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Biosynthesis of β-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene units

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

A green callus culture of *Croton sublyratus* Kurz established from the leaf explants appeared to actively synthesize two well-known phytosterols, β-sitosterol and stigmasterol. The phytosterol biosynthesis was highly active during the linear phase of the culture. Feeding of [1-^{13}C]glucose into the callus culture at this growth phase showed that the label from glucose was highly incorporated into both phytosterols. Isolation of the labeled products followed by ^{13}C NMR analysis revealed that the phytosterols had their ^{13}C–labeling patterns consistent with the acquisition of isoprene units via both the mevalonate pathway and the deoxyxylulose pathway with relatively equal contribution. Since the biosynthesis of phytosterol has so far been reported to be mainly from the classical mevalonate pathway, this study provides a new evidence on the biosynthesis of phytosterols via the novel deoxyxylulose pathway.

Keywords: *Croton sublyratus* Kurz; Euphorbiaceae; Callus culture; Mevalonate pathway; Deoxyxylulose pathway; β-Sitosterol; Stigmasterol; Mixed isoprene units

1. Introduction

β-Sitosterol (**1**) and stigmasterol (**2**) are two major phytosterols generally present in plant cell membranes. Inspite of their similarity in structure, β-sitosterol has been shown to be a membrane reinforcer whereas stigmasterol is not (Krajewsky-Bertrand et al., 1992; Schuler et al., 1991). Biosynthetically, it has been proposed recently that the isoprene building blocks of the phytosterols are originated mainly from the mevalonate pathway rather than the deoxyxylulose pathway (the non-mevalonate pathway) which has been shown to be involved in the biosynthesis of various terpenoid natural products (reviewed in Eisenreich et al., 1998; Rohmer, 1999). The involvement of the mevalonate pathway in the phytosterol biosynthesis has been demonstrated in a number of cases. It has been found that mevalonate is utilized efficiently for sterol biosynthesis in duckweed (*Lemna gibba*) whereas 1-deoxy-D-xylulose is incorporated poorly (Schwender et al., 1997). Use of [1-^{13}C] glucose as precursor has shown that three higher plants, duckweed, barley (*Hordeum vulgare*) and carrot (*Daucus carota*) possess two distinct routes for isoprene biosynthesis: one produces their cytoplasmic sterols via the mevalonate pathway and the other produces their chloroplast-bound terpenoids via the deoxyxylulose pathway (Lichtenthaler et al., 1997). In *Catharanthus roseus* cell cultures, it has been shown that label from 1-deoxy-D-xylulose is incorporated mainly into phytol and carotenoids with a poor incorporation into sitosterol (Arigoni et al., 1997).

This paper describes labeling experiments with [1-^{13}C] glucose using a green callus culture of *Croton sublyratus*. The data show that the labeled glucose is efficiently incorporated into β-sitosterol and stigmasterol and that the two distinct isoprene biosynthetic pathways are involved to a similar extent in the formation of the two phytosterols.

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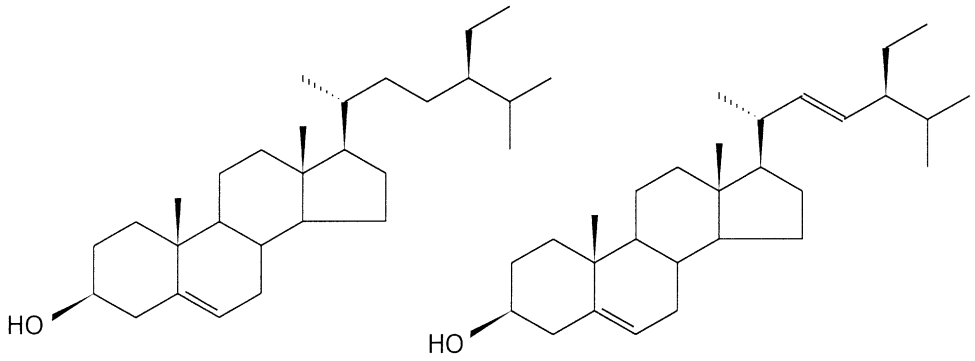

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2. Results and discussion

A green callus culture of *C. sublyratus* used in this study appeared to produce significant amount of phytosterols which could be detected by a simple technique of TLC-densitometry. The resulting TLC-densitochromatogram of the non-polar fraction extracted from the green callus showed the presence of a dominant peak co-chromatographed with authentic β-sitosterol (Fig. 1A). No plaunotol which is an antipeptic ulcer diterpenoid produced in *C. sublyratus* plant (Ogiso et al., 1978) was detected in this callus culture although either gellen gum or agarose was tried to add into our MS medium. These gelling agents have been shown to induce plaunotol formation in the callus culture of this plant (Morimoto and Murai, 1989). In addition, no geranylgeraniol which has been reported to be an immediate precursor of plaunotol (Tansakul and De-Eknamkul, 1998) was detected in our callus culture. This acyclic diterpenoid has been detected in *C. sublyratus* cell

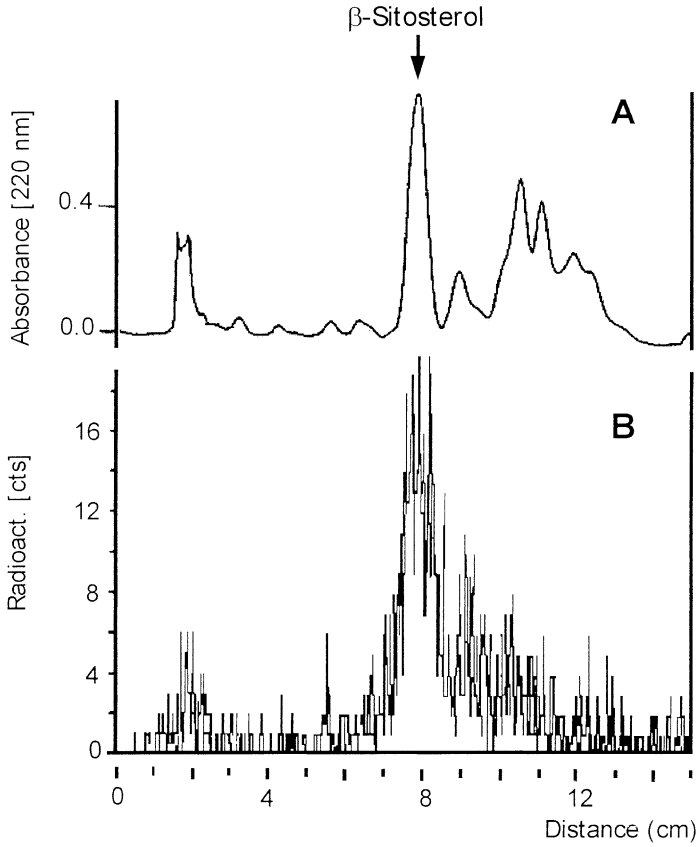

Fig. 1. TLC-densitometric chromatogram (A) and TLC-radiochromatogram (B) of the same non-polar fraction extracted from 20-day-old callus cultures of *Croton sublyratus* after being fed with 1 μCi [1-^{14}C]glucose solution for 3 days.

suspension cultures maintained in B5 medium (Kitaoka et al., 1989). Therefore, it seemed that the phytosterol biosynthetic pathway rather than the diterpenoid pathway was highly expressed in the green callus cultures of *C. sublyratus* and it is, thus, a good material for studying the biosynthesis of phytosterols.

Preliminary feeding experiments with radioactively labeled [1-^{14}C]glucose was first carried out in order to observe the efficiency of glucose to be incorporated into the molecule of β-sitosterol. By TLC-radioscan, it was found that the radiochromatogram of the same non-polar fraction also showed a major peak of radio-intensity at

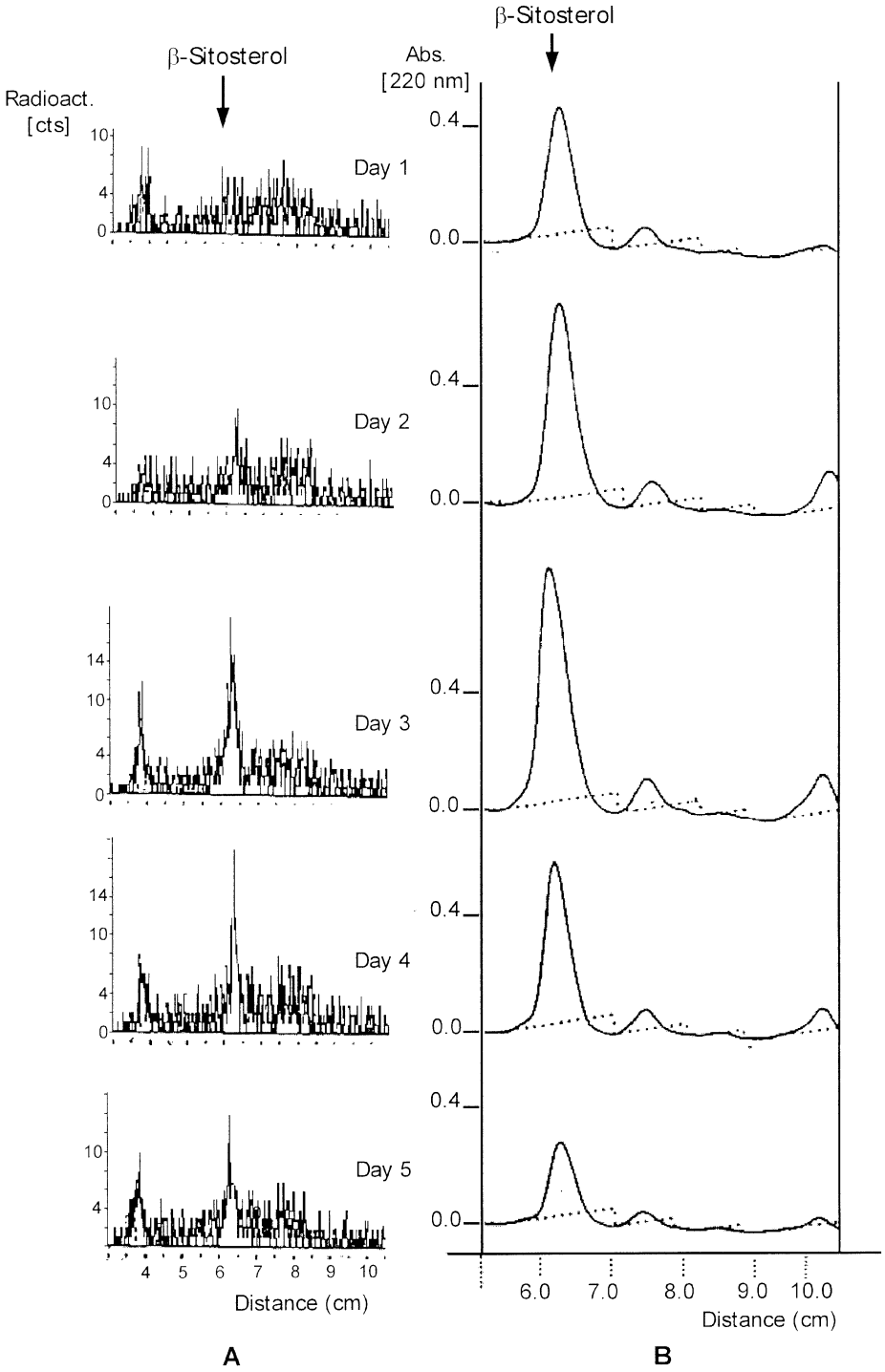

Fig. 2. TLC-radiochromatograms (A) and TLC-densitometric chromatograms (B) of the same organic fractions obtained from a time-course study of glucose incorporation (8 mg unlabeled glucose) into the phytosterol peak. One μCi of [1-^{14}C]glucose was added to the glucose solution for radio-monitoring of the glucose incorporation. The change of phytosterol level in the callus cultures during glucose feeding was monitored by TLC-densitometry.

the same position as β-sitosterol (Fig. 1B). This suggested that the label from [1-^{14}C]glucose was effectively incorporated into the molecule of β-sitosterol in *C. sublyratus* callus cultures. It should be noted that these incorporation experiments were carried out by feeding the callus tissues with the solution containing only [1-^{14}C]glucose (1 μCi). This condition was found to be more effective for glucose uptake and incorporation than the condition of using MS medium for the glucose feeding. Whether a nutritional stress occurring during the feeding period had an effect on the high label incorporation into β-sitosterol is still not clear.

Subsequently, feeding of high amount of glucose into the same green callus (8 mg glucose/5 mg callus) was performed in order to observe the optimal period of glucose incoporation into the phytosterol. In this experiment, 5 g of 20-day-old callus tissues were put into a 50-ml sterile jar containing 2 ml 0.4% (w/v) glucose. Radioactively labeled [1-^{14}C]glucose (1 μCi) was also added to the glucose solution for time-course monitoring. It was found that more than 90% glucose was taken up by the callus tissues within 2 days and the label from glucose was effectively incorporated into the phytosterol after 3 days of incubation. As illustrated in Fig. 2, both TLC-radiochromatograms (Fig. 2A) and TLC-densitochromatograms (Fig. 2B) of the non-polar fractions showed a maximum radio-intensity and peak area of β-sitosterol at day 3 of the incubation time. Thereafter, both parameters appeared to decrease continuously. Therefore, it was clear that the incubation time during high glucose feeding had a significant effect on the degree of glucose incorporation into the phytosterol. This finding led us to use the incubation time of 3 days for subsequent incorporation experiments of [1-^{13}C]glucose feeding.

In addition to the feeding time, a time-course of phytosterol production during the growth cycle of *C. sublyratus* callus cultures was also studied. This experiment was carried out in order to observe the culture age that is highly active in phytosterol biosynthesis. The results of the study are shown in Fig. 3. It can be seen that the phytosterol production was very active from days 15 to 23 during a 30-day growth cycle of *C. sublyratus* callus culture. The highest peak of the production appeared to be on day 21. This eight-day growth period corresponded to the linear phase of the growth cycle. Based on these results, the callus cultures with the age of 20 days were used for subsequent incorporation experiments.

Using the optimized conditions of glucose feeding, the 20-day-old callus cultures were then fed for 3 days with [1-^{13}C]glucose in comparison with unlabeled glucose (control). The intense phytosterol peaks obtained from both feedings were then isolated by preparative TLC and were analyzed by ^{1}H and ^{13}C NMR spectroscopy. The resulting isolate was, however, identified as a mixture of β-sitosterol and stigmasterol as reported previously

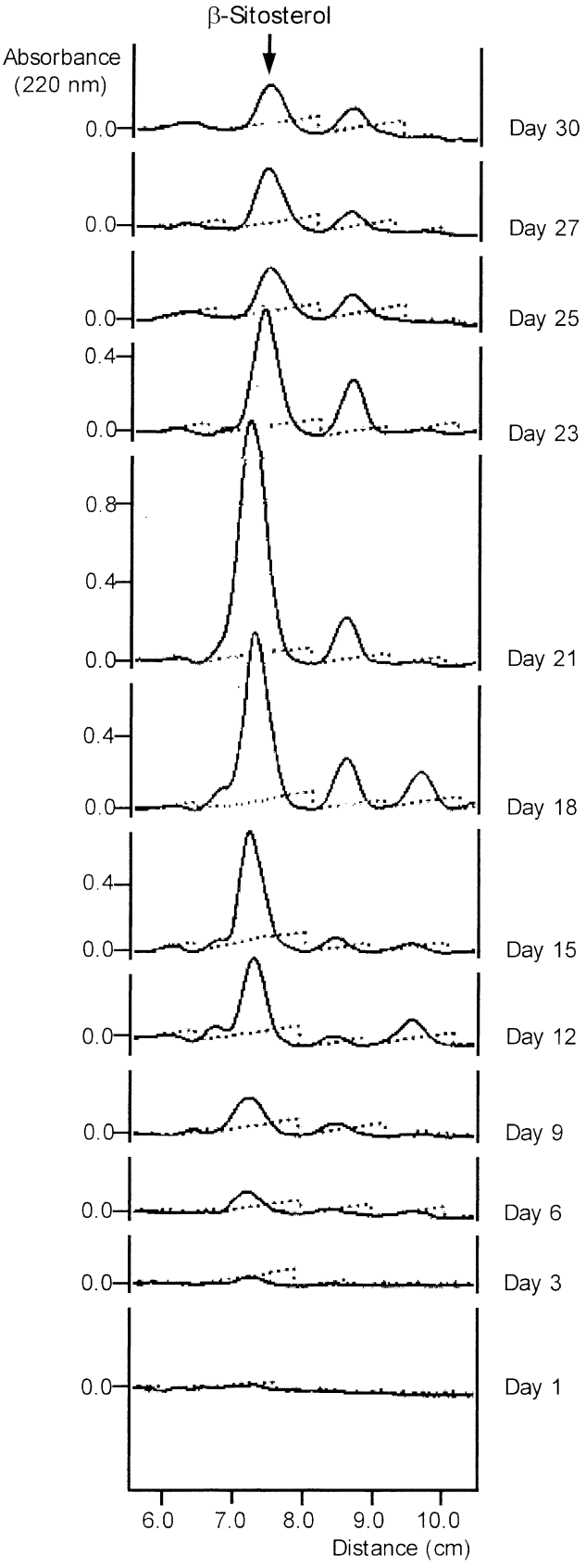

Fig. 3. TLC-densitometric chromatograms showing time-course of phytosterol formation during a 30-day growth cycle of *Croton sublyratus* callus cultures.

(Khalil and Idler, 1980; Iribarren and Pomillio, 1985; Heupel et al., 1986; Wright et al., 1978). In the ^{1}H NMR spectrum, the signals at δ 5.03 (0.45 H, *dd*, $J=15.2$, 8.4 Hz) and δ 5.17 (0.45 H, *dd*, $J=15.2$, 8.4 Hz) were assigned to H-22 and H-23 of stigmasterol and δ 5.36 (1.06 H, *d*, $J=4.8$ Hz) was assigned to H-6 of both β-sitosterol and stigmasterol data not shown. The integration of H-6, H-22 and H-23 appeared to be in the ratio of 1.06:0.45:0.45. Therefore, it could be deduced that the mixture of β-sitosterol and stigmasterol isolated from the callus culture was in the ratio of 1.3:1.0.

For ^{13}C NMR data, comparison of the two ^{13}C NMR spectra of the phytosterol mixtures obtained from the separate feedings of [1-^{13}C]glucose and unlabeled glucose found that the phytosterols obtained from the labeled glucose showed ^{13}C-enrichment in most of their carbons except the carbon numbers 4, 10, 20 and 25 (Fig. 4). Quantitative analysis on the degree of ^{13}C-enrichment of each carbon was then calculated by comparison of ^{13}C-signal intensity between the ^{13}C-labeled and unlabeled isolates. This ^{13}C-enrichment is best expressed as a value of S:C ratio of each carbon, where S (sample) is the ^{13}C NMR signal intensity of a phytosterol carbon obtained from [1-^{13}C] glucose incorporation and C (control) is that obtained from the unlabeled glucose incorporation under the same condition. These values of relative ^{13}C-signal intensity, however, must be obtained based on a carbon that is not involved in any labeling process either by the mevalonate pathway or the deoxycelluse pathway (e.g. carbon 4, 10, 20 or 25). In this study, the peak intensity of C-4 carbon of β-sitosterol which gave clear enrichment values of S:C of all ^{13}C-signal peaks (Table 1) was chosen to have 100% intensity. The results showed that 23 out of the 29 of both phytosterols were labeled by [1-^{13}C]glucose incorporation whereas less than 6 carbons were not labeled (Fig. 5). Quantitatively, those ^{13}C-labeled carbons were found to have their significant degree of enrichment ranging from 1.20 to 1.87. These values were, however, lower than expectation based on

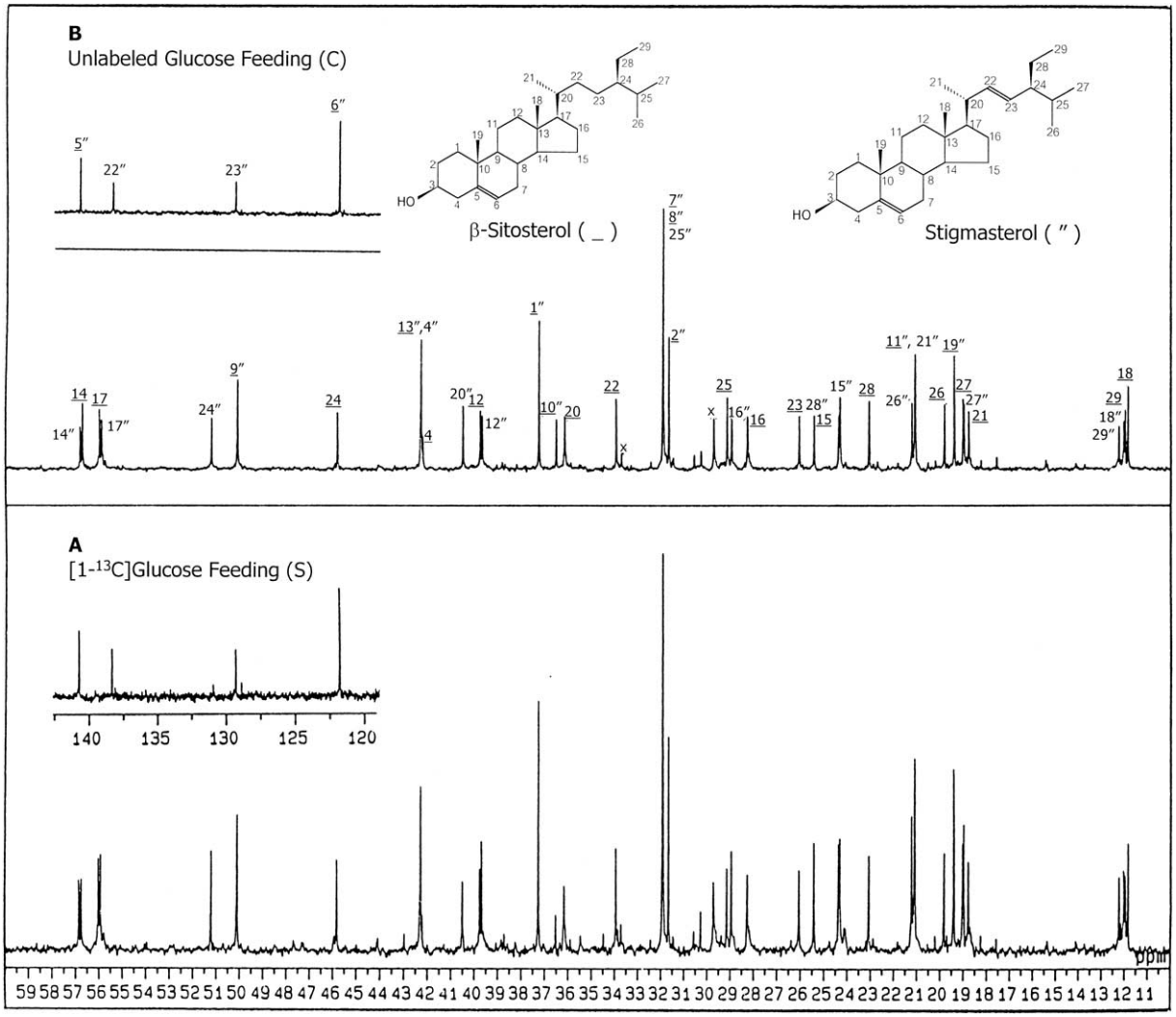

Fig. 4. Comparison of ^{13}C NMR spectra of a mixture of β-sitosterol and stigmasterol isolated from 20-day-old callus cultures which had been fed with either [1-^{13}C]glucose (A) or unlabeled glucose (B).

our preliminary studies on [1-^{14}C]glucose feeding experiments (Figs. 1 and 2). Since pre-existing phytosterols in the intracellular pools of the callus culture were also found to be present in relatively high level by the time of carrying out [1-^{13}C]glucose feeding (Fig. 3), it was highly possible that the ^{13}C-labeled phytosterols formed during the feeding were diluted by the endogenous compounds which caused the low enrichment values.

It should be noted that the carbons 28 and 29 of the phytosterols which are not part of the isoprene unit were also observed to be enriched in this study (Fig. 5). This can be explained by considering the biosynthetic acquisition of the two carbons. It has been well established that in the process of phytosterol biosynthesis in higher plants, three methyl groups of the steroid nucleus (two at C4 and one at C14) are removed and two methyl groups are transferred to the carbons 28 and 29 (review in Brown, 1998). Since the removed methyl groups are also labeled from [1-^{13}C]glucose feeding by the process of isoprene and squalene formation (Fig. 6),

Table 1
^{13}C NMR analysis of β-sitosterol and stigmasterol from *Croton sublyratus* callus cultures after being fed with [1-^{13}C]glucose (S) and unlabeled glucose (C) under the same condition[a]

Carbon No.[b]	Chemical shift (ppm)	^{13}C-Signal intensity (4 = 100%)[c]		S:C ratio
		[1-^{13}C] Glucose (S)	Unlabeled Glucose (C)	
5″	140.72	208.583	164.501	1.27
22″	138.31	151.776	92.317	1.64
23″	129.25	147.417	97.932	1.51
6″	121.71	343.317	285.837	1.20
3″	71.80	524.807	306.677	1.71
14″	56.85	196.482	130.788	1.50
14	56.73	199.821	198.482	1.01
17	56.02	256.639	181.118	1.42
17″	55.93	267.608	149.450	1.79
24″	51.22	278.353	158.729	1.75
9″	50.10	378.073	267.348	1.41
24	45.81	254.633	172.200	1.48
13″,4″	42.26	459.641	389.155	1.18
4	42.19	100.000	100.000	1.00
20″	40.48	194.252	196.076	0.99
12	39.74	229.714	178.964	1.28
12″	39.66	303.910	166.963	1.82
1″	37.22	701.300	447.656	1.57
10″	36.48	100.045	156.244	0.64
20	36.12	183.115	171.254	1.06
22	33.91	285.479	212.504	1.34
7″,8″,25″	31.87	1120.448	786.411	1.42
2″	31.63	600.235	397.609	1.51
25	29.11	230.824	217.183	1.06
16″	28.91	277.703	159.846	1.74
16	28.22	213.412	160.318	1.33
23	26.02	226.465	173.215	1.31
28″	25.39	301.244	175.841	1.71
15″	24.34	297.255	164.140	1.81
15	24.29	312.381	216.436	1.44
28	23.04	264.997	204.357	1.30
26″	21.20	377.692	201.486	1.87
11″,21″	21.07	540.952	346.312	1.56
26	19.80	271.204	194.283	1.40
19″	19.39	509.345	339.179	1.50
27	19.01	298.342	212.182	1.41
27″	18.96	351.742	197.295	1.78
21	18.76	248.482	176.777	1.41
29″	12.23	205.613	130.379	1.58
18″	12.03	223.193	145.690	1.53
29	11.97	207.955	179.215	1.16
18	11.84	298.958	249.324	1.20

[a] Each intensity value was obtained based on the intensity of C4 of β-sitosterol which is not involved in the label incorporation either from the mevalonate pathway or the deoxyxylulose pathway.

[b] Assignments are based on the reported values of β-sitosterol and stigmasterol (Wright et al., 1978) and the assigned carbons are shown in the structures of Fig. 4.

[c] Recalculated from the original data of intensity (% of max at 31.87 ppm) and used the intensity of 4 at 42.19 ppm as 100% instead.

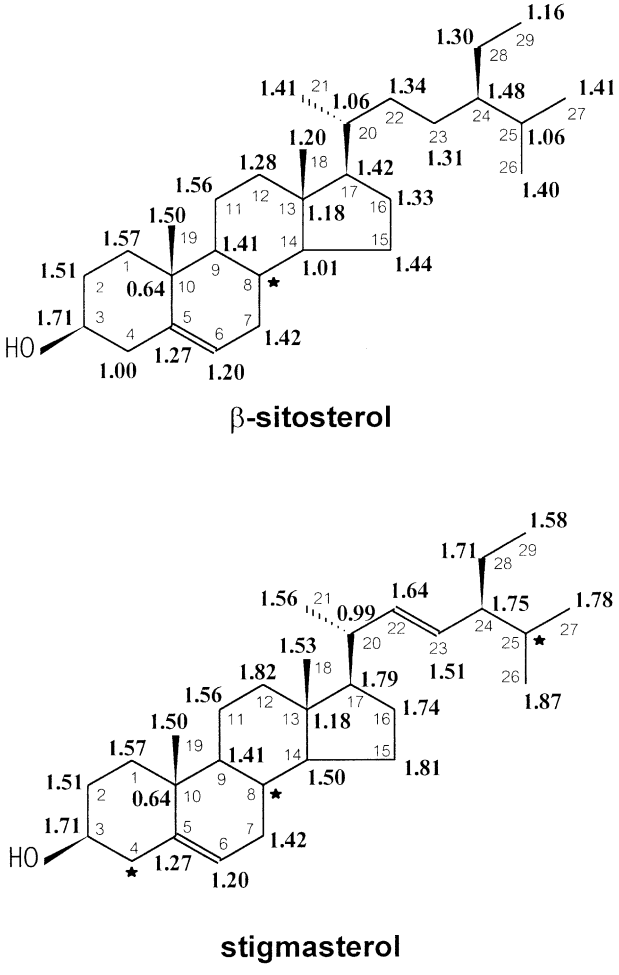

Fig. 5. Summary of the degree of ^{13}C-enrichment of various carbon atoms of β-sitosterol and stigmasterol isolated as a mixture from *Croton sublyratus* callus cultures after being fed with either [1-^{13}C]glucose (S) or unlabeled glucose (C). The value indicated at a particular carbon is the S:C ratio obtained from Table 1. The values of the carbon with * could not be determined owing to signal overlapping of different carbons.

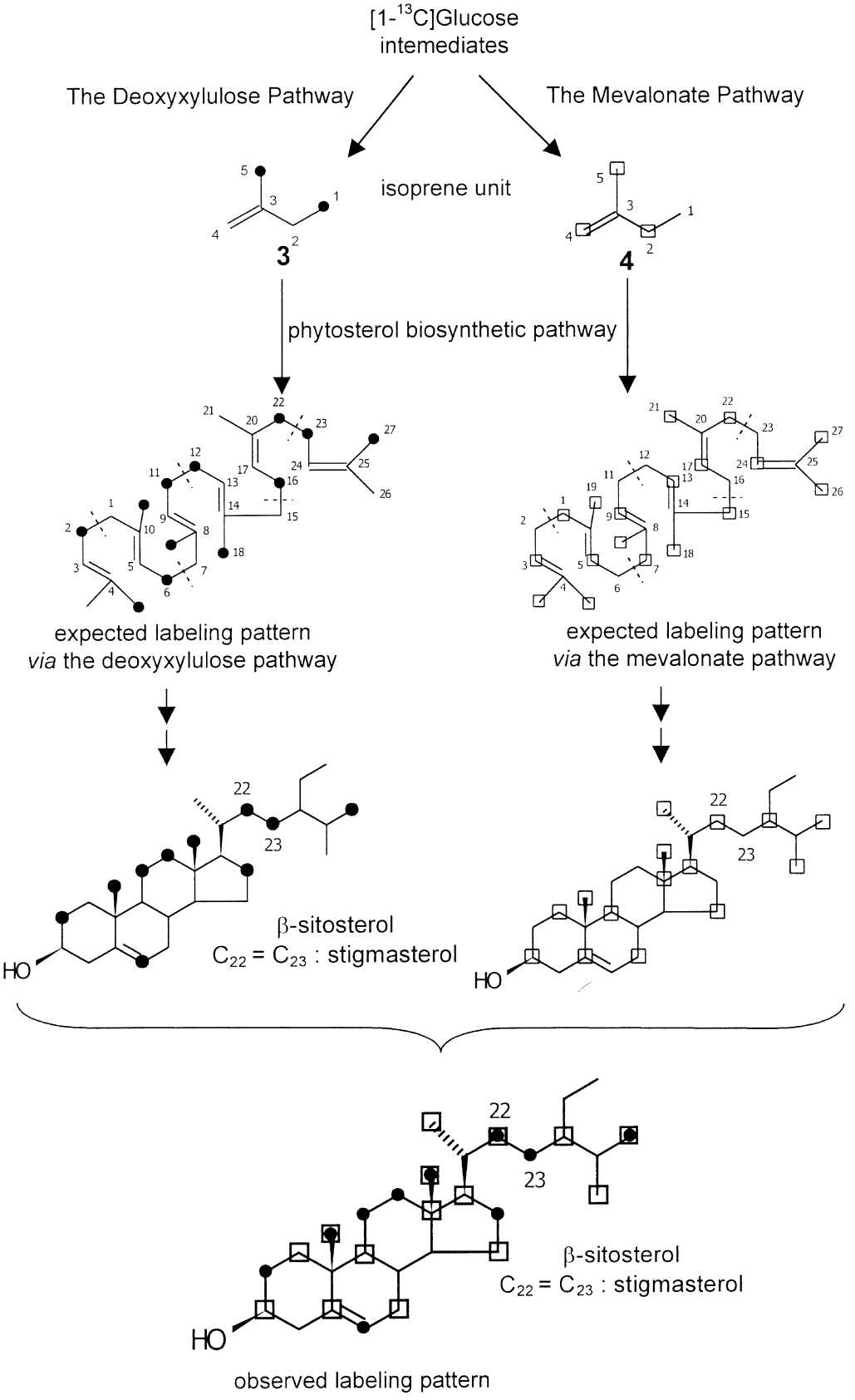

Fig. 6. Proposed biosynthetic conversion of [1-^{13}C]glucose during incorporation of the labeled carbon into the molecules of β-sitosterol and stigmasterol by *Croton sublyratus* callus cultures, ● the label carbon contributed from the deoxyxylulose pathway; □ from the mevalonate pathway and ◘ from both pathways.

the intracellular pool of methyl groups should also be labeled. Utilization of this one-carbon pool [through (*S*)-adenosyl-L-methionine] should also lead to the label of C28 and C29 of the phytosterol molecules.

Analysis on the possibility of the two biosynthetic pathways of isoprene contributed to the observed ^{13}C-labeling patterns led to the conclusion that both the mevalonate pathway and the deoxyxylulose pathway are involved in the formation of the phytosterols in *C. sublyratus* callus cultures. Fig. 6 summarizes the major steps of [1-^{13}C]glucose conversion to β-sitosterol and stigmasterol. The labeled glucose is first catabolized by the glycolytic pathway to generate various intermediates. These include acetyl CoA which is the precursor for the mevalonate pathway and glyceraldehyde-3-phosphase and pyruvate which are the precursors for the deoxyxylulose pathway. (for reviews, see Rohmer, 1999; Dewick, 1999). Both pathways lead to the formation of the same isoprene unit but having two different labeling patterns; the mevalonate pathway gives the isoprene labeling pattern **3** whereas the deoxyxylulose pathway gives the isoprene labeling pattern **4** (Schwarz, 1994; Rohmer, 1998; Eisenreich et al., 1998). Separate utilization of the two isoprenes for phytosterol biosynthesis would lead to two different labeling patterns of the products. In our case, however, the observed phytosterol labeling pattern appeared to be the combination of both patterns (Fig. 6). This clearly indicates that the mevalonate pathway and the deoxyxylulose pathway are both operative in *C. sublyratus* callus cultures, and their isoprene end products are both used for the phytosterol biosynthesis. The observed labeling pattern also suggests that the different C5-building blocks from either of the two pathways are mixed in a completely random fashion before being utilized for the phytosterol biosynthesis. It is possible that the subcellular compartmentation of the two isoprenoid pathways (the mevalonate pathway in the cytoplasm and the deoxyxylulese pathway in the plastids) is not fully developed in the callus cultures and thus allow the different origin of isoprenes be mixed more readily.

Analysis on the degree of ^{13}C-enrichment of some phytosterol carbons allowed us to estimate the relative degree of contribution of the two isoprene pools to the phytosterol formation in *C. sublyratus* callus cultures. This was done by comparing the average values of ^{13}C-enrichment (S:C) of the carbons labeled solely by the mevalonate pathway with those of the carbons labeled solely by the deoxyxylulose pathway. Table 2 shows S:C values of the selected carbons of both β-sitosterol and stigmasterol labeled by each pathway. It can be seen that the relative contribution of the mevalonate pathway: the deoxyxylulose pathway in labeling the phytosterols were approximately 1.07:1.00. This means that both isoprene pathways contribute almost equally to the biosynthesis of β-sitosterol and stigmasterol. This observed high degree of contribution of the deoxyxylulose pathway appears to be in contrast to the previous reports which have demonstrated the major role of the mevalonate pathway in the phytosterol biosynthesis (Schwender et al., 1997; Lichtenthaler et al., 1997; Arigoni et al., 1997). As mentioned earlier, it is very likely that a metabolite exchange between the two isoprene-producing pathways might be taken place in the green callus cultures of *C. sublyratus*. This cross-talk between the two isoprenoid pathways has been proposed for *Catharanthus roseus* cell cultures which produce β-carotene, lutein, phytol and sitosterol (Arigoni et al., 1997). In that case, a minor contribution form the deoxyxylulose pathway was found for sterol biosynthesis. A detailed study of the metabolite partitioning of the two pathways will be important of the understanding of this physiological relationship.

Table 2
Analysis on the relative contribution of the mevalonate pathway and the deoxyxylulose pathway in the biosynthesis of the phytosterols in *Croton sublyratus* callus cultures[a]

Carbon No.	Degree of ^{13}C-enrichment (S:C)	
	β-Sitosterol	Stigmasterol
Selected carbons contributed solely by the mevalonate pathway		
15	1.44	1.81
17	1.41	1.79
24	1.48	1.75
27	1.41	1.78
Average degree of ^{13}C-enrichment	1.43	1.78
Selected carbons contributed solely by the deoxyxylulose pathway		
12	1.28	1.82
16	1.33	1.74
23	1.31	1.51
Average degree of ^{13}C-enrichment	1.31	1.69
Relative pathway contribution		
the mevalonate pathway : the deoxyxylulose pathway	1.09:1.00	1.05:1.00 (average = 1.07:1.00)

[a] The carbon atoms selected for the analysis were the ones being labeled solely by either the mevalonate pathway or deoxyxylulose pathway and their ^{13}C NMR signals had no signal overlapping.

3. Experimental

3.1. Chemicals

[1-^{14}C]Glucose with specific activity of 56.0 mCi mmol^{-1} and radioactive activity of 308 μCiml^{-1} was purchased from Amersham (Buckinghamshire, England). [1-^{13}C]Glucose (99% ^{13}C-abundance) obtained from Isotec (Miamisburg, OH) was kindly provided by Professor M.H. Zenk, Biocenter, University of Halle, Halle (Saale), Germany.

3.2. Plant material

The leaves of *Croton sublyratus* Kurz (Euphorbiaceae) used in this study were collected from the plants cultivated by the Institute of Biotechnology and Genetic Engineering of Chulalongkorn University, Bangkok, Thailand. A voucher specimen of this plant was deposited in the Herbarium, Royal Forest Department in Bangkok, Thailand under the number 21867.

3.3. Callus culture

Young fully expanded leaves of *C. sublyratus* were soaked in 70% alcohol for 3 min, followed by sterilization in 0.5% (w/v) sodium hypochlorite for 5 min and washing five times with sterile distilled water. The sterile leaves were excised into pieces (ca. 5×5 mm^2) and placed on to MS agar medium containing 3% (w/v) sucrose, 20 ml/l 2,4-D and 1.0 mg/l kinetin and solidified with 0.8% (w/v) agar. The leave explants were maintained under 16-h photoperiod (2000 lux) at 25 ± 2 °C. Under these use μE conditions, callus tissues were formed in 2 weeks followed by their rapid proliferation. After 4 weeks, the calli formed were maintained by subculturing for every 3 weeks on MS agar medium containing 3% (w/v) sucrose, 1.0 mg/l 2,4-D, 0.1 mg/l BA and 0.1 mg/l NAA. The callus culture appeared to be friable with greenish colour of the tissues.

3.4. A time-course study of phytosterol formation in C. sublyratus callus cultures

Green callus tissues of 20-day-old (0.05 g each) were transferred to 50-ml jars containing MS agar medium supplemented with 3% (w/v) sucrose, 1.0 mg/l 2,4-D, 0.1 mg/l BA and 0.1 mg/l NAA. The jars were maintained at 25 ± 2 °C under controlled 16-h photoperiod (2000 lux). Two jars were taken every 2 days for fresh weight recording and sample preparation for phytosterol analysis. The sample preparation was usually carried out by extracting 1 g fresh weight tissues with 2 ml 95% ethanol containing 1% NaOH under reflux for 1 h. The ethanolic extract was then partitioned with 1 ml *n*-hexane for three times and the combined organic phases were evaporated to dryness and the products separated by thin layer chromatography (silica gel 60 F_{254}, Merck) developed in chloroform:*n*-propanol 24:1. A β-sitosterol standard was also developed in the same TLC plate to locate the position of the phytosterol. To obtain TLC-chromatograms, the TLC plate was scanned by TLC-densitometer (Shimadzu CS-930) using the wavelength of 220 nm.

3.5. Optimization of feeding conditions

For optimization of feeding conditions, tracer experiments were first performed with radioactively labeled [1-^{14}C]glucose. Five grams of 20-day-old callus tissues were fed with 1 μCi [1-^{14}C]glucose (in 2-ml H_2O) in a 50-ml sterile jar for 3 days or for various intervals during a time-course study. The tissues were then extracted under reflux for 1 h with 10 ml 95% ethanol containing 1% (w/v) NaOH. The ethanolic extract was concentrated and partitioned with 1-ml *n*-hexane three times. The combined organic phase was evaporated to dryness and redissolved with 40 μl absolute ethanol. The dissolved products were separated by thin layer chromatography (silica gel 60 F_{254}, Merck) using a solvent system of chloroform-*n*-propanol (24:1). The TLC plate was then subjected to both, a radio-scan (Linear Analyzer LB 284, Berthold) to produce radio-chromatograms and a densitometric scan (Shimadzu CS-930) using the wavelength of 222 nm to produce densitometric chromatograms. Both chromatograms were compared and analyzed for the efficiency of glucose incorporation into phytosterols.

3.6. [1-^{13}C]Glucose incorporation experiments

Incorporation of label from glucose into phytosterols by *C. sublyratus* callus cultures was performed in parallel with the solutions of 0.4% (w/v) [1-^{13}C]glucose and 0.4% (w/v) unlabeled glucose. Five grams fresh weight of 20-day-old callus tissues were placed on 2 ml of each glucose solution in a 50-ml jar and incubated for 3 days under the described culturing conditions. Sixty jars were incubated for each feeding of the labeled and unlabeled glucose. In order to monitor the uptake and incorporation of glucose into the phytosterols, 1 μCi [1-^{14}C]glucose was added into one jar of the unlabeled glucose. The glucose solution and callus tissues were taken everyday during the incubation to determine the radioactivity remaining in the glucose solution and to observe the radio-intensity of the phytosterol peak in a TLC plate as described earlier.

3.7. Isolation of β-sitosterol and stigmasterol mixture

The callus tissues (300 g fresh weight) fed with either [1-^{13}C]glucose or unlabeled glucose for 3 days were separated from the remaining glucose solution and extracted under reflux for 1 h with 500 ml 95% ethanol containing 1% (w/v) NaOH. The extract was filtered and evaparated under reduced pressure to a volume of 200 ml before being extracted 3 times with 200 ml *n*-hexane. The organic extracts were combined and evaporated to dryness. The residue was dissolved in absolute ethanol and further purified by preparative TLC (silica gel 60 F_{254}) using benzene–ethylacetate (5:1, v/v) as mobile phase. Under these conditions, β-sitosterol and stigmasterol were co-chromatographed on the TLC plate with the same Rf value of 0.54. The phytosterol mixture was eluted from the silica gel by absolute ethanol and

taken to dryness under reduced pressure. The obtained products, from both the labeled and unlabeled feedings, were purified once more using the same preparative TLC procedure prior to analysis for their ^{13}C abundance at each carbon atom by NMR spectroscopy.

3.8. NMR measurements

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ using a JEOL JNM-A500 spectrometer, 500 MHz (^{1}H) and 125 MHz (^{13}C). ^{1}H-Decoupled ^{13}C NMR spectra of samples from incorporation experiments with [1-^{13}C]glucose (99% ^{13}C abundance) and unlabeled glucose (natural ^{13}C abundance, 1.1% ^{13}C) were recorded under identical conditions. Relative ^{13}C abundance of individual carbon atoms was then calculated by comparison of ^{13}C-signal integrals between ^{13}C-labeled and unlabeled material.

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