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Stereochemical fate of C-26 and C-27 during the conversion of isofucosterol to sitosterol and of 24-methylenecholesterol to campesterol and dihydrobrassicasterol in *Oryza sativa* cell cultures

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Abstract

Administration of pro-*R*-methyl-^{13}C-labeled isofucosterol to cultured cells of *Oryza sativa* revealed that the pro-*R* and pro-*S* methyls at C-25 become the pro-*R* and pro-*S* methyls at C-25 of sitosterol, respectively. Similar administration experiments using pro-*S*-methyl-^{13}C-labeled 24-methylenecholesterol established that the pro-*R* and pro-*S* methyls at C-25 of 24-methylenecholesterol become the pro-*R* and pro-*S* methyls of campesterol, and the pro-*S* and pro-*R* methyls of dihydrobrassicasterol, respectively. These results are compatible with our recently proposed '*syn*-S_E2' mechanism' for double bond isomerization of $\Delta^{24(28)}$ into $\Delta^{24(25)}$.

Keywords: *Oryza sativa*; Sterol; Biosynthesis; Gramineace; Isofucosterol; Sitosterol; 24-Ethyldesmosterol; 24-Methyldesmosterol

1. Introduction

In the biosynthesis of the side chain of sitosterol (**1**), campesterol (**2**) and dihydrobrassicasterol (**3**), common 24-alkylsterols in higher plants, $\Delta^{24(28)}$-olefinic sterols, e.g., isofucosterol (**4**) and 24-methylenecholesterol (**5**) isomerize to $\Delta^{24(25)}$-olefinic sterols, e.g., 24-ethyldesmosterol (**6**) and 24-methyldesmosterol (**7**), which are then reduced to furnish **1**–**3** (Scheme 1) (Nes and McKean, 1977). Concerning the two methyl groups (C-26 and C-27) on the prochiral C-25 center of the phytosterols, it has been reported that the pro-*S* methyl of **1**, **2**, **4** and **5** and the pro-*R* methyl of **3** originate from C-2 of mevalonate, while the other methyls are derived from C-6 of mevalonate (Seo et al., 1978, 1984, 1986, 1990, 1992; Nes et al., 1992; Guo et al., 1996; Fujimoto et al., 1997a, 1998a). The metabolic origins of C-26 and C-27 were deduced mainly on the basis of administration experiments using [1,2-$^{13}C_2$]acetate. A contrasting finding that the pro-*R* methyl on C-25 of **4** derives from C-2 of mevalonate was reported with *Pinus pinea* (Nicotra et al., 1981).

We recently established that the (*E*)-methyl of **6** becomes the pro-*S* methyl of **1** (Fujimoto et al., 1998a), and that the corresponding methyl of **7** becomes the pro-*S* methyl of **2** and the pro-*R* methyl of **3** (Fujimoto et al., 1997b) in *Oryza sativa* cell cultures. Thus, reduction of **6** and **7** should proceed with the *anti*-addition of hydrogen atoms. On the basis of these findings with *O. sativa* cell cultures, combined with the observations of the origin of C-26 and C-27 of **4** and **5** reported for other plants (Seo et al., 1990, 1992; Fujimoto et al., 1998b), the double bond isomerization is suggested to follow a mechanism in which the pro-*S* methyls of **4** and **5** become the (*E*)-methyls of **6** and **7**. The purpose of the present investigation is to substantiate this hypothesis. Because sterols **6** and **7** are not present in isolable amount in *O. sativa* cell cul-

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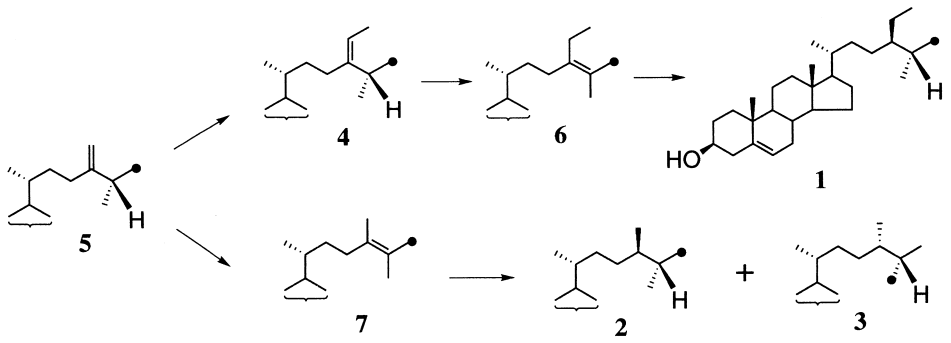


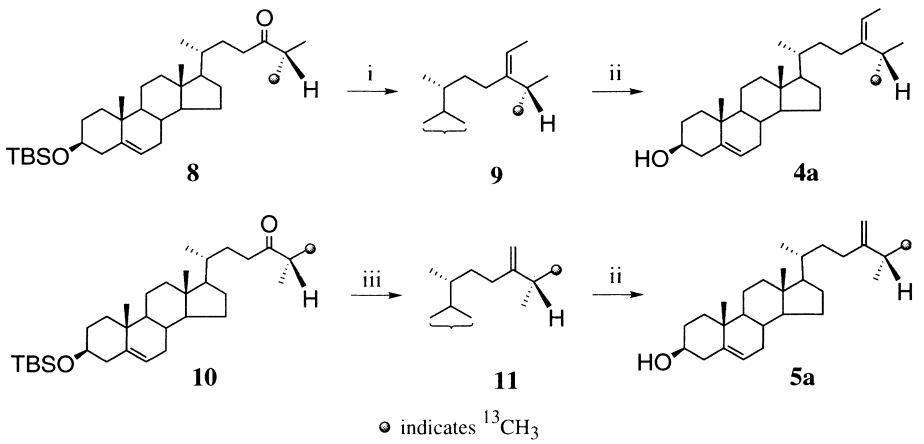
Scheme 1. Biosynthetic pathway of the side chain of common plant sterols **1**–**3**. Dots designate the carbons derived from C-2 of mevalonate.

tures, no direct correlation between **4** and **6**, and **5** and **7** is possible. On the other hand, an analysis of the metabolic correlation of C-26 and C-27 between **4** and **1**, and between **5** and **2/3**, should be informative particularly since the stereochemical course of **6** to **1**, and **7** to **2/3** has been firmly established (Fujimoto et al., 1997b, 1998a).

2. Results and discussion

We considered that the metabolic correlation of interest might be obtained conveniently by administering stereospecifically ^{13}C-labeled substrates combined with ^{13}C-NMR spectroscopic analyses. The requisite stereochemically defined ^{13}C-labeled sterols, pro-*R*-methyl-^{13}C-labeled isofucosterol (**4a**) and pro-*S*-methyl-^{13}C-labeled 24-methylenecholesterol (**5a**), were synthesized according to Scheme 2 from stereospecifically ^{13}C-labeled 24-oxocholesterol derivatives for which we had previously described a convenient synthetic method (Fujimoto et al., 1990). Thus, the pro-*R*-methyl-^{13}C-labeled 24-ketone **8** (83% of the ^{13}C label resided at the pro-*R* methyl and 17% at the pro-*S* methyl) was reacted with triphenylethylidenephosphorane (Dusza, 1960) to afford the ethylidene **9** which upon desilylation gave desired **4a** [78% of the ^{13}C label resided at the pro-*R* methyl (δ 21.08) and 22% at the pro-*S* methyl (δ 21.00), containing ca. 15% of fucosterol] (Seo et al., 1990). The small amount of fucosterol contamination was discounted, as it is not converted into sitosterol in *O. sativa* cell cultures (Okuzumi and Fujimoto, unpublished data). The known pro-*S*-methyl-^{13}C-labeled 24-ketone **10** (85% of the ^{13}C label resided at the pro-*S* -methyl and 15% at the pro-*R* -methyl) was reacted with triphenylmethylenephosphorane (Bergmann and Dusza, 1957) to give exomethylene derivative **11** which upon desilylation under acidic conditions furnished **5a** [80% of the ^{13}C label resided at the pro-*S* methyl (δ 21.81) and 20% at the pro-*R* methyl (δ 21.99)] (Fujimoto et al., 1997a).

The pro-*R*-methyl-^{13}C-labeled isofucosterol **4a** was administered to *O. sativa* cell cultures as described previously (Yamada et al., 1997), and the resulting sterol



Reagents: i) $Ph_3P^+EtBr^-$, *n*-BuLi, ether, 100°C, ii) HCl, THF, iii) $Ph_3P^+EtBr^-$, *n*-BuLi, THF, 100°C

Scheme 2. Synthesis of pro-*R*-methyl-^{13}C-labeled isofucosterol (**4a**) and pro-*S*-methyl-^{13}C-labeled 24-methylenecholesterol (**5a**).

fraction obtained was separated by reversed-phase HPLC to furnish sitosterol (**1**). The ^{13}C-NMR spectrum of **1** (Fig. 1) showed an enriched signal (δ 19.04) due to the pro-*R* methyl at C-25, accompanied by a weakly enriched signal (δ 19.82) due to the pro-*S* methyl at C-25 (Horibe et al., 1989). The intensities (4:1) of the two peaks were approximately equal to those of the substrate. It is therefore established that the pro-*R* methyl at C-25 of **4** becomes the pro-*R* methyl of **1**, while the pro-*S* methyl of **4** becomes the pro-*S* methyl of **1**. This finding is consistent with the aforementioned biosynthetic origin of the pro-*S* (derived from C-2 of mevalonate) and pro-*R* methyl groups at C-25 of **4** in *Catharanthus roseus* (Fujimoto et al., 1998b) and *Physalis perviana* (Seo et al., 1990).

Similar administration experiments using pro-*S*-methyl-^{13}C-labeled 24-methylenecholesterol (**5a**) afforded, after HPLC separation, campesterol (**2**) and dihydrobrassicasterol (**3**) as an inseparable mixture. The ^{13}C-NMR spectrum (Fig. 2) of the mixture showed enriched signals at δ 18.26 (the pro-*S* methyl of **2**) and 17.60 (the pro-*R* methyl of **3**), accompanied by weakly enriched signals at δ 20.19 (the pro-*R* methyl of **2**) and 20.50 (the pro-*S* methyl of **3**) (Colombo et al., 1990). These data clearly indicate that the pro-*S* methyl of **5** is converted to the pro-*S* methyl of **2** and the pro-*R* methyl of **3**. The fact that the ratio of the two major enriched signals in Fig. 2 was closely similar to that obtained upon incubation of (*E*)-methyl-^{13}C-labeled 24-methyldesmosterol (Fujimoto et al., 1997b) supports the view that conversion proceeds via 24-methyldesmosterol as an obligatory intermediate, ruling out a mechanism of direct reduction of the $\Delta^{24(28)}$-double bond leading to **2** and **3**.

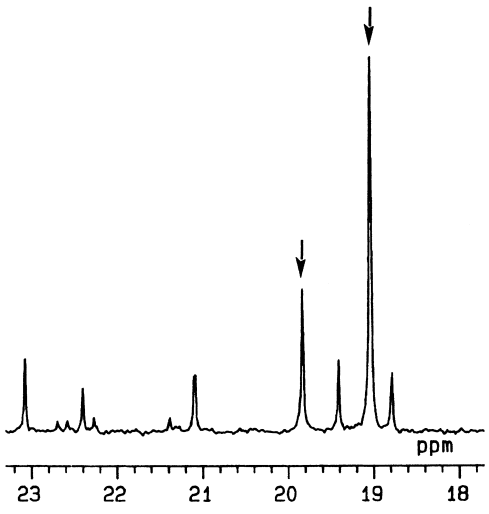


Fig. 1. ^{13}C-NMR spectrum (125 MHz, in $CDCl_3$) of sitosterol derived from pro-*R*-methyl-^{13}C-labeled isofucosterol (**4a**). The pro-*R* and pro-*S* methyls at C-25 of **1** resonate at δ 19.04 and 19.82, respectively.

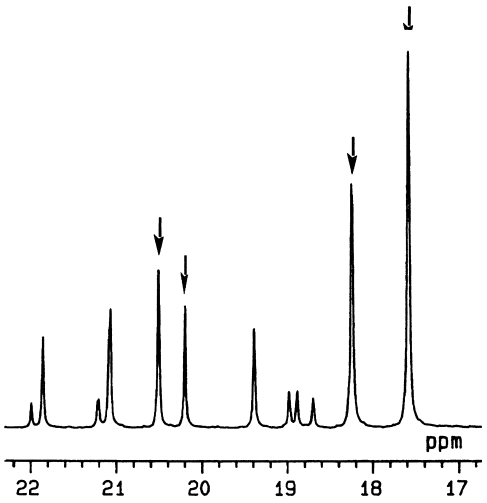


Fig. 2. The ^{13}C-NMR spectrum (125 MHz, in $CDCl_3$) of a mixture of campesterol and dihydrobrassicasterol derived from pro-*S*-methyl-^{13}C-labeled 24-methylenecholesterol. The pro-*R* and pro-*S* methyls at C-25 of **2** resonate at δ 20.19 and 18.26, respectively, and the corresponding signals of **3** resonate at δ 17.60 and 20.50.

In the present paper, it has been unequivocally established that the pro-*S* methyl (one derived from C-2 of mevalonate) of **4** is converted to the pro-*S* methyl of **1** in *O. sativa* cell cultures. Further, it has been elucidated that the pro-*S* methyl (one derived from C-2 of mevalonate) of **5** becomes the pro-*S* methyl of **2** and the pro-*R*-methyl of **3**. These findings, combined with our earlier observations (Fujimoto et al., 1997a, 1998b) in the conversion from **6** to **1** and **7** to **2** and **3**, allow us to depict the metabolic correlation of C-26 and C-27 in *O. sativa* cell cultures as illustrated in Scheme 1.

We recently demonstrated that C-28 hydrogen of **4** become the pro-*R* hydrogen at C-28 of **1**, proposing a '*syn*-S_E2' mechanism' (Fig. 3, R = Me) for the double bond isomerization of **4** to **6** (Okuzumi et al., 1999). A similar mechanism is suggested to be operating in the conversion of **5** to **7** (Fig. 3, R = H). In conclusion, the present study has provided further experimental basis for our proposal that isomerization of the $\Delta^{24(28)}$-olefinic sterols (**4** and **5**) to the $\Delta^{24(25)}$-olefinic sterols (**6** and **7**) follow a '*syn*-S_E2' mechanism' and the sub-

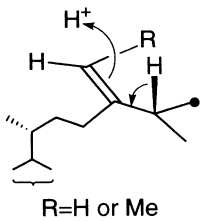


Fig. 3. Postulated stereostructure (*syn*-S_E2' mechanism) for the double bond isomerization from $\Delta^{24(28)}$ to $\Delta^{24(25)}$.

sequent reduction proceeds via an '*anti*-addition mechanism'. These mechanisms appear to be general for phytosterol biosynthesis in higher plants, although such a thorough study including a $\Delta^{24(25)}$-olefinic sterol is limited.

3. Experimental

3.1. General

Cell cultures of *O. sativa* were maintained as described previously (Yamada et al., 1997). ^{1}H-NMR (500 MHz) spectra were obtained on a JEOL Alpha 500 spectrometer in $CDCl_3$ solutions and chemical shifts (δ) are reported in ppm downfield from tetramethylsilane (used as internal reference). ^{13}C-NMR (125 MHz) spectra were recorded on the same spectrometer and chemical shifts are referenced to the signal (δ 77.0) of $CDCl_3$. HPLC separations were performed on a Shimadzu LC-6A with a SPD-6A UV detector, equipped with Shim-pack CLC-ODS column (6 mm i.d. × 15 cm).

3.2. Synthesis of pro-R-methyl-^{13}C-labeled isofucosterol (**4a**)

To a suspension of triphenylethylphosphonium bromide (464 mg, 1.2 mmol) in dry ether (5 ml) placed in a pressure flask was added *n*-BuLi (1.50 M hexane soln., 653 μl) at room temperature under N_2 and the mixture was stirred for 30 min. To this ylide solution, the ketone **8** (260 mg, 0.49 mmol) was added and the mixture was heated at 100°C for 2 h. After cooling, the mixture was diluted with ether and dil. HCl. The organic layer was washed with sat. $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated. The crude product was subjected to silica gel chromatography with hexane–AcOEt (100:1) as eluent to give the TBS ether **9** (67 mg), and then with hexane–AcOEt (10:1) to recover **8** (169 mg). The former was dissolved in THF (2 ml) with the resulting solution stirred for 2.5 h after addition of conc. HCl (200 μl). Extractive workup gave a product, which was recrystallized from MeOH to afford **4a** (32 mg, 11%) as white crystals, mp 113–115°C. EI-MS *m/z*: 413 (M^+), 398, 380, 314, 299, 281, 271, 255, 229. ^{1}H-NMR δ: 0.68 (*s*, 18-H_3), 0.95 (*d*, J = 6.5 Hz, 21-H_3), 0.97 (*dd*, J = 6.5 Hz, $^1J_{C-H}$ = 125 Hz, pro-*R*-Me), 0.98 (*t*, J = 6.5 Hz, $^2J_{C-H}$ = 6.5 Hz, pro-*S*-Me), 1.01 (*s*, 19-H_3), 1.59 (3H, *d*, J = 7.0 Hz, 29-H_3), 2.82 (*m*, 25-H), 3.53 (*m*, 3-H), 5.11 (*q*, J = 7.0 Hz, 28-H), 5.18 (0.15H, *q*, J = 7.0 Hz, H-28 of fucosterol), 5.35 (*m*, 6-H). ^{13}C-NMR δ: 11.85 (C-18), 12.74 (C-29), 18.80 (C-21), 19.39 (C-19), 21.00 (pro-*S*-Me, enriched signal), 21.08 (pro-R-Me, enriched signal), 21.08 (C-11), 22.22 (pro-*R*-Me of fucosterol, enriched signal), 24.29 (C-15), 27.89 (C-23), 28.22 (C-16), 28.59 (δ, $^2J_{C-C}$ = 35 Hz, C-25), 31.66 (C-2), 31.91 (C-7 and C-8), 35.95 (C-22), 36.16 (C-20), 36.50 (C-10), 37.24 (C-1), 39.77 (C-12), 42.30 (C-4), 42.33 (C-13), 50.12 (C-9), 55.99 (C-17), 56.75 (C-14), 71.80 (C-3), 116.45 (C-28), 121.71 (C-6), 140.75 (C-5), 145.88 (C-24).

3.3. Synthesis of pro-S-methyl-^{13}C-labelled 24-methylenecholesterol (**5a**)

To a suspension of triphenylmethylphosphonium bromide (235 mg, 0.66 mmol) in dry THF (1.6 ml) placed in a pressure flask was added *n*-BuLi (1.50 M hexane soln., 436 μl) at room temperature under N_2 and the mixture was stirred for 30 min. To this ylide solution, the ketone **10** (170 mg, 0.33 mmol) was added and the mixture was heated at 100°C overnight. After cooling, it was diluted with ether and dil. HCl. The organic layer was washed with sat. $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated. The crude product was chromatographed over silica gel with hexane–AcOEt (15:1) to give the TBS ether **11** (107 mg), and then with hexane–AcOEt (3:1) to give **5a** (21 mg). The former compound was desilylated as described for **9**. The resulting **5a** was mixed with the above **5a** and recrystallized from MeOH to yield pure **5a** (77 mg, 58% from **10a**) as white crystals, mp 138–140°C. EI-MS *m/z*: 399 (M^+), 384, 381, 366, 314, 299, 281, 271, 253, 229. ^{1}H-NMR δ: 0.69 (*s*, 18-H_3), 0.95 (*d*, J = 6.5 Hz, 21-H_3), 1.01 (*s*, 19-H_3), 1.02 (*t*, J = 6.5 Hz, $^2J_{C-H}$ = 6.5 Hz, pro-*R*-Me), 1.03 (*dd*, J = 6.5 Hz, $^1J_{C-H}$ = 126 Hz, pro-*S*-Me), 3.49 (*m*, 3-H), 4.66 (*brs*, 28-Ha), 4.71 (*brs*, 28-Ha), 5.35 (1H, *m*, 6-H). ^{13}C-NMR δ: 11.85 (C-18), 18.70 (C-21), 19.39 (C-19), 21.08 (C-11), 21.86 (pro-*S*-Me, enriched signal), 21.99 (pro-*R*-Me, enriched signal), 24.27 (C-15), 28.21 (C-16), 30.97 (C-23), 31.66 (C-2), 31.91 (C-7 and C-8), 33.79 (*d*, $^2J_{C-C}$ = 35 Hz, C-25), 34.69 (C-22), 35.74 (C-20), 36.50 (C-10), 37.25 (C-1), 39.77 (C-12), 42.30 (C-4), 42.35 (C-13), 50.12 (C-9), 55.99 (C-17), 56.77 (C-14), 71.80 (C-3), 105.94 (C-28), 121.68 (C-6), 140.77 (C-5), 156.9 (C-24).

3.4. Precursor administration experiments

To cultured cells of *O. sativa* (2 weeks, four 500 ml-flasks, each containing 250 ml of N6 medium (Chu et al., 1975) supplemented with sucrose 30 g/l, proline 2.8 g/l, casein hydrolysate 300 mg/l, 2,4-D 2 mg/l), a solution of **4a** (30 mg) in acetone (1 ml) and Tween 80 (1 ml) was added evenly through a membrane filter. Incubation was continued for another 2 weeks and the cells were collected by filtration. The sterol fraction was extracted and separated from the wet cells as described previously (Yamada et al., 1997). HPLC separation

(conditions: solvent, MeOH; flow rate 1.0 ml/min; retention time 11.9 min) of the sterol fraction gave pure **1** (4 mg). Administration of compound **5a** (50 mg) was similarly carried out to afford a mixture of **2** and **3** (27 mg) after HPLC separation (conditions: solvent, MeOH; flow rate 1.0 ml/min; retention time 11.9 min for **2/3**).

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