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# Biosynthesis of abietane diterpenoids in cultured cells of *Torreya nucifera* var. *radicans*: biosynthetic inequality of the FPP part and the terminal IPP

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**Abstract**—Labelling experiments with [1-$^{13}$C] glucose, [1-$^{13}$C] sodium acetate and [1,2-$^{13}C_2$] sodium acetate proved that the fundamental isopentenyl diphosphate (IPP) unit, constituting GGPP in the abietane diterpenoids, were biosynthesized via the GAP/pyruvate pathway, as well as the acetate/mevalonate pathway in the cultured cells of *Torreya nucifera* var. *radicans*. In addition, the terminal IPP showed the preferential labelling from the GAP/pyruvate pathway and unfavorable labelling from the acetate/mevalonate pathway, suggesting that the biosynthetic route leading to the terminal IPP involved the GAP/pyruvate pathway more and the acetate/mevalonate pathway less, compared with the FPP part. Furthermore, it was also found that the utilization of the two pathways in the formation of the FPP part and the terminal IPP was dependent on the exogenous precursors through the simultaneous administration of [1-$^{13}$C] sodium acetate and unlabelled glucose.


## 1. Introduction

Abietane diterpenoids are a class of natural products widespread in the plant kingdom. They are attracting a great deal of attention due to their varied biological activities. For example, they are the principal constituents of tree rosin responding to wounding and attack by insect pests and pathogens, such as abietic acid.[1,2] The active component in *Salvia miltiorrhiza* that inhibits platelet aggregation is also found to be an abietane diterpenoid.[3]

The generally accepted pathway for the biosynthesis of the abietane diterpenoids is shown in Fig. 1. A series of condensation reactions between isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP), produce geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) in turn. Cyclization of the all *trans*-GGPP gives rise to (+)-copalyl diphosphate, and then cyclization of (+)-copalyl diphosphate accompanied by elimination of the diphosphate gives pimaradiene. The 1,2-shift of the methyl group from C-13 to C-15 results in the formation of the abietane skeleton. Variation in the position of the deprotonation in the abietane skeleton and subsequent decorative reactions would account for the great variety of the abietane diterpenoids.

On the other hand, as for the fundamental IPP unit of the isoprenoid biosynthesis, it had been accepted that the acetate/mevalonate pathway is the only and common route leading to IPP in all living organisms. Recently, a totally different pathway, in which mevalonate is not a precursor and where IPP is formed from glyceraldehyde 3-phosphate (GAP) and pyruvate, was discovered[4] in bacteria[5,6] and green alga,[7] and later found to also be operative in the biosynthesis of plastidic isoprenoids,[8,9] isoprenes[10] and monoterpenoids[11] in higher plants. In the diterpenoids, taxol[12] and marrubiin[13] have also been verified to be biosynthesized via the GAP/pyruvate pathway, not the acetate/mevalonate pathway. In the case of the abietane diterpenoids, the pathway utilized for the formation of the IPP unit has not yet been clarified.

In a previous paper, we have already reported the isolation of the abietane diterpenoids from the cultured cells of *T. nucifera* var. *radicans*.[14] Here, through the feeding experiments with $^{13}$C-labelled glucose and sodium acetate, it was found that the acetate/mevalonate pathway and the GAP/pyruvate pathway showed different degrees of contributions to the biosynthesis of the FPP part and the terminal IPP, although both of them participated in the formation of the four IPP units in the abietane diterpenoids in the cultured cells of *T. nucifera* var. *radicans*.

## 2. Results and discussion

According to the time course of the major abietane diterpe-

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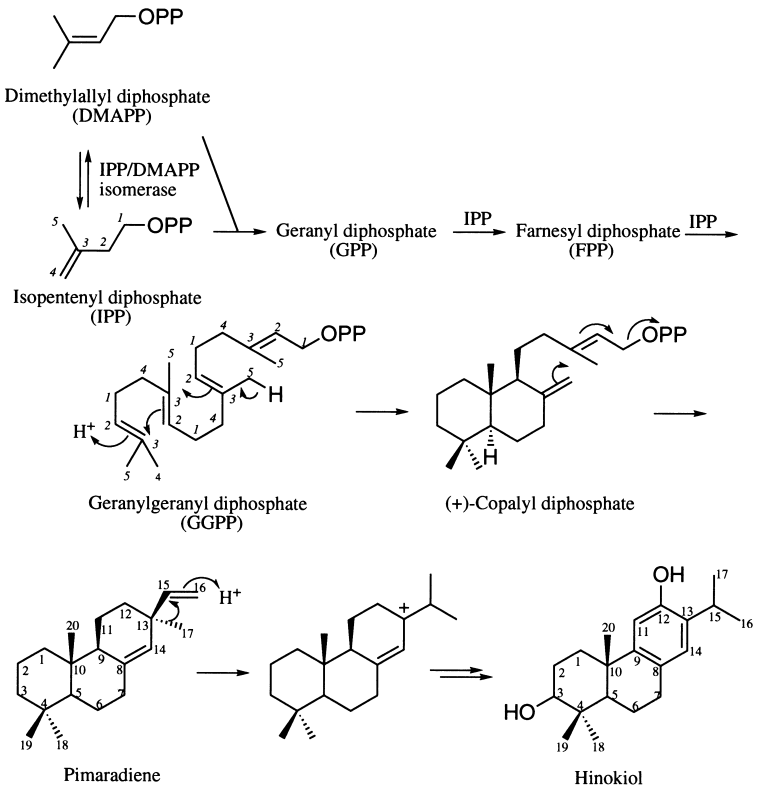


**Figure 1.** Biosynthetic pathway of hinokiol. The numbers in italic represent the carbon positions in IPP; the roman numbers represent the carbon positions in hinokiol.

noid, hinokiol (data not shown), the cell cultures of *T. nucifera* var. *radicans* were fed with $^{13}C$-labelled precursors during the third week, the rapid production phase of hinokiol. $^{13}C$-labelled hinokiol was isolated from the harvested fresh cells and the relative $^{13}C$ abundance ($\%^{13}C$) and the fraction of the double-labelled isotopomers ($\%^{13}C^{13}C$) were calculated as described in Section 4.

At first, the participation of the GAP/pyruvate pathway in the formation of the IPP unit in hinokiol was proved from the incorporation experiment with [1-$^{13}C$] glucose. As shown in Table 1 and Fig. 2, C-2, C-6, C-11, C-14, C-16, C-17, C-19 and C-20 were significantly labelled with the average relative $^{13}C$ abundance of 4.0%, in which C-2, C-6, C-11 and C-16 (or C-17) are biosynthetically equivalent to

**Table 1.** $^{13}C$ NMR analysis of hinokiol from cultured cells of *T. nucifera* var. *radicans* after feeding with [1-$^{13}C$] glucose, [1-$^{13}C$] sodium acetate, [1,2-$^{13}C_2$] sodium acetate, [1-$^{13}C$] sodium acetate+unlabelled glucose and [1-$^{13}C$] glucose+unlabelled sodium acetate

| Carbon | $\delta$ (ppm) | [1-$^{13}C$] glucose ($\%^{13}C$) | [1-$^{13}C$] $CH_3COONa$ ($\%^{13}C$) | [1,2-$^{13}C_2$] $CH_3COONa$ ($\%^{13}C^{13}C$ ($J$ Hz)) | [1-$^{13}C$] $CH_3COONa$ +unlabelled glucose ($\%^{13}C$) | [1-$^{13}C$] glucose+ unlabelled $CH_3COONa$ ($\%^{13}C$) |
|---|---|---|---|---|---|---|
| C-1 | 37.68 | 2.3 | 1.6 | Trace | **2.4** | 2.5 |
| C-2 | 28.9 | **4.3** | **3.6** | 37 (36) | **4.5** | **4.0** |
| C-3 | 78.1 | 1.8 | 1.5 | 31 (36) | 1.6 | 1.3 |
| C-4 | 37.74 | 1.1 | **2.2** | 23 (36) | **4.5** | 1.7 |
| C-5 | 50.5 | 1.5 | 1.2 | 28 (34) | 1.8 | 1.6 |
| C-6 | 19.8 | **4.0** | **3.6** | 32 (34) | **4.4** | **3.8** |
| C-7 | 30.6 | 2.0 | 1.6 | Trace | **2.1** | 2.1 |
| C-8 | 125.9 | 1.1 | **4.7** | 28 (59) | **3.6** | 1.8 |
| C-9 | 148.4 | 1.5 | 1.7 | 30 (60) | 1.4 | 1.2 |
| C-10 | 39.7 | 1.5 | **2.7** | 26 (34) | **2.4** | 1.1 |
| C-11 | 111.7 | **4.4** | **3.9** | 34 (60) | **5.0** | **4.3** |
| C-12 | 154.0 | 2.2 | **2.7** | | **2.8** | 2.6 |
| C-13 | 133.3 | 1.5 | **2.6** | | 1.6 | 1.4 |
| C-14 | 127.1 | **4.5** | 1.8 | 30 (59) | 1.7 | **3.9** |
| C-15 | 27.5 | 1.7 | 1.4 | 10 (33) | 1.5 | 1.7 |
| C-16$_{pro(S)}$[a] | 23.3 | **4.2** | **2.4** | 14 (33) | 1.6 | **3.9** |
| C-17$_{pro(R)}$ | 23.2 | **3.3** | 1.1 | | 1.6 | **3.0** |
| C-18 | 29.0 | 2.1 | 1.6 | Trace | **2.3** | 2.1 |
| C-19 | 16.4 | **3.5** | 1.2 | 32 (36) | 1.1 | **2.9** |
| C-20 | 25.4 | **4.0** | 1.7 | 30 (34) | 1.8 | **3.5** |

[a] *Pro*(*S*) and *pro*(*R*) were assigned by comparison with the data in the literature.[19]

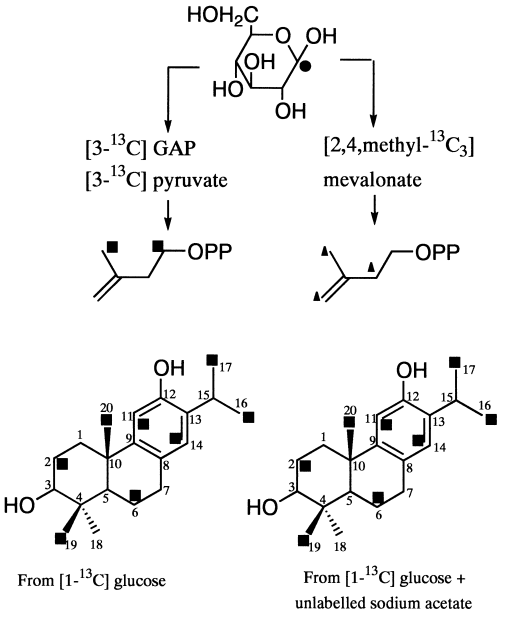


**Figure 2.** The expected labellings in IPP units from [1-$^{13}$C] glucose via the two distinct pathways; the observed labelling pattern in hinokiol from [1-$^{13}$C] glucose and [1-$^{13}$C] glucose+unlabelled sodium acetate.

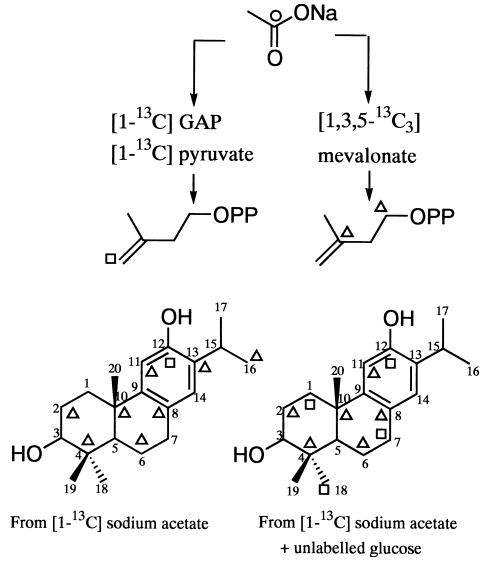


**Figure 3.** The expected labellings in IPP units from [1-$^{13}$C] sodium acetate via the two distinct pathways; the observed labelling pattern in hinokiol from [1-$^{13}$C] sodium acetate and [1-$^{13}$C] sodium acetate+unlabelled glucose.

position 1 of the IPP unit, and C-19, C-20, C-14 and C-17 (or C-16) are biosynthetically equivalent to position 5 of the IPP unit. This labelling pattern exactly coincided with that expected from the GAP/pyruvate pathway. In addition, C-18, C-1, C-7 and C-12, biosynthetically equivalent to position 4 of the IPP unit, showed a $^{13}$C enrichment of 2.0–2.3%. This phenomenon was also observed in similar feeding experiments and was considered to reflect the somewhat imperfect stereocontrol of the IPP/DMAPP isomerase.[15–17] In the case of the acetate/mevalonate pathway, the $^{13}$C labelling from [1-$^{13}$C] glucose should occur at the carbon atoms corresponding to positions 2, 4 and 5 of the IPP unit. However, the absence of the labelling pattern from the acetate/mevalonate pathway could not totally exclude the participation of the acetate/mevalonate pathway, as such a labelling pattern was also not observed in β-sitosterol (data not shown), known to be biosynthesized via the acetate/mevalonate pathway.[8]

Direct confirmation of the involvement of the acetate/mevalonate pathway in the formation of the IPP unit in hinokiol was obtained by examining the incorporation of acetate. After feeding with [1-$^{13}$C] sodium acetate, a significant $^{13}$C enrichment at positions 1 (C-2, C-6, C-11 and C-16) and 3 (C-4, C-10, C-8 and C-13) of the four IPP units in hinokiol was observed (Table 1, Fig. 3). This labelling pattern is very characteristic of the IPP unit formed via the acetate/mevalonate pathway. Furthermore, it should be noted that the position 4 (C-12) of the fourth IPP unit was also labelled with the relative $^{13}$C abundance of 2.7%. This labelling was also observed in similar feeding experiments,[7] and can be explained by the GAP/pyruvate pathway. The $^{13}$C labelling at C-1 of sodium acetate can be transformed via the glyoxylate cycle, oxaloacetate, into [1-$^{13}$C] pyruvate and [1-$^{13}$C] GAP, and then incorporated into position 4 of the IPP unit via the GAP/pyruvate pathway. This unique feature in the fourth IPP unit suggested that the contribution of the GAP/pyruvate pathway to the biosynthesis of the fourth IPP unit was more than that to the FPP part, although both of the two distinct pathways participated in the formation of each IPP unit in hinokiol. Additionally, the labelling at C-16, *pro*(*S*)-methyl, demonstrated that C-17, *pro*(*R*)-methyl, is position 5 of the fourth IPP unit, the methyl group migrated from C-13. Such a stereoselectivity of the methyl shift from C-13 to C-15 is also observed in another two abietane diterpenoids, cryptotanshinone[18] and ferruginol,[19] in the cell cultures of *S. miltiorrhiza*.

The greater contribution of the GAP/pyruvate pathway means the less involvement of the acetate/mevalonate pathway in the biosynthesis of the fourth IPP unit. However, the comparatively low label of the fourth IPP unit from the acetate/mevalonate pathway was not clearly observed in the feeding experiment with [1-$^{13}$C] sodium acetate. As shown in Table 1, although position 1 (C-16) of the fourth IPP unit was 2.4% labelled, much lower than the corresponding carbons (C-2, C-6 and C-11) in the FPP part, position 3 (C-13) of the fourth IPP unit was 2.6% labelled, almost the same as the corresponding C-4 and C-10. Therefore, a feeding experiment with [1,2-$^{13}$C$_2$] sodium acetate was further performed in which the joint incorporation of the $^{13}$C–$^{13}$C unit from sodium acetate was only observed in the IPP unit formed via the acetate/mevalonate pathway, and can be assessed by the highly sensitive analysis of the satellite signals arising by $^{13}$C–$^{13}$C coupling. In accordance with the expected incorporation pattern of the $^{13}$C$_2$ unit (Fig. 4), the $^{13}$C satellites arising by the $^{13}$C–$^{13}$C coupling appeared and showed the same coupling constant to each pair of expected coupling carbon atoms (Table 1). The appearance of singlet signals for C-13 and C-17, originating from positions 3 and 5 of the fourth IPP unit, respectively, is attributable to the 1,2-shift of the methyl group from C-13 to C-15 during the formation of the abietane skeleton. However, it is noteworthy that an average 30% of the

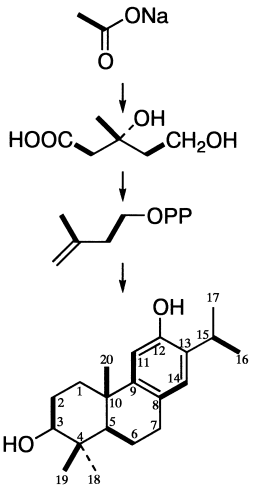


**Figure 4.** The expected incorporation pattern of the $^{13}C_2$ unit in hinokiol from [1,2-$^{13}C_2$] sodium acetate via the acetate/mevalonate pathway.

$^{13}$C-coupled satellite signal intensity relative to the overall $^{13}$C signal intensity was observed in the FPP part, whereas it was only 14 and 10% at positions 1 (C-16) and 2 (C-15) in the fourth IPP unit, respectively. This result proved that the acetate/mevalonate pathway was indeed less involved in the biosynthesis of the fourth IPP unit (the terminal IPP) compared with the FPP part. In addition, the appearance of the weak $^{13}$C satellites accompanying the $^{13}$C signals of C-18, C-1 and C-7 was attributed to the imperfect stereocontrol of the IPP/DMAPP isomerase.

In higher plants, it has been generally accepted that the GAP/pyruvate pathway for the formation of the IPP unit is localized inside the plastid, whereas the acetate/mevalonate pathway is localized in the cytosol.[20] Although the subsequent biosynthesis and accumulation of hinokiol may occur in other compartments, the biosynthesis of GGPP is considered to occur in the plastid because GGPP synthase has been purified from the plastid of various plants.[21] However, our present study showed that both the acetate/mevalonate pathway and the GAP/pyruvate pathway participated in the formation of the IPP unit in hinokiol; besides, the GAP/pyruvate pathway was comparatively more involved in the formation of the terminal IPP, whereas the acetate/mevalonate pathway was comparatively more involved in the formation of the FPP part. These results suggested that the importing rate of IPP and FPP may be different, although both IPP and FPP formed via the acetate/mevalonate pathway in the cytosol can be imported into the plastid. Namely, due to the fast import of FPP and the slow import of IPP, FPP from the acetate/mevalonate pathway occupied a high ratio, whereas IPP from the acetate/mevalonate pathway occupied a low ratio in the overall FPP and IPP for the biosynthesis of GGPP leading to hinokiol in the plastid. Additional investigations into the permeability of the membrane of the intact plastid to IPP and FPP may help to prove this hypothesis.

The biosynthetic inequality of the FPP part and the terminal IPP in GGPP, observed in the abietane diterpenoids in the cultured cells of *T. nucifera* var. *radicans*, also took place in the Bryophyta plant, liverworts[22,23] and hornworts.[24] The preferential labellings of the FPP part from mevalonate were observed in all compounds formed from GGPP, including chlorophyll, β-carotene and diterpenoid, in liverworts and hornworts. This suggests that the phenomenon may be common and dependent on the specific state of the plastid in the Bryophyta plant. In the Gymnospermae plant, such a biosynthetic inequality of the FPP part and the terminal IPP was also observed in the ginkgolides in *Ginkgo biloba* on the one hand,[25] while the four IPP units were indistinguishable in the taxol in *Taxus chinensis* on the other hand.[12] However, it should be noted that the ginkgolides also originate from the abietane skeleton, although they do not belong to the abietane diterpenoids. Therefore, whether this phenomenon is a common feature for the abietane diterpenoids in the Gymnospermae plant needs additional investigations, such as the biosynthetic site of GGPP leading to the abietane skeleton in plastids.

Finally, there was still another problem we had to consider. We administered the respective $^{13}$C-labelled precursors of the two pathways, sodium acetate or glucose, in order to prove their existence in the above experiments. Therefore, it was possible that exogenous sodium acetate or glucose unilaterally enhanced the contribution of the acetate/mevalonate pathway or the GAP/pyruvate pathway for the biosynthesis of the FPP part and the terminal IPP. That is also to say, the utilization of the two pathways in the biosynthesis of the FPP part and the terminal IPP may be variable with the addition of different exogenous precursors. With regards to this possibility, we simultaneously administered glucose and sodium acetate with the same amount as in the previous experiments. Compared with the feeding experiment using only [1-$^{13}$C] sodium acetate, the $^{13}$C labellings from both the GAP/pyruvate pathway and the acetate/mevalonate pathway were observed in the FPP part, whereas only the $^{13}$C labellings from the GAP/pyruvate pathway appeared in the terminal IPP in the feeding experiment with [1-$^{13}$C] sodium acetate plus unlabelled glucose (Table 1 and Fig. 3). This result demonstrated that the GAP/pyruvate pathway was activated by the simultaneous administration of glucose, resulting in the enhanced participation of the GAP/pyruvate pathway in formation of the FPP part, and the weakened contribution of the acetate/mevalonate pathway to the formation of the terminal IPP. Thus it could be suggested that the utilization of the two pathways in the formation of the FPP part and the terminal IPP was affected by the quantity and quality of the exogenous precursors. Moreover, when [1-$^{13}$C] glucose plus unlabelled sodium acetate was administered, both the FPP part and the terminal IPP showed identical $^{13}$C labelling patterns from the GAP/pyruvate pathway with that observed in the feeding experiment using only [1-$^{13}$C] glucose (Table 1 and Fig. 2). The absence of the incorporation of [1-$^{13}$C] glucose via the acetate/mevalonate pathway suggested that [1-$^{13}$C] glucose had hardly contributed to the acetate/mevalonate pathway, because unlabelled sodium acetate was fully utilized by the acetate/mevalonate pathway as its direct precursor. Based on these above results, it could also be inferred that the involvement of the two pathways in the biosynthesis of the FPP part and the terminal IPP was influenced by many factors, for example, the activities of the relevant metabolic pathways such as the glycolytic pathway, glyoxylate cycle and so on. Namely, the plants can select the

utilization of the two pathways in the biosynthesis of the abietane diterpenoids, in order to adapt to any variations in the external or internal surroundings.

## 3. Conclusion

First, both the acetate/mevalonate and the GAP/pyruvate pathways participated in the biosynthesis of the fundamental IPP unit in the abietane diterpenoids in the cultured cells of *T. nucifera* var. *radicans*. Second, the FPP part and the terminal IPP showed biosynthetic inequality due to the different contributions of the two pathways. The GAP/pyruvate pathway contributed more, while the acetate/mevalonate pathway contributed less to the formation of the terminal IPP, compared with the FPP part. Third, the utilization of the two pathways in the biosynthesis of the FPP part and the terminal IPP would be variable based on the quality and quantity of the exogenous precursors.

## 4. Experimental

### 4.1. General experimental procedures

D-[1-$^{13}$C] Glucose (99% isotopic abundance), [1-$^{13}$C] sodium acetate (99% isotopic abundance) and [1,2-$^{13}C_2$] sodium acetate (99% isotopic abundance) were purchased from Sigma–Aldrich chemical co., USA. NMR spectra were determined using a JEOL Lambda-500 spectrometer. HPLC was performed on a Shimadzu model LC-10 system with an RID-10A detector, and μ Bondasphere 5 μ C-18 100 Å (19×150 mm$^2$) column at a flow rate of 3 ml min$^{-1}$. Column chromatography (CC) was carried out using Silicagel 60 (Merck 9385). TLC was conducted on Kieselgel 60 F254 plates (Merk). In the feeding experiments using [1-$^{13}$C] sodium acetate and [1-$^{13}$C] glucose, the $^{13}$C NMR spectra of the $^{13}$C-labelled and natural abundance samples were recorded under identical conditions: 125 MHz; repetition time 3 s (acq. time 0.96 s, delay time 2.03 s); pulse width 5.25 μs. The relative $^{13}$C abundance of individual carbon atoms (%$^{13}$C) was calculated by comparison of the $^{13}$C signal integral between the $^{13}$C-labelled and natural abundance samples. The values were referenced to 1.1% for the carbon with the lowest $^{13}$C enrichment. In the feeding experiment with [1,2-$^{13}C_2$] sodium acetate, the fraction of the double-labelled isotopomers (%$^{13}$C$^{13}$C) was calculated from the $^{13}$C NMR spectra as the fraction of $^{13}$C–$^{13}$C-coupled satellite signals compared with the integral of the entire $^{13}$C signal of the respective carbon atom.

### 4.2. Cell culture conditions

The cells of *T. nucifera* var. *radicans* were subcultured on Murashige and Skoog's agar medium[26] modified with 10 mg l$^{-1}$ 2,4-dichlorophenoxyacetic acid, 1 g l$^{-1}$ casamino acid, 7% coconut milk and $K^+$ instead of $NH_4^+$ (D10CCM-$NH_4^+$). After 4 weeks of subculture, the cells (1.5 g/flask) were transplanted into 100 ml flasks containing 40 ml D10CCM-$NH_4^+$ liquid medium and cultured at 25°C in the dark at 50 rpm on a rotary shaker. Fourteen flasks of such suspension cultures were used in each feeding experiment.

### 4.3. Precursor administration

$^{13}$C-Labelled or unlabelled precursors, dissolved in distilled water (conc. 0.48 g ml$^{-1}$ for glucose; 0.12 g ml$^{-1}$ for sodium acetate) and sterilized in an autoclave (121°C, 15 min), were administered to two-week-old cell cultures, and then the incubation was continued for another week. In the feeding experiments using only [1-$^{13}$C] glucose or [1-$^{13}$C] sodium acetate, the administered amounts were 6 and 1.5 g l$^{-1}$ of the medium, respectively. In the feeding experiments with [1-$^{13}$C] glucose plus unlabelled sodium acetate or [1-$^{13}$C] sodium acetate plus unlabelled glucose, the administered amounts of glucose and sodium acetate were the same as above. In the feeding experiments with [1,2-$^{13}C_2$] sodium acetate, 0.5 g of [1,2-$^{13}C_2$] sodium acetate was diluted with 1.0 g of unlabelled sodium acetate per litre of the medium.

### 4.4. Extraction and isolation

The harvested fresh cells were extracted three times with MeOH at room temp. Evaporation of the solvent under reduced pressure gave the MeOH extract. The extract was then partitioned between EtOAc and $H_2O$. Removal of the solvent from the EtOAc phase under reduced pressure yielded the EtOAc extract. The EtOAc extract was subjected to silica gel CC and eluted with $CHCl_3$–MeOH (99:1) to give two frs. 1 and 2. Further purification of fr. 2 by HPLC (80% MeOH) afforded hinokiol. The identification of hinokiol was made by comparison with the data described in the literature.[27]

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