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24β-ETHYLSTEROL BIOSYNTHESIS: INCORPORATION OF L-[ME-$^2H_3$]METHIONINE IN TISSUE CULTURES OF *TRICHOSANTHES KIRILOWII*

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Key Word Index—*Trichosanthes kirilowii;* Cucurbitaceae; tissue culture; biosynthesis; phytosterols; 22-dihydrochondrillasterol; 22-dihydro-25-dehydrochondrillasterol; [Me-$^2H_3$]methionine.

Abstract—A maximum of five deuterium atoms was incorporated into the 24β-ethylsterols, 22-dihydro-25-dehydrochondrillasterol and 22-dihydrochondrillasterol, in tissue cultures of *Trichosanthes kirilowii* incubated with L-[Me-$^2H_3$]methionine. The results proved that a 24-ethylidene intermediate is not involved in the biosynthesis of 24β-ethylsterols in *T. kirilowii*.

INTRODUCTION

Sterols in higher plants generally have a methyl or an ethyl group at C-24 in the side-chain, whilst those in fungi and animals have a methyl group and a hydrogen, respectively. The C-24 alkyl groups have α or β stereochemical orientation and are known to be formed by transmethylation from *S*-adenosylmethionine [1–4]. Recently, we found in 24-methylsterols in higher plants that both campesterol (24α) and dihydrobrassicasterol (24β) are biosynthesized through a 24-methylene intermediate [5] and a 24-methyl-24(25)-ene intermediate [6]. The direction of reduction of the 24(25)-double bond seems to lead to opposite stereochemistry at C-24 and C-25 of campesterol and dihydrobrassicasterol [6].

Sterols having a 24β-ethyl side-chain mostly appear in photosynthetic algae, but some vascular plants contain 24β-ethylsterols [7–9]. The mechanism of 24β-ethyl side-chain formation has been studied in lower plants and found to vary with the organism. Poriferasterol in the Chrysophyte alga *Ochromanas malhamensis* [10] and clionasterol in the yellow-green alga *Monodus subterraneus* [11] are biosynthesized through a 24-ethylidene intermediate (**8**) (**8**→**9**). However, intermediate **8** does not participate in 24β-ethyl side-chain formation in some chlorophytes [12–14] and the fungus-like microorganism (*Dictyostelium discoideum*) [15]. In higher plants, the mechanism of 24β-ethyl side-chain formation is less clear. We report herein the results of incorporation of L-[Me-$^2H_3$]methionine into 22-dihydro-25-dehydrochondrillasterol (**1**) and 22-dihydrochondrillasterol (**2**) in suspension cultures of *Trichosanthes kirilowii*.

RESULTS AND DISCUSSION

L-[Me-$^2H_3$]Methionine was administered to 11-day-old suspension cultures of *T. kirilowii* grown in Linsmaier-Skoog medium. After 10 days incubation, [^{2}H]-labelled 22-dihydro-25-dehydrochondrillasterol (**1**) and 22-dihydrochondrillasterol (**2**) were isolated by HPLC from methanolic extracts of cells.

Mass spectral analysis clearly demonstrated the high incorporation of two, three and five deuterium atoms into **1** (*m/z* 412 $[M]^+$, 414 $[M+2]^+$, 415 $[M+3]^+$ and 417 $[M+5]^+$) and **2** (*m/z* 414 $[M]^+$, 416 $[M+2]^+$, 417 $[M+3]^+$ and 419 $[M+5]^+$) (Figs 1 and 2). Ion clusters containing the side-chain moiety $[M-15]^+$ also gave peaks indicating incorporation of a maximum of five deuterium atoms. The peaks at *m/z* 271 and 255 in **1** and *m/z* 273 and 255 in **2**, which correspond to ions which had lost the side-chain, did not show any peak indicating deuterium incorporation. The composition of deuterated and non-deuterated molecules is shown in Table 1. The data indicate that incorporation of deuterated methionine in the first and second methylations is *ca* 40%. Based on these findings, intermediates with the 24-ethylidene side-chain (**8**) clearly do not participate in the biosynthesis of 24β-ethylsterols in *T. kirilowii*.

We have already investigated the labelling patterns of **1** and **2** biosynthesized from [2-$^{13}C^2H_3$]acetate and [1,2-$^{13}C_2$]acetate in the same callus and concluded that 24β-ethylsterols are synthesized by reduction of the 25(26)-double bond in *T. kirilowii* [16]. Thus, the biosynthetic mechanism of 24β-ethylsterols in this callus can be summarized as shown in Scheme 1 (**3**→**4**→**5**→**6**→**7**). This pathway for 24β-ethylsterols seems to be similar to that operating in some *Chlorophytes*, *Trebouxia* sp. [14], *Chrollera* sp. [12, 13] and *Dictyostelium* [15], and also in the higher plant *Clerodendrum campbelli* [17], although in the latter case, introduction of deuterium atoms from methionine is yet to be determined. The 24-ethylidene intermediate (**8**) has been shown to be involved in 24α-ethylsterol biosynthesis in *Hordeum vulgare* [18] and *Physalis peruviana* [19], but ruled out in 24β-ethylsterol (**7**) synthesis in *T. kirilowii*.

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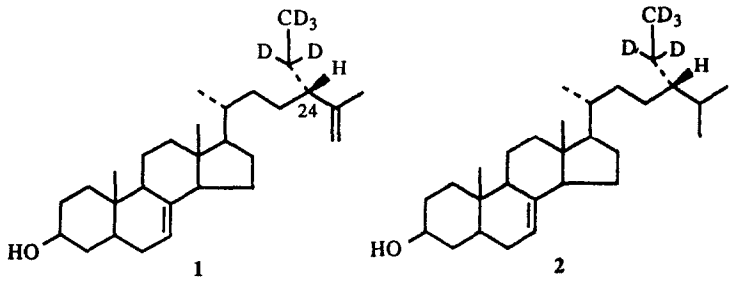


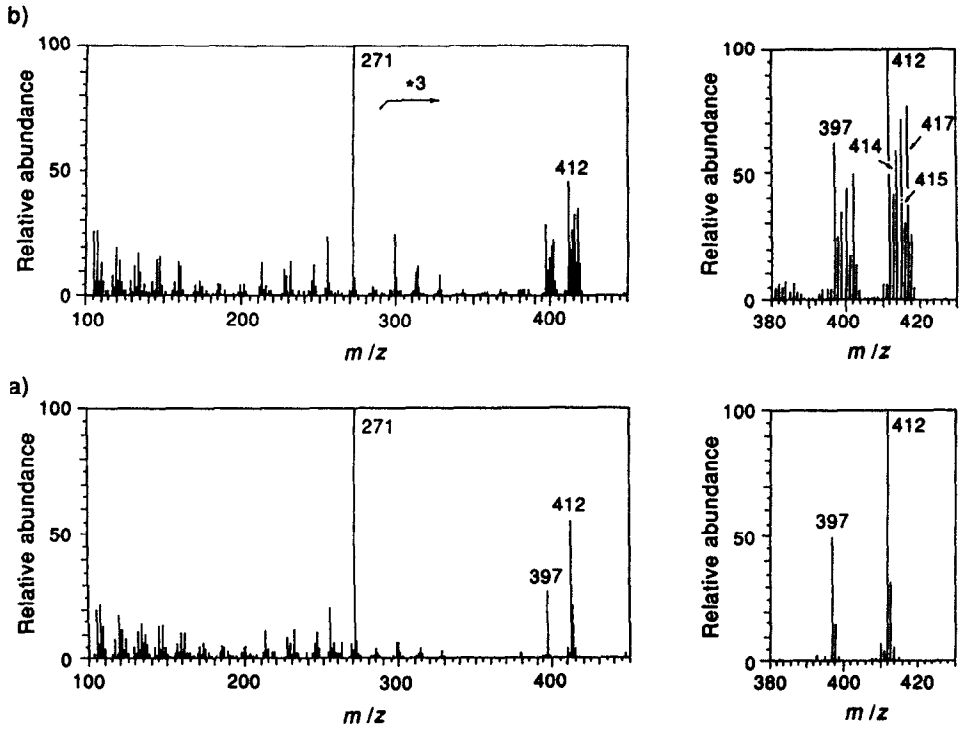


Fig. 1. Mass spectra of (a) non-labelled and (b) labelled 22-dihydro-25-dehydrochondrillasterol (1) isolated from suspension cultures of *T. kirilowii* grown in the absence and presence of L-[Me-$^2H_3$]methionine, respectively.

Table 1. Incorporation of deuterium atoms from L-[Me-$^2H_3$]methionine into side-chains of 24β-ethylsterols 1 and 2 synthesized in suspension cultures of *T. kirilowii*

Compound	Composition of molecular ion (mol %)*					
	D_0†	D_1	D_2	D_3	D_4	D_5
22-Dihydro-25-dehydrochondrillasterol (1)	34.2	3.2	16.7	18.9	3.2	23.9
22-Dihydrochondrillasterol (2)	39.0	2.8	12.1	16.1	4.5	25.5

*Molecular % was obtained by correction of natural abundance isotope.
†D_{0-5} indicates the number of deuterium atoms in the side-chain, 0–5, respectively.

EXPERIMENTAL

L-[Me-$^2H_3$]Methionine (98 atom % of $^2H_3$) was purchased from CEA Service des Molécule (France). ^{1}H NMR spectra were measured at 200 MHz in $CDCl_3$ using TMS as int. standard; *J* values are expressed in Hz. Typical conditions were as follows: spectral width 3201 Hz, acquisition time 3.0 sec, pulse delay 2 sec, and pulse width 12.0 μsec.

Feeding of L-[Me-$^2H_3$]*methionine to tissue cultures.* Established tissue cultures of *T. kirilowii* Maxim. var *japonica* were used [20]. Cell suspension cultures were precultured for 11 days in Linsmaier-Skoog medium (300 ml flask^{-1}) supplemented with

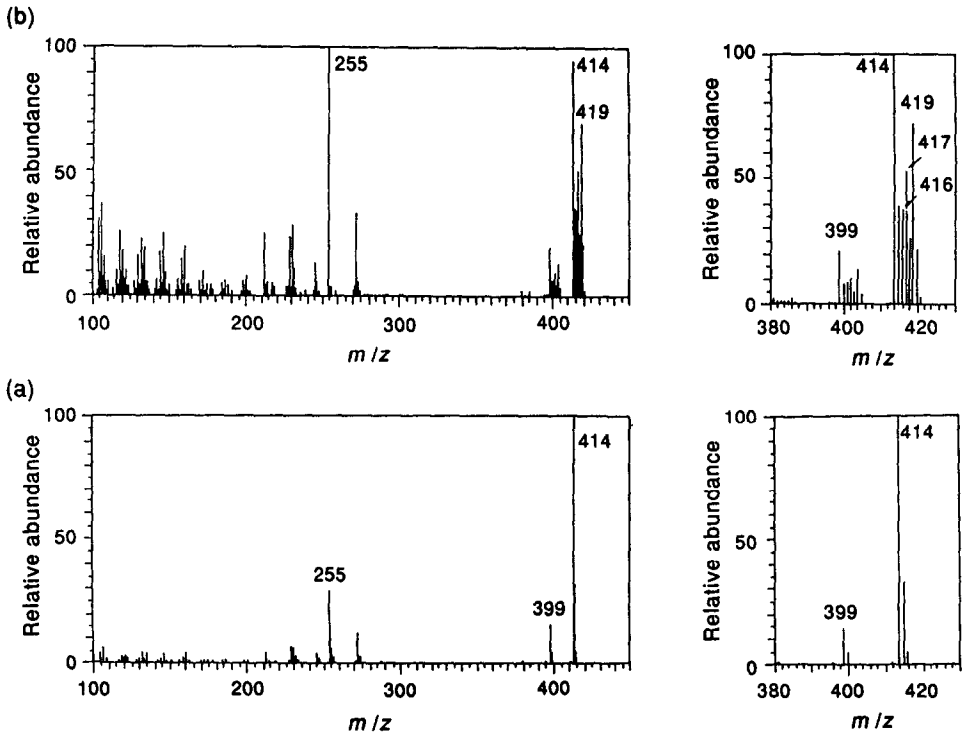
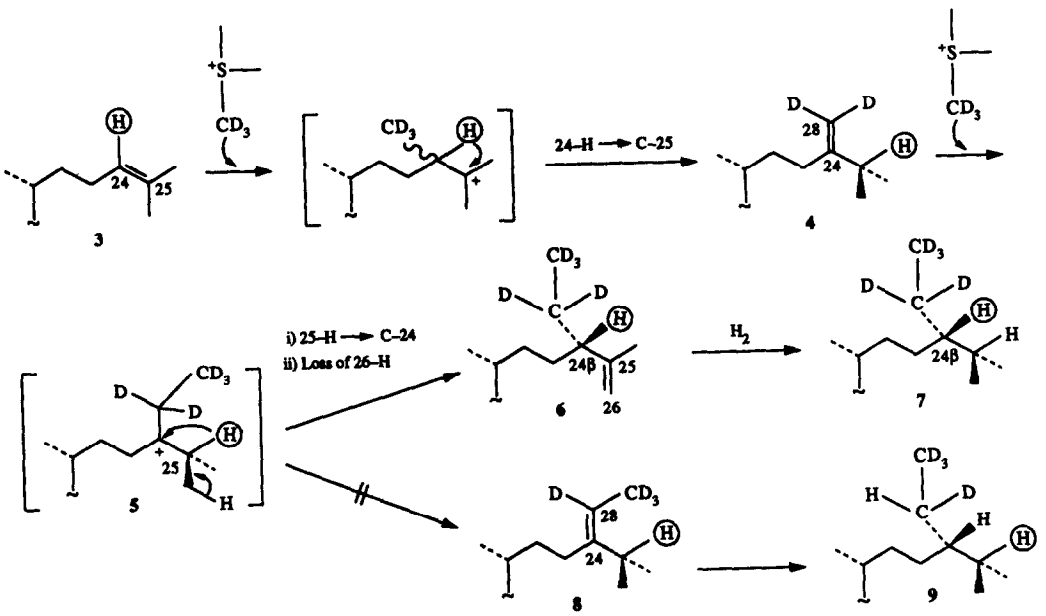


Fig. 2. Mass spectra of (a) non-labelled and (b) labelled 22-dihydrochondrillasterol (2) isolated from suspension cultures of *T. kirilowii* grown in the absence and presence of L-[Me-$^2H_3$]methionine, respectively.



Scheme 1. Proposed mechanism of C-24 alkylation in the biosynthesis of 24β-ethylsterols in tissue cultures of *T. kirilowii* incubated with L-[Me-$^2H_3$]methionine.

10^{-6} M 2,4-D and kinetin (0.02 ppm) at 25° on a rotary shaker (110 rpm). L-[Me-$^2H_3$]Methionine (100 mg) dissolved in H_2O (1 ml) was added to the suspension. After 10 days of incubation, cells (17 g fr. wt) were collected and extracted with hot MeOH (2 × 150 ml). The MeOH extracts (0.7 g) were applied to prep. silica gel TLC plates and developed with hexane–$CHCl_3$–EtOAc (4:1:1). The area corresponding to Δ^7-sterol (R_f 0.22–0.27) (2.4 mg) was extracted and further processed using reverse phase

HPLC. The YMC pack A312 ODS(S5) column (150 × 6 mm i.d.) was eluted with MeOH (0.8 ml min^{-1}) monitored at 210 nm. 22-Dihydro-25-dehydrochondrillasterol (**1**, 0.4 mg, R_t 22 min) and 22-dihydrochondrillasterol (**2**, 0.8 mg, R_t 28 min) were isolated. ^{1}H NMR spectra of **1**; δ 0.527 (3H, *s*, H-18), 0.793 (3H, *s*, H-19), 0.801 (3H, *t*, $J = 7.2$ Hz, H-29), 0.908 (3H, *d*, $J = 6.2$ Hz, H-21), 1.567 (3H, *dd*, $J = 1.4$ and 0.8 Hz, H-27), 3.594 (1H, *m*, H-3), 4.645 (1H, *br d*, $J = 2.8$ Hz, H-26), 4.730 (1H, *d*, $J = 2.8$ and 1.4 Hz, H-26), and 5.160 (1H, *br s*, $W_{1/2}$9, H-7), and **2**; δ0.536 (3H, *s*, H-18), 0.796 (3H, *s*, H-19), 0.813 (3H, *d*, $J = 6.7$ Hz, H-26), 0.833 (3H, *d*, $J = 6.9$ Hz, H-27),, 0.854 (3H, *t*, $J = 7.4$ Hz, H-29), 0.931 (3H, *d*, $J = 6.1$ Hz, H-21), 3.604 (1H, *m*, H-3), and 5.169 (1H, *br s*, $W_{2/1}$ 8, H-7) were identical with those in a previous report [14].

Non-labelled specimens of 22-dihydro-25-dehydrochondrillasterol (0.4 mg) and 22-dihydrochondrillasterol (1.3 mg) were isolated from cells (27.9 g fr. wt) grown in the same medium (300 ml $flask^{-1}$) as that mentioned above without L-[Me-$^2H_3$]methionine as a control run. Cell growth was diminished by 2.2 mM methionine.

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