene units from both the MVA pathway and the DXP pathway.[11] With young whole leaves of *C. stellatopilosus*, on the other hand, feeding of the same labeled glucose clearly showed that the isoprene units in the skeleton of plaunotol originated exclusively from the DXP pathway.[13]

In this Letter, we report our research on the cell suspension cultures of *C. stellatopilosus* with, again, emphasis on the results of incorporation experiments using two different labeled precursors of [1-^{13}C]glucose and [2-^{13}C]sodium acetate. We also compare the results obtained with our previous work on the callus cultures[11] which were carried out under similar experimental conditions and using the same source of plant material of *C. stellatopilosus*. The suspension culture was induced as described previously.[12] It was maintained in Murashige and Skoog (MS) medium[14] supplemented with 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg/L of benzyladenine (BA) and 3% w/v sucrose. Growth curves and product formation during the growth cycle of the suspension culture were first established. Thus, fresh cells of various growth phases were harvested, weighed and 2 g of the biomass was extracted with 5 ml of 95% (v/v) ethanol by heating at 60 °C for 1 h. The filtrate was evaporated and re-dissolved in 2 ml of 50% (v/v) ethanol. A solution of 10% (w/v) NaOH was added to a final concentration of 1% (v/v) and the mixture was heated at 70 °C for 30 min. The lipophilic components in the solution were partitioned with *n*-hexane (3 ml each, ×5) in order to examine whether there were any terpenoids or phytosterols produced by the cultured cells. The *n*-hexane fraction, after being concentrated, was then subjected to GC analysis.

Using a chemically bonded fused silica capillary column of methylsiloxane (HP1, 30 m × 0.32 mm i.d., 0.25 μm film thickness), GC separation of some acyclic terpenoids and phytosterols was achieved with the temperature program being set from 220 °C (hold 3 min) to 280 °C (rate 10 °C/min and hold 16 min), injector temperature 280 °C, oven temperature 300 °C, detector temperature 280 °C, sample size 1.0 μl, flow 1.0 ml/min helium and splitless injection. Under these conditions, the elution times of the non-polar compounds: phytol, plaunotol, geranylgeraniol (GGOH), campesterol, stigmasterol and β-sitosterol were 6.1, 6.2, 6.4, 17.9, 18.6, and 20.1 min, respectively. Based on this GC analysis, growth curves and product formation curves of *C. stellatopilosus* cell cultures were obtained

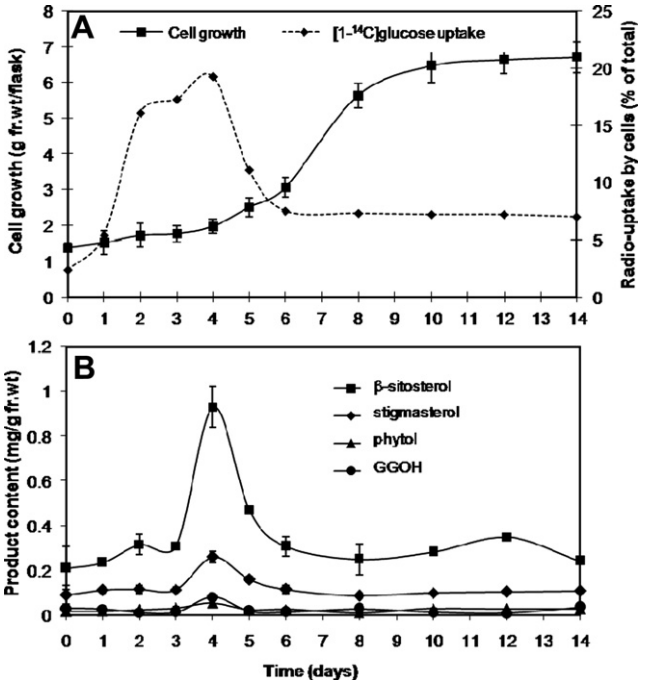

Fig. 1. Growth curve (A) and product formation curves (B) during a 14-day culture cycle of *C. stellatopilosus* cell suspension cultures. The ability of the cultured cells to take up glucose was monitored by radioactively labeled [1-^{14}C]glucose uptake (A).

(Fig. 1). It can be seen that the cultured cells produced relatively high quantities of β-sitosterol and stigmasterol, compared to the very low levels of GGOH and phytol. No plaunotol was detected in the cell culture although its immediate precursor, GGOH, was formed during the culture cycle. Interestingly, the formation of all the products showed their peaks of maximal contents on day 4, the period during which the cultured cells are entering their rapid growth phase (Fig. 1). This suggested that these compounds are involved in the primary metabolism relating to cell growth, especially the phytosterols which are known to be components of plant cell membranes.[15]

For incorporation experiments, preliminary studies were first carried out with radioactively labeled [1-^{14}C]glucose in order to monitor the efficiency of glucose the uptake into the cultured cells. [1-^{14}C]glucose with 3.3×10^{-3} μCi/ml (specific activity of 56.0 mCi/mmol, Amersham, Buckinghamshire, England) was fed into the freshly subcultured cells. The suspension culture was grown at 25 ± 2 °C, in the light (16 h/day), with shaking at 120 rpm. Cells were harvested during the culture cycle by centrifugation. The radioactivities present both in the cells and the supernatant were counted by liquid scintillation. The percentage of radio-uptake by the cells during the culture cycle was calculated based on the distribution of the radioactivity in the cells and the medium. The results showed that uptake of [1-^{14}C]glucose by the cells was highest from day 2 to day 4 with 16% to 20% uptake compared with less than 7% for the other period of cell growth (Fig. 1). Based on these results and the time-course of product formation, we

decided to perform the labeling experiments with freshly cultured cells, and allowed the uptake and metabolic conversion of the labeled precursors to run for 4 days. Two kinds of stable isotopic precursors, [1-^{13}C]glucose and [2-^{13}C]sodium acetate, were used in the experiments for comparison of their label incorporation. After the feedings, the cultured cells were harvested and the phytosterols were isolated for the analysis of their ^{13}C-labeling patterns.

[1-^{13}C]Glucose (20% w/v, 99% ^{13}C enrichment in 80% w/v of unlabeled D-glucose) and [2-^{13}C]sodium acetate (50% w/v, 99% ^{13}C enrichment in 50% w/v of unlabeled sodium acetate)(Cambridge Isotope Laboratories, Inc, USA) were fed separately into *C. stellatopilosus* suspension cultures. For the control, separate feedings with unlabeled glucose and sodium acetate as cold precursors were also carried out in parallel. After day 4, the cells from these feedings were harvested (750–820 g fresh weight obtained). Isolation of the phytosterols containing β-sitosterol and stigmasterol was performed by extracting the cells with 700 ml of 95% ethanol (Merck KGaA) by heating at 60 °C for 1 h. The extracts were filtered and evaporated to dryness under vacuum. The residues were then re-dissolved in 50% (v/v) ethanol in the presence of 1% (v/v) NaOH and heated at 60 °C for 30 min. After cooling to room temperature, the extracts were partitioned five times with 150 ml of *n*-hexane. The *n*-hexane layers were combined and concentrated to dryness under reduced pressure. The crude residue was dissolved in *n*-hexane and further purified by column chromatography on silica gel 60 eluting with dichloromethane: methanol; 98:2. Fractions were analyzed by TLC on using dichloromethane: methanol; 98:2 as mobile phase and a mixture of anisaldehyde/H_2SO_4 as a visualization reagent. The R_f values of both the phytosterols, β-sitosterol and stigmasterol, were 0.42. The fractions containing the phytosterols were combined and re-crystallized from chloroform: methanol; 7:3, to afford 120.2 mg

Table 1
^{13}C NMR analysis and relative ^{13}C-enrichment of β-sitosterol and stigmasterol from *C. stellatopilosus* cell suspension cultures after being fed with [1-^{13}C]glucose and unlabeled glucose (control) under the same conditions

Carbon no.[a]	Chemical shift (ppm)		^{13}C-signal intensity[b] (based on integration C20 = 1.00)				Enrichment ratio	
	β-Sitosterol	Stigmasterol	[1-^{13}C]Glucose		Unlabeled glucose		β-Sitosterol L/U	Stigmasterol L/U
			β-Sitosterol (L)	Stigmasterol (L)	β-Sitosterol (U)	Stigmasterol (U)		
1*	37.21		2.05		1.22		1.68	1.68
2*	31.62		1.16		1.36		0.85	0.85
3*	71.80		2.27		1.57		1.45	1.45
4	42.18	42.29*	0.32	2.45	0.32	2.04	1.00	1.20
5*	140.72		1.21		0.74		**1.68**	**1.68**
6*	121.71		1.18		1.34		0.85	0.85
7*	31.87		3.72		3.01		**1.45**	**1.45**
8*	31.87		3.72		3.01		2.45	0.32
9*	50.08		2.28		1.48		**1.64**	**1.64**
10*	36.48		0.83		0.90		0.88	0.88
11*	21.07		0.29		0.28		1.24	1.24
12	39.73	39.64	0.92	0.33	1.06	0.35	0.87	0.94
13*	42.29		2.45		2.04		1.20	1.20
14	56.73	56.83	0.93	0.15	1.04	0.25	0.89	0.60
15	24.28	24.34	1.62	0.35	0.98	0.28	**1.65**	**1.25**
16	28.23	28.92	1.06	0.18	1.13	0.28	0.94	0.64
17	56.00	55.90	1.27	0.28	0.70	0.22	**1.81**	**1.27**
18	11.84	12.03	1.85	0.40	1.10	0.34	**1.68**	**1.18**
19*	19.38		1.93		1.21		**1.60**	**1.60**
20	36.12	40.50	1.00	0.18	1.00	0.19	1.00	0.95
21	18.75	21.05	1.31	1.34	0.76	1.10	**1.72**	**1.22**
22	33.90	138.32	1.76	0.33	0.98	0.23	**1.80**	**1.43**
23	25.99	129.22	0.77	0.16	0.84	0.22	0.92	0.73
24	45.78	51.21	1.84	0.32	1.07	0.22	**1.72**	**1.46**
25	29.08	31.87*	0.88	3.72	0.97	3.01	0.91	1.24
26	19.81	21.20	1.71	0.42	0.92	0.31	**1.86**	**1.35**
27	19.00	18.95	1.99	0.44	0.86	0.31	**2.31**	**1.42**
28	23.02	25.40	1.39	0.17	0.90	0.16	**1.54**	1.06
29	11.96	12.25*	1.49	0.27	0.90	0.20	**1.65**	**1.35**

* The carbons with signals overlapping either the same carbon of the two phytosterols (carbon nos. 1–3, 5–11, 13 and 19) or of different carbons (carbon nos. 4 and 25 of stigmasterol with carbon nos. 13 and 7 + 8 of both phytosterols, respectively).

[a] Assignments are based on the values of β-sitosterol and stigmasterol reported by Wright et al.[16] and the assigned carbons are shown in the structure of Figure 2.

[b] Each intensity value was obtained based on the intensity of C20 of β-sitosterol (set to 1.0) which is not involved in the label incorporation either from the MVA pathway or DXP pathway.

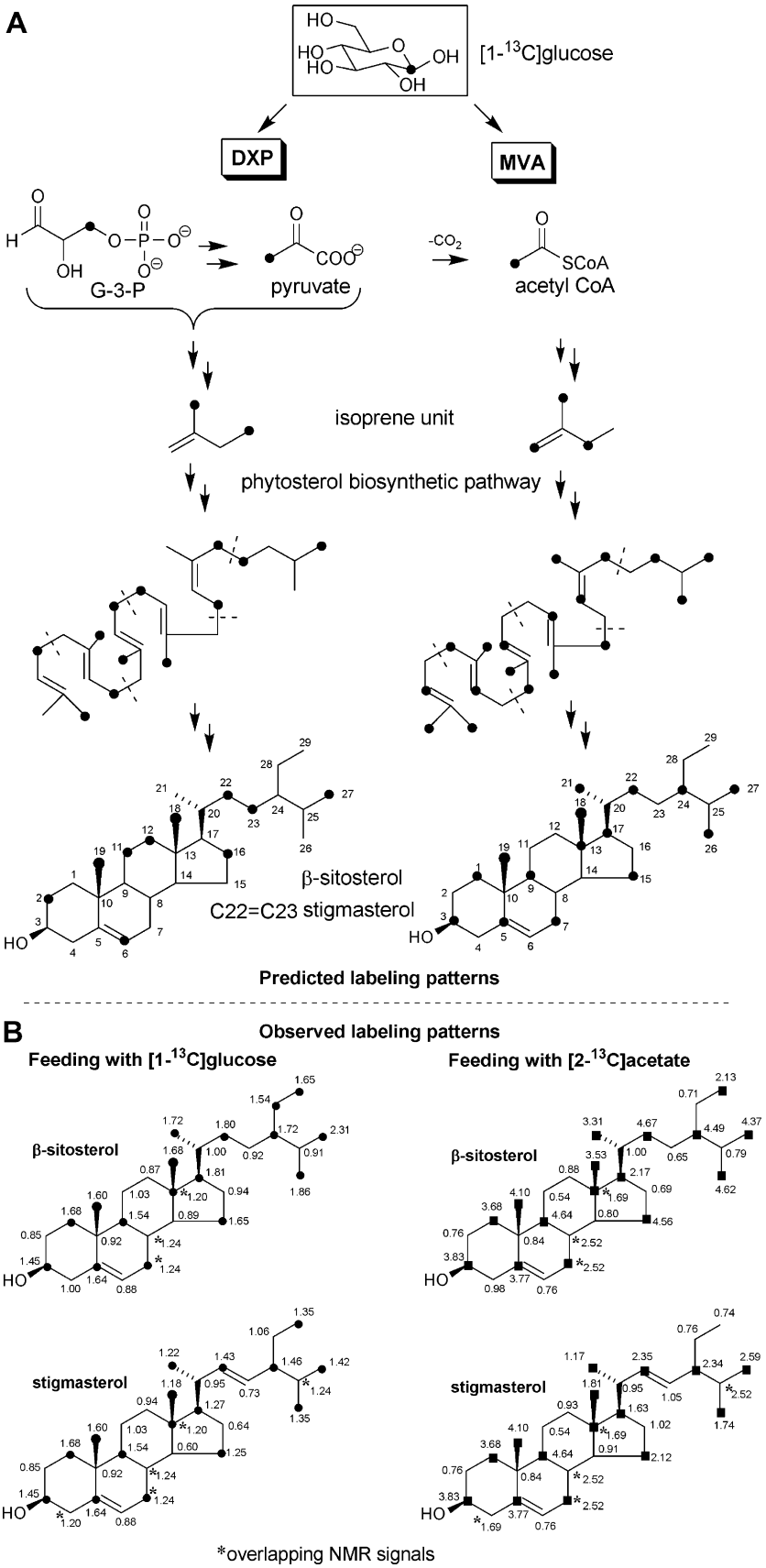

Fig. 2. Comparison of the ^{13}C-labeling patterns of *C. stellatopilosus* phytosterols between the predicted patterns based on [1-^{13}C]glucose conversion via either the DXP pathway or the MVA pathway (A) and the actual labeling patterns obtained from the incorporation experiments using both [1-^{13}C]glucose (labeled by ●) and [2-^{13}C]sodium acetate (labeled by ■) (B). The value indicated at a particular carbon is the ^{13}C-enrichment ratio obtained from Table 1. The value of the carbon denoted * is the summation of NMR-signal overlapping of different carbons.

and 102.6 mg of phytosterols for [1-^{13}C]glucose and [2-^{13}C]sodium acetate feedings, respectively.

Structural analysis of both the labeled and unlabeled phytosterols was performed by ^{13}C NMR spectroscopy (125 MHz $CDCl_3$). The resulting isolate was, again, identified as a mixture of β-sitosterol and stigmasterol as reported previously.[11,16] The ^{13}C-NMR spectra obtained from the incorporation experiments using [1-^{13}C]glucose and [2-^{13}C]sodium acetate (99% ^{13}C abundance) and unlabeled glucose and sodium acetate (1.1% ^{13}C natural abundance) were recorded under the same conditions. The NMR signals were separately integrated for each carbon atom. Quantitative analysis on the degree of ^{13}C-enrichment of each carbon was then calculated by using the ratio between the ^{13}C-signal intensity of the ^{13}C-labeled isolates (label feedings) and the ^{13}C-signal intensity of the unlabeled isolates (unlabel feedings) as described previously.[11] These values of relative ^{13}C-signal intensity were normalized using the peak intensity of the C20 carbon of β-sitosterol since this carbon is not involved in any labeling process either by the MVA pathway or by the DXP pathway.

Table 1 shows the results of the ^{13}C-enrichment of β-sitosterol and stigmasterol obtained from the feedings with [1-^{13}C]glucose and [2-^{13}C]sodium acetate in comparison to their parallel unlabeled feedings. Analysis of the labeling patterns suggested that the common skeleton of β-sitosterol and stigmasterol is derived exclusively from the MVA pathway. This phytosterol skeleton can be predicted to have different labeling patterns from the labeled glucose depending on whether its biosynthesis proceeds via the DXP pathway or the MVA pathway (Fig. 2). Our [1-^{13}C]glucose feedings showed clearly that the resulting labeling patterns of the two phytosterols (with enrichment 1.2–2.3-fold for β-sitosterol and 1.2–1.5-fold for stigmasterol) were consistent with isoprenoid origin from the MVA pathway. This result was confirmed by the incorporation experiment using [2-^{13}C]sodium acetate which also showed the same labeling pattern (with enrichment 1.7–4.7-fold for β-sitosterol and 1.3–3.0-fold for stigmasterol) (Fig. 2). It should also be noted that C28 and C29 of β-sitosterol and stigmasterol which are not part of the isoprene unit were also labeled. It is likely that the labels are the result of removal of the two labeled methyl groups of C4 followed by re-utilization of the same labeled carbons to form C28 and C29 during the course of phytosterol biosynthesis as proposed previously.[17]

The results from this study, which shows clearly the sole operation of the MVA pathway in *C. stellatopilosus* cell suspensions, were in contrast with those found previously from its callus culture.[11] In that case, the isoprene units in the molecules of β-sitosterol and stigmasterol appeared to be supplied equally from both the MVA and DXP pathways. This raised a question on the potential expression of the DXP pathway in non-differentiated tissues, especially on the relationship between the structural development of chloroplasts, the location site of the DXP pathway,[4] and the pathway expression. To answer this question, the cultured cells and callus tissue samples were prepared[18] for electron microscopic observation. The results showed clearly that the callus cells contained partially developed chloroplasts (Fig. 3A and B), whereas the cell suspension contained no such organelle (Fig. 3C and D). It is, therefore, likely that cellular differentiation toward chloroplast development is a prerequisite for the expression of the DXP pathway, and might be the reason for the observed results of the mixed isoprene origin in the callus cultures and the sole mevalonate pathway in the cell suspension cultures. To confirm this conclusion, comparative studies with respect to gene expression of the DXP pathway in various cell types with different degrees of differentiation (e.g., cell suspension, callus culture, and whole plant tissues), and their chloroplast development are underway in our laboratory.

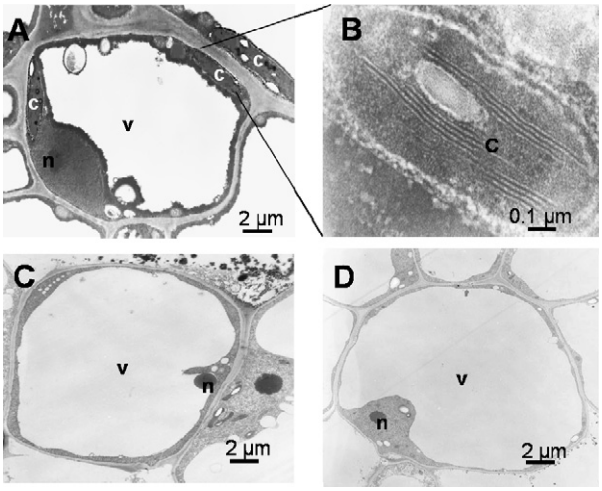

Fig. 3. Electron micrographs of in vitro cultures of *C. stellatopilosus*: (A) a callus cell,(×4,000) and (B) its magnified chloroplast (×60,000), (C) and (D) suspension cultured cells (×4,000). v, vacuole; n, nucleus; and c, chloroplast.

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References and notes

1. Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals*; Interdruck Graphischer Großbetrieb: Leipzig, 1984; pp 211–213.
2. Dewick, P. M. *Medicinal Natural Products: A Biosynthetic Approach*; John Wiley and Sons, Ltd: West Sussex, 2001; pp 252–255.
3. Arigoni, D.; Schwarz, M. In *Comprehensive Natural Product Chemistry*; Cane, D., Ed.; Pergamon: Oxford, 1999; Vol. 2, pp 45–68.
4. Schwarz, M. K. Ph.D. Thesis Nr. 10951, ETH, Zurich, Switzerland, **1994**.

5. Arigoni, D.; Sagner, S.; Latzel, C.; Eisenreich, W.; Bacher, A.; Zenk, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10600–10605.
6. Lichtenthaler, H. K.; Schwender, J.; Disch, A.; Rohmer, M. *FEBS Lett.* **1997**, *400*, 271–274.
7. Eisenreich, W.; Sagner, S.; Zenk, M. H.; Bacher, A. *Tetrahedron Lett.* **1997**, *38*, 3889–3892.
8. Disch, A.; Schwender, J.; Müller, C.; Lichtenthaler, H. K.; Rohmer, M. *Biochem. J.* **1998**, 381–388.
9. Esser, J.; Chayamarit, K. *Thai For. Bull. Bot.* **2001**, *29*, 51–57.
10. Ogiso, A.; Kitazawa, E.; Kurabayashi, M.; Sato, A.; Takahashi, S.; Noguchi, H.; Kuwano, H.; Kobayashi, S.; Mishima, H. *Chem. Pharm. Bull.* **1978**, *26*, 3117–3123.
11. De-Eknamkul, W.; Potduang, B. *Phytochemistry* **2003**, *62*, 389–398.
12. Wungsintaweekul, J.; Sriyapai, C.; Kaewkerd, S.; Tewtrakul, S.; Kongduang, D.; De-Eknamkul, W. *Z. Naturforsch.* **2007**, *62C*, 389–396.
13. Wungsintaweekul, J.; De-Eknamkul, W. *Tetrahedron Lett.* **2005**, *46*, 2125–2128.
14. Murashige, T.; Skoog, F. *Physiol. Plant.* **1962**, *15*, 473–497.
15. Hartmann, M. A.; Benveniste, P. *Methods Enzymol.* **1987**, *148*, 632–650.
16. Wright, J. L. C.; Mc Innes, A. G.; Shimizu, S.; Smith, D. G.; Walter, J. A.; Idler, D.; Khalil, W. *Can. J. Chem.* **1978**, *56*, 1898–1903.
17. Brown, G. D. *Nat. Prod. Rep.* **1998**, *15*, 653–696.
18. Glauert, A. M. *Fixation, Dehydration and Embedding of Biological Specimens*; North-Holland: Amsterdam, The Netherlands, 1984; p. 208.