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Bioorganic & Medicinal Chemistry 14 (2006) 732–738
Bioorganic & Medicinal Chemistry

Metabolic conversion of 24-methyl-Δ^{25}-cholesterol to 24-methylcholesterol in higher plants

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Received 6 July 2005; revised 26 August 2005; accepted 27 August 2005
Available online 6 October 2005

Abstract—Feeding of chemically synthesized [27-^{13}C]codisterol ([27-^{13}C]**2**), [27-^{13}C]24-epicodisterol ([27-^{13}C]**3**), [23,24-$^{2}H_2$]codisterol ([23,24-$^{2}H_2$]**2**), and [26,27-$^{2}H_6$]24-methyldesmosterol ([26,27-$^{2}H_6$]**8**) to *Oryza sativa* cell cultures, followed by MS and NMR analysis of the biosynthesized dihydrobrassicasterol (**9**)/campesterol (**10**), revealed that both (24*R*)- and (24*S*)-epimers of 24-methyl-Δ^{25}-cholesterol (**2**/**3**) were converted to **9** and **10** via the common intermediate 24-methyldesmosterol (**8**).

1. Introduction

The final step of plant sterol biosynthesis involves hydrogenation of either Δ^{24}, $\Delta^{24(28)}$, or Δ^{25} olefinic sterols (Scheme 1). It has been established that the tetrasubstituted Δ^{24} sterol (**12**) serves for the hydrogenation substrate to form 24α-ethylsterol, for example, sitosterol (**13**), whereas the corresponding methyl analog (**8**) is reduced to 24β-methylsterol, for example, dihydrobrassicasterol (**9**), as well as 24α-methylsterol, for example, campesterol (**10**), in higher plants.[1–5] The 24-exomethylene-sterol (**4**) is a well-known intermediate of ergosterol biosynthesis in fungi,[6,7] while the 24-ethyl-Δ^{25}-olefinic compound (**5**) is hydrogenated to give poriferasterol (**6**) in *Trebouxia* sp.[8] and chondrillasterol (**7**) in *Trichosanthes kirilowii* (Cucurbitaceae).[9] However, no hydrogenation of a major sterol of *Ajuga* species (Labiatae), clerosterol (24β-ethyl-Δ^{25}-cholesterol) appears to occur.[10]

As for 24-methyl-Δ^{25} sterols, codisterol [(24*S*)-24-methylcholesta-5,25-dien-3β-ol] (**2**) and 24-epicodisterol (**3**) were only rarely found in higher plants and marine sponges,[11] and information on their metabolism is lacking (dashed arrow in Scheme 1).[12] The same is true of *Oryza sativa*, one of our stock plant cell cultures which contains a 1:1 mixture of dihydrobrassicasterol (**9**) and campesterol (**10**), but is devoid of **2**/**3**.[13] Here, we have investigated on the possibility of **2** and **3** as alternative biogenetic precursors of dihydrobrassicasterol (**9**) and/or campesterol (**10**) in higher plants. Feeding of the chemically synthesized [27-^{13}C]codisterol ([27-^{13}C]**2**), [27-^{13}C]24-epicodisterol ([27-^{13}C]**3**), [23,24-$^{2}H_2$]codisterol ([23,24-$^{2}H_2$]**2**), and [26,27-$^{2}H_6$]24-methyldesmosterol ([26,27-$^{2}H_6$]**8**) to cultured cells of *O. sativa*, followed by MS and NMR analysis of the resulting metabolites, revealed that both **2** and **3** were converted to a mixture of **9** and **10** via 24-methyldesmosterol (**8**).

2. Results and discussion

The requisite labeled sterols were prepared in a slight modification of our previous method used for the synthesis of Δ^{24}- and Δ^{25}-sterols (Scheme 2).[1,14] The known (24*R*)-ester **15**[15] was alkylated with [^{13}C]methyl iodide. The C-25 methylated ester was converted to [27-^{13}C]**2** in four steps, that is, reduction with $LiAlH_4$, catalytic hydrogenation, dehydration, and final regeneration of Δ^{5}-3β-ol. The corresponding 24-epimer, [27-^{13}C]**3**, was prepared starting with the (24*S*)-ester **16**[15] in the same manner. For the preparation of [23,24-$^{2}H_2$]**2**, the known (22*R*)-acetylenic alcohol **17**[15,16] was first converted to the 23,24-di-deuterated analog of **15** in two steps (reduction with $LiAl^{2}H_4$[16] followed by quenching with $^{2}H_2O$ and orthoester Claisen rearrangement). The deuterated

Keywords: Steroids and sterols; Biosynthesis; Campesterol; Dihydrobrassicasterol; Codisterol; *Oryza sativa*.
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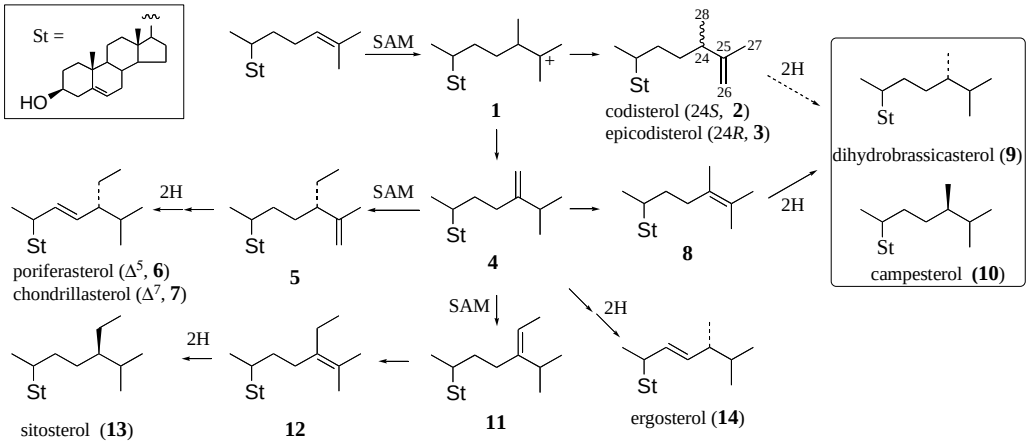
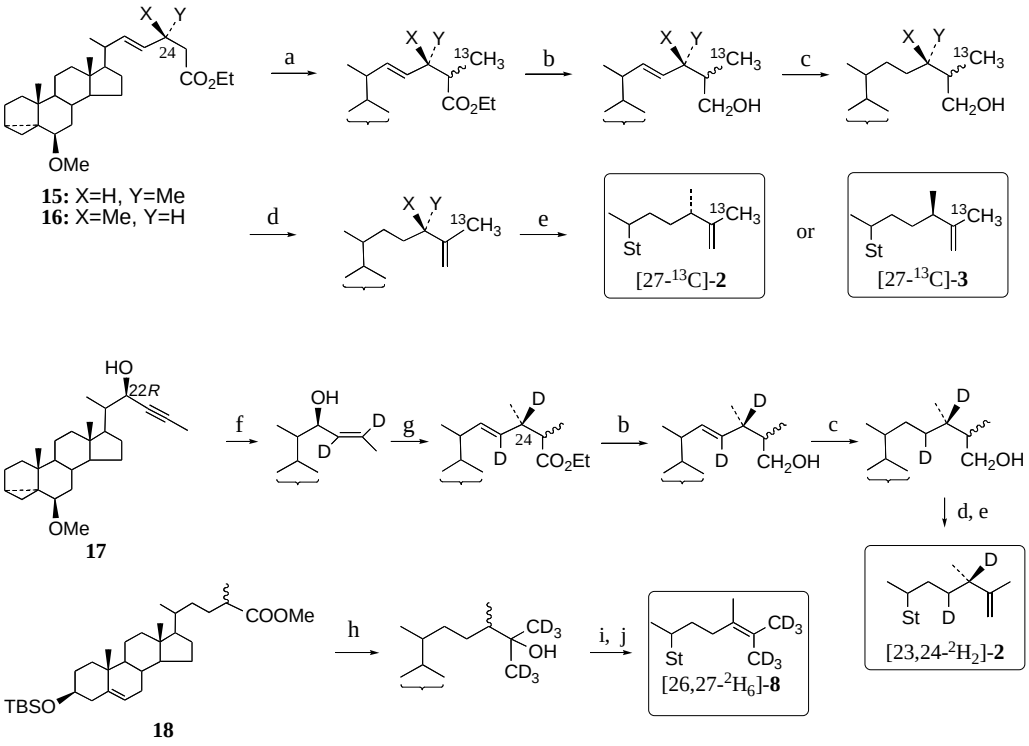
doi:10.1016/j.bmc.2005.08.061



Scheme 1. Biosynthetic pathway of phytosterol (24-alkylsterol). St, Δ^5-, Δ^7-, or $\Delta^{5,7}$-3β-hydroxysterol skeleton; 2H, hydrogenation; SAM, *S*-adenosylmethionine.



Scheme 2. Preparation of labeled substrates. St denotes 3β-hydroxy-Δ^5-sterol skeleton. Reagents: (a) LDA, $^{13}CH_3I$, (b) $LiAlH_4$, (c) H_2-Pd/C, (d) *m*-NO_2-PhSeCN, Bu_3P, then H_2O_2, (e) aq dioxane, TsOH, (f) $LiAlD_4$/THF, then D_2O, (g) $CH_3CH_2C(OEt)_3$, $EtCO_2H$, (h) CD_3MgBr, (i) $POCl_3$, (j) HCl.

ester was subjected to the procedure described above to give [23,24-$^2H_2$]**2**. [26,27-$^2H_6$]**8** was prepared starting from the methyl ester **18**[17] according to the procedure used for the synthesis of [26,27-$^{13}C_2$]24-methyldesmosterol[1] with C^2H_3MgI as a source of the 26,27-C^2H_3 groups.

Feeding experiments of the labeled substrates to *O. sativa* cell cultures were carried out as described previously.[1] The biosynthesized 24-methylcholesterols (**9**/**10**) obtained as a mixture (24*S*/24*R* = ca. 1:1) after final HPLC fractionation were subjected to spectroscopic analysis as such.[18] The recovered substrates were also analyzed by NMR and MS spectroscopy, which established no configurational interconversion on C-24 and C-25 during incubation.

The ^{13}C NMR spectra of 24-methylsterols (**9**/**10**) biosynthesized from [27-^{13}C]**2** and [27-^{13}C]**3** are illustrated in Figure 1. The signals[19] of the pro-*R*-Me (δ 17.6) of **9** and the pro-*S*-Me (δ 18.3) of **10** were enriched compared to those of non-labeled samples, whereas the signal intensities for the pro-*S*-Me (δ 20.5) of **9** and the pro-*R*-Me (δ 20.2) of **10** were the same as those of endogenous samples. These data indicated that both codisterol **2** and 24-epicodisterol **3** were metabolized to 24-meth-

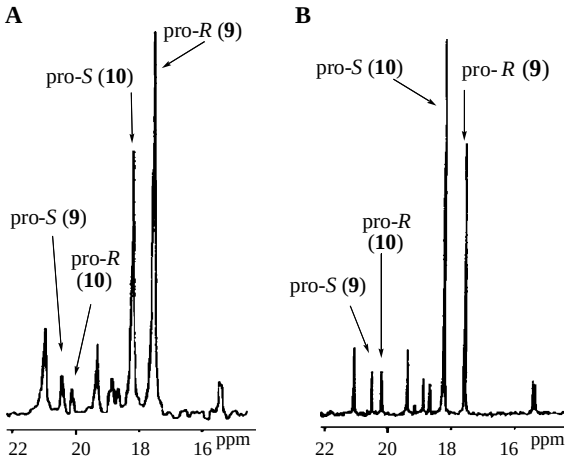


Figure 1. Partial ^{13}C NMR spectra (125 MHz, $CDCl_3$) of 24-methylcholesterols **9/10** derived from [27-^{13}C]codisterol (A) and from [27-^{13}C]24-epicodisterol (B).

ylsterols (**9/10**) in a stereospecific manner.[20] It should be noted that both [27-^{13}C]**2** (24β) and [27-^{13}C]**3** (24α) were converted to give a mixture of **9** (24β) and **10** (24α). These results ruled out a direct reductive conversion of **2** and **3** to **9/10** and suggested that the Δ^{25}-sterols first isomerize into the common intermediate Δ^{24}-sterol (**8**) which should be a substrate for hydrogenation. However, there remains a possibility that the direct hydrogenation of Δ^{25} is cooperative as a minor pathway since dihydrobrassicasterol (**9**) was more ^{13}C-enriched than campesterol (**10**) when [27-^{13}C]codisterol was fed, and vice versa when [27-^{13}C]epicodisterol was fed (Fig. 1).

This possibility can be elucidated by tracing the metabolic fate of the 24-hydrogen atom of **2** and/or **3**. For this purpose, a feeding experiment of [23,24-$^2H_2$]**2** was carried out. The resulting 24-methylcholesterol (**9/10**) was analyzed by GC–MS. As can be seen in Figure 2B, the biosynthesized **9/10** clearly showed a molecular ion peak at *m/z* 401 for a mono-deuterated species, along with a molecular ion peak at *m/z* 400 for the endogenous **9/10**, whereas a molecular ion peak (*m/z* 402) for di-deuterated species was negligible. Analysis of dehydration ($M^+ - H_2O$) ions supported the above results. It is thus likely that 24-^2H of [23,24-$^2H_2$]**2** was eliminated and 23-^2H retained during the bioconversion. The location of the retained deuterium atom in the biosynthesized 24-methylcholesterols (**9/10**) was unambiguously determined by ^2H-decoupled ^{13}C NMR analysis. The spectrum (Fig. 3) showed weak but significant signals due to deuterium isotope-shifts in addition to the signals of the endogenous 24-methylcholesterols. The assignments of the signals are summarized in Table 1 which clearly indicated that the C-24 deuterium atom of [23,24-$^2H_2$]**2** was eliminated during the bioconversion into **9/10**, whereas the C-23 deuterium atom of the substrate was retained at the same position. This implies that the direct reductive conversion of **2/3** to **9/10** is not operative in *O. sativa* cell cultures and the double-bond isomerization from Δ^{25} to Δ^{24} is an obligatory step for **2** and **3** to be reduced. This is in marked contrast to the 24β-ethylsterol biosynthesis in *Trebouxia* sp. and

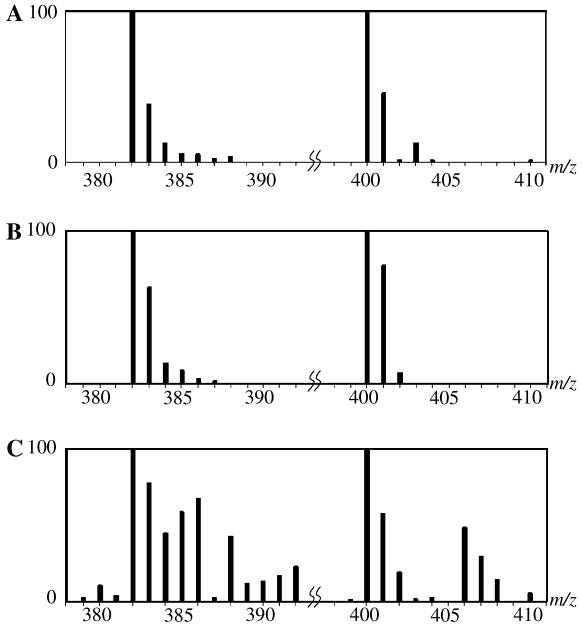


Figure 2. Partial mass spectra of 24-methylcholesterols **9/10**. A, non-labeled; B, biosynthesized from [23,24-$^2H_2$]codisterol; C, biosynthesized from [26,27-$^2H_6$]24-methyldesmosterol. The spectra were scanned at a relatively early part of the GC peak wherein the deuterium-labeled compound was more enriched than the center of the peak due to the isotope effect.

T. kirilowii, in which the Δ^{25} precursor (**5**) is directly hydrogenated without isomerization to Δ^{24}-olefin (**12**).[8,9]

24-Methyl-Δ^{25}-sterols (**2** and **3**) would be formed by deprotonation of a C-25 cationic species (**1**, Scheme 1) generated by the methyl transfer from SAM onto Δ^{24}-sterol. However, isomerization of the 24-double bond of 24-methyldesmosterol (**8**) into the 25-double bond might be an alternative way to yield **2/3**. To explore this possibility, [26,27-$^2H_6$]**8** was fed to *O. sativa* cell cultures. The mass spectrum of the biosynthesized 24-methylcholesterol **9/10** (Fig. 2C) exhibited a molecular ion peak at *m/z* 406 due to $^2H_6$-24-methylcholesterol in addition to a molecular ion peak at *m/z* 400 for the

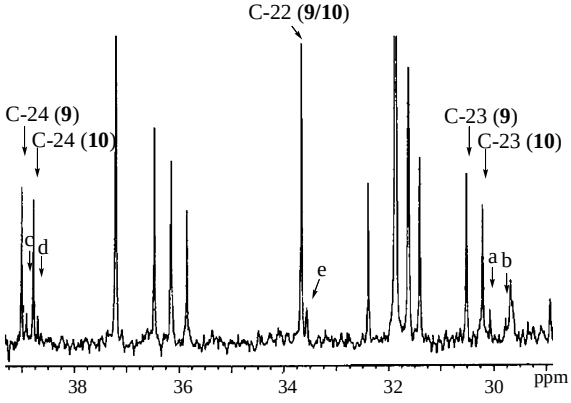
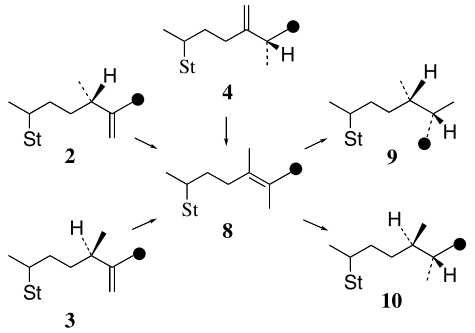


Figure 3. Partial {^1H}{^2H}-^{13}C NMR spectrum (125 MHz, $CDCl_3$) of 24-methylcholesterols **9/10** derived from [23,24-$^2H_2$]**2**. For the assignments of peaks a–e, see Table 1.

Table 1. Assignments of pertinent ^{13}C signals of 24-methylcholesterol **9/10** biosynthesized from [23,24-$^{2}H_2$]**2**

Carbon	δ		Upfield shift
	Non-labeled	^{2}H-labeled	
C-24 of **9**	39.02	38.92 (c)	0.10
C-24 of **10**	38.79	38.70 (d)	0.09
C-23 of **9**	30.52	30.08 (a)	0.44
C-23 of **10**	30.22	29.78 (b)	0.47
C-22 of **9/10**	33.67	33.57 (e)	0.15

Note. (a)–(e) refer to indicated points in Fig. 1.



Scheme 3. Proposed mechanism of the metabolic conversion of codisterol **2** and 24-epicodisterol **3** to 24-methylcholesterol **9/10** in *O. sativa*. Dots represent correlations of C-26 and C-27.

endogenous 24-methylcholesterol, whereas ions at *m/z* 405, 404, and 403 were negligible. The results revealed that [26,27-$^{2}H_6$]**8** was converted to **9/10** without any loss of deuterium atom, thus ruling out the possibility of the double-bond isomerization from Δ^{24} to Δ.[25]

We previously established in the ^{13}C-labeling study with cell cultures of *O. sativa* and *Catharanthus roseus* that the *pro*-(*S*)-Me of 24-exomethylenesterol (**4**) becomes stereospecifically the (*E*)-Me of 24-methyldesmosterol **8**[21] and reduction of **8** with *anti*-addition mechanism yields **9** and **10**.[2] The ^{13}C-labeling pattern (Fig. 1) of 24-methylcholesterols (**9** and **10**) derived from [27-^{13}C]**2** and **3** in the present study is identical with those of **9** and **10** derived from [(*E*)-Me-^{13}C]**8** previously.[2,21] This implies that the conversion of **2** and **3** to **8** takes place in such a stereospecific manner that the ^{13}C-labeled carbons (C-27) of **2** and **3** become the (*E*)-Me of **8** (Scheme 3).

3. Conclusion

We have investigated the metabolism of codisterol (**2**) and epicodisterol (**3**) in cell cultures of a higher plant for the first time and found that the C-24 epimers of 24-methyl-Δ^{25}-cholesterol were indifferently converted to both (24*S*)- and (24*R*)-24-methylcholesterols (**9/10**). Detailed investigation of the conversion mechanism established that neither **2** nor **3** is a substrate for the reduction, but both **2** and **3** first isomerize to the common intermediate 24-methyldesmosterol (**8**), which is then reduced to give **9** and **10**. The gene, *Dwarf1*, which encodes a multifunctional enzyme catalyzing the conversion of 24-exomethylene-sterol (**4**) to **8** as well as the reduction of **8** to **9/10**, has been characterized in *Arabidopsis thaliana*.[22] Database search showed that there is no homologous gene in *A. thaliana*. This is the case also in *O. sativa*, which has only one ortholog of *Arabidopsis Dwarf1*.[23] It is therefore suggested that the isomerization from Δ^{25} to Δ^{24} and the subsequent reduction would be catalyzed by DWARF1 enzyme of a poor substrate specificity. The metabolism of (24*R*)- and (24*S*)-24-ethyl-Δ^{25}-cholesterols in higher plants is under investigation in our laboratory.

4. Experimental

4.1. Preparation of the labeled substrates

4.1.1. General. ^{1}H NMR (500 MHz) spectra were recorded on a JEOL JNM-DX 500 spectrometer in $CDCl_3$ solution and chemical shifts (δ) are reported in ppm downfield from tetramethylsilane (used as an internal reference). ^{13}C NMR (125 MHz) spectra are obtained on the same spectrometer and chemical shifts were referenced to the signal (δ 77.0) of $CDCl_3$. ^{13}C-{^{1}H}{^{2}H} NMR (125 MHz) spectra are recorded on a Bruker DRX500 spectrometer. GC–MS analyses were performed on a JEOL AUTOMASS SUN200 instrument with HP-1 capillary column (0.32 mm × 30 m) using He gas (1 mL/min), with an oven temperature from 250 to 260 °C (1 °C/min). HRMS were recorded with a JEOL JMS-700 spectrometer. HPLC was performed on a Shimadzu LC-6A apparatus equipped with a UV detector using a reversed-phase column, PREP-ODS(H) (25 cm × 20 mm i.d.). TLC analyses were performed on commercial glass plates bearing a 0.25 mm layer of Merck DC-Fertigplatten Kieselgel 60 F_{245}. Column chromatography was done with Merck Kieselgel 60 (70–230 mesh). Tetrahydrofuran (THF) was distilled before use from benzophenone ketyl under N_2 atmosphere. Diethyl ether was distilled from $LiAlH_4$ before use. [^{13}C]Methyl iodide (99% ^{13}C), lithium aluminum deuteride (98% ^{2}H), deuterium oxide (99% ^{2}H), and $^{2}H_3$-methylmagnesium bromide (98% ^{2}H) were purchased from Merck.

4.1.2. Synthesis of [27-^{13}C]codisterol ([27-^{13}C]2). *n*-Butyl lithium (1.54 M solution in toluene, 11.3 mL, 17.0 mmol) was added to a solution of freshly distilled diisopropylamine (2.5 mL, 17.8 mmol) in THF (169 mL) at 0 °C under N_2 atmosphere and the solution was stirred for 0.5 h. The resultant LDA solution was added to a solution of the (24*R*)-ester **15**[15] (3.87 g, 8.47 mmol) in THF (170 mL) at −78 °C and the mixture was stirred for 1 h. [^{13}C]Methyl iodide (1.14 mL, 17.0 mmol) was added to the enolate solution and the mixture was stirred at −78 °C for 2 h. The reaction mixture was diluted with satd NH_4Cl and extracted with ethyl acetate. The organic layer was washed with satd $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was chromatographed on silica gel (hexane/ethyl acetate = 30:1) to give ethyl [27-^{13}C]-(22*E*,24*S*)-6β-methoxy-3α,5-cycloergost-22-en-26-oate (3.82 g, 89%) as a colorless oil. ^{1}H NMR δ: 0.42 (dd, J = 8.5, 5.4 Hz, H-4a), 0.64 (t,

J = 4.0 Hz, H-4b), 0.72 (s, H_3-18), 1.00 (d, J = 8.5 Hz, H_3-21), 1.01 (d, J = 8.5 Hz, H_3-28), 1.02 (s, H_3-19), 1.08 (dd, J = 107, 6.4 Hz, H_3-27), 1.26 (t, J = 7.8 Hz, OCH_2CH_3), 2.76 (s, H-6), 3.23 (s, OCH_3), 4.12 (q, J = 7.8 Hz, OCH_2CH_3), 5.11 (m, H-22), 5.23 (*m*, H-23). ^{13}C NMR δ: 13.71 and 14.65 (enriched 25*R*- and 25*S*-$^{13}CH_3$).

A solution of the methylated ester (3.80 g, 8.06 mmol) in THF (80 mL) was stirred at room temperature for 0.5 h after addition of $LiAlH_4$ (758 mg, 19.9 mmol). Extractive (ethyl acetate) work-up gave the crude product, which was chromatographed on silica gel (hexane/ethyl acetate = 15:1) to afford the C-26 alcohol (3.01 g, 86%) as a colorless oil. A mixture of the alcohol (1.00 g, 2.32 mmol) and 5% Pd/C (200 mg) in ethyl acetate (58 mL) was stirred at room temperature for 17 h under hydrogen atmosphere. The catalyst was filtered off through a pad of Celite and the filtrate was concentrated under reduced pressure. The crude product was chromatographed on silica gel (hexane/ethyl acetate = 15:1) to give [27-^{13}C]-(24*S*)-6β-methoxy-3α,5-cycloergostan-26-ol (900 mg, 90%). ^{1}H NMR δ: 0.43 (dd, J = 8.5, 5.3 Hz, H-4a), 0.65 (t, J = 4.0 Hz, 4-Hb), 0.72 (s, H_3-18), 0.88 (d, J = 8.5 Hz, H_3-*28*), 0.91 (d, J = 7 Hz, H_3-*21*), 0.92 (dd, J = 125, 6.4 Hz, H_3-27), 1.02 (s, H_3-19), 2.77 (s, H-6), 3.32 (s, OCH_3), 3.45 and 3.63 (m, H_2-26). ^{13}C NMR δ: 11.05 and 13.94 (enriched 25*R*- and 25*S*-$^{13}CH_3$).

A mixture of the saturated alcohol (768 mg, 1.78 mmol), 2-nitrophenylselenocyanate (2.0 g, 10.8 mmol), and tri-*n*-butylphosphine (2.2 mL, 10.8 mmol) in THF (25 mL) was stirred at room temperature for 30 min under N_2 atmosphere. The reaction mixture was stirred, after addition of 30% H_2O_2 solution (35 mL), for another 1 h and extracted with ethyl acetate. The organic layer was washed with sat $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was chromatographed on silica gel (hexane/ethyl acetate = 30:1) to afford [27-^{13}C]-(24*S*)-6β-methoxy-3α,5-cycloergost-25-ene (570 mg, 78%) as a colorless oil. ^{1}H NMR δ: 0.43 (dd, J = 8.5, 5.3 Hz, H-4a), 0.65 (t, J = 4 Hz, H-4b), 0.71 (s, H_3-18), 0.90 (d, J = 6.5 Hz, H_3-21), 0.99 (d, J = 6.5 Hz, H_3-28), 1.02 (s, H_3-19), 1.63 (d, J = 125 Hz, H_3-27), 2.77 (s, H-6), 3.32 (s, OCH_3), 4.66 (m, H_2-26). ^{13}C NMR δ: 18.68 (enriched 27-^{13}C).

A solution of the 25-ene (570 mg, 1.38 mmol) and *p*-toluensulfonic acid monohydrate (223 mg, 0.322 mmol) in dioxane (27.5 mL) and water (27.5 mL) was stirred at 75 °C for 2.5 h and then cooled to room temperature. The mixture was extracted with ether and the organic layer was washed with satd $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was chromatographed on silica gel (hexane/ethyl acetate = 10:1) and crystallization from methanol to give [27-^{13}C]**2** (422 mg, 77%) as colorless needles, mp 143–144 °C. ^{1}H NMR δ: 0.67 (s, H_3-18), 0.91 (d, J = 6.5 Hz, H_3-21), 0.99 (d, J = 7.4 Hz, H_3-28), 1.01 (s, H_3-19), 1.65 (d, J = 111 Hz, H_3-27), 3.51 (m, H-3), 4.65 (m, H_2-26), 5.35 (m, H-6). ^{13}C NMR δ: 11.83 (C-18), 18.52 (C-21), 18.66 (enriched C-27), 19.39 (C-28), 20.12 (C-19), 21.07 (C-11), 24.27 (C-15), 28.15 (C-2), 31.15 (C-16), 31.65 (C-23), 31.89 (C-7), 31.89 (C-8), 33.66 (C-22), 35.65 (C-20), 36.49 (C-10), 37.24 (C-4), 39.76 (C-1), 41.55 (C-12), 42.3 (C-13), 42.3 (C-24), 50.12 (C-9), 55.97 (C-17), 56.75 (C-14), 71.78 (C-3), 109.32 (C-26), 121.69 (C-6), 140.74 (C-5), 150.05 (C-25). HREIMS found *m*/*z*: 399.3584 (M^+), calcd for $C_{27}{}^{13}CH_{46}O$: 399.3582. The labeled codisterol was found be a mixture of 92% of [27-^{13}C]codisterol and 8% of [27-^{13}C]epicodisterol on the basis of the signal intensity of the enriched carbons at δ: 18.66 and 19.14.

4.1.3. Synthesis of [27-^{13}C]24-epicodisterol ([27-^{13}C]3). [27-^{13}C]24-Epicodisterol ([27-^{13}C]**3**) was synthesized from the (24*S*)-ester **16**[15] in the same procedure as described for the synthesis of [27-^{13}C]**2**. [27-^{13}C]**3**: mp 142–143 °C; ^{1}H NMR δ: 0.67 (s, H_3-18), 0.92 (d, J = 6.5 Hz, H_3-21), 0.99 (d, J = 7.4 Hz, H_3-28), 1.01 (s, H_3-19), 1.63 (d, J = 130 Hz, H_3-27), 3.48 (m, H-3), 4.65 (m, H_2-26), 5.36 (m, H-6). ^{13}C NMR δ: 11.83 (C-18), 18.79 (C-21), 19.14 (enriched C-27), 19.39 (C-28), 19.56 (C-19), 20.12 (C-11), 24.27 (C-15), 28.17 (C-2), 31.35 (C-16), 31.66 (C-23), 31.90 (C-7), 31.90 (C-8), 33.67 (C-22), 35.84 (C-20), 36.49 (C-10), 37.24 (C-4), 39.75 (C-1), 41.46 (C-12), 42.30 (C-13), 42.30 (C-24), 50.12 (C-9), 55.93 (C-14), 56.76 (C-17), 71.78 (C-3), 108.92 (C-26), 121.68 (C-6), 140.75 (C-5), 150.86 (C-25). HREIMS found *m*/*z*: 399.3579 (M^+), calcd for $C_{27}{}^{13}CH_{46}O$: 399.3582. The labeled epicodisterol was found be a mixture of 91% of [27-^{13}C]epicodisterol and 9% of [27-^{13}C]codisterol on the basis of the signal intensity of the enriched carbons at δ: 19.14 and 18.66.

4.1.4. Synthesis of [23,24-$^2H_2$]codisterol ([23,24-$^2H_2$]2). $LiAl^2H_4$ (2.10 g, 49.9 mmol) was added to a solution of the (22*R*)-acetylenic alcohol **17**[15,16] (1.09 g, 2.83 mmol) in THF (28 mL) and the mixture was refluxed under N_2 atmosphere for 18 h. The mixture was cooled with ice water and ^2H_2O (6.0 mL) was carefully added in portions. The mixture was extracted with ethyl acetate and the organic layer was washed with satd $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was chromatographed on silica gel (hexane/ethyl acetate = 25:1) to give [23,24-$^2H_2$]-(23*E*)-6β-methoxy-26,27-dinor-3α,5-cyclocholest-23-en-22-ol (347 mg, 32%) as a pale yellow oil. ^{1}H NMR δ: 0.43 (dd, J = 8.5, 5.3 Hz, H-4a), 0.65 (t, J = 4.0 Hz, H-4b), 0.72 (s, H_3-18), 0.90 (d, J = 5.5 Hz, H_3-21), 1.02 (s, H_3-19), 1.71 (s, H_3-25), 2.77 (s, H-6), 3.33 (s, OCH_3), 4.19 (m, H-22). EIMS *m*/*z*: 388 (M^+), 373 ($[M\text{-}CH_3]^+$).

A solution of the allylic alcohol (326 mg, 0.89 mmol), triethyl orthopropionate (1.90 mL, 4.45 mmol), and propionic acid (40 μL, 0.53 mmol) in xylene (20 mL) was stirred at 140 °C for 3 h under N_2. Concentration of the mixture under reduced pressure gave ethyl [23,24-$^2H_2$]-(22*E*,24*S*)-6β-methoxy-3α,5-cycloergost-22-en-26-oate (371 mg, 93%) as a pale yellow oil. ^{1}H NMR δ: 0.43 (dd, J = 8.5, 5.4 Hz, H-4a), 0.65 (t, J = 4.0 Hz, H-4b), 0.73 (s, H_3-18), 0.95 (d, J = 7.5 Hz, H_3-21), 0.96 (d, H_3-28), 1.02 (s, H_3-19), 1.25 (t, J = 7.8 Hz, OCH_2CH_3),

1.26 (s, H_3-27), 2.77 (s, H-6), 3.26 (s, OCH_3), 4.12 (q, J = 7.8 Hz, OCH_2CH_3), 5.25 (m, H-22, H-23).

The unsaturated ester was converted to [23,24-$^2H_2$]**2** as described for the synthesis of [27-^{13}C]**2**. [23,24-$^2H_2$]**2**: white needles, mp 143–144 °C. ^{1}H NMR δ: 0.67 (s, H_3-18), 0.91 (d, J = 7.4 Hz, H_3-21), 0.99 (s, H_3-28), 1.01 (s, H_3-19), 1.63 (s, H_3-27), 3.51 (m, H-3), 4.65 (m, H-26), 5.35 (m, H-6). ^{13}C NMR δ: 11.83 (C-18), 18.63 (C-27), 18.71 (C-21), 19.39 (C-19), 19.98 (C-28), 21.07 (C-11), 24.27 (C-15), 28.15 (C-2), 28.15 (C-16), 30.61 (t, J_{CD} = 19 Hz, C-23), 31.89 (C-7), 31.89 (C-8), 33.54 (C-22), 35.63 (C-20), 36.5 (C-10), 37.24 (C-1), 39.76 (C-12), 41.01 (t, J_{CD} = 19 Hz, C-24), 42.3 (C-4), 42.3 (C-13), 50.12 (C-9), 55.98 (C-17), 56.75 (C-14), 71.8 (C-3), 109.29 (C-26), 121.71 (C-6), 140.75 (C-5), 150.19 (C-25). ^{13}C-{^{1}H}{^{2}H} NMR δ: 30.53 (C-23), 40.99 (C-24). HREIMS found m/z: 400.3670 (M^+), calcd for $C_{28}H_{44}{}^2H_2O$: 400.3672.

4.1.5. Synthesis of [26,27-$^2H_6$]24-methyldesmosterol ([26,27-$^2H_6$]8). C^2H_3MgI (1.0 M solution in diethyl ether, 3.3 mL, 3.3 mmol) was added to a solution of the TBS ether **18**[17] (420 mg, 0.79 mmol) in THF (2.6 mL) at room temperature under N_2 atmosphere and the mixture was stirred for 2 h. Extractive (ethyl acetate) work-up gave the crude product, which was chromatographed on silica gel (hexane/ethyl acetate = 15:1), to afford [26,27-$^2H_6$]3β-*t*-butyldimethylsilyloxyergost-5-en-25-ol (402 mg, 62%) as a colorless oil. ^{1}H NMR δ: 0.057 (s, H_6-Me_2Si), 0.67 (s, H_3-18), 0.89 (s, H_9-*t*-Bu), 0.92 (d, J = 6.5 Hz, H_3-21), 1.00 (s, H_3-19), 3.48 (m, H-3), 5.31 (br s, H-6).

$POCl_3$ (0.56 mL, 6.0 mmol) was added to a solution of the C-25 alcohol (300 mg, 0.56 mmol) in pyridine (8.0 mL) and the mixture was stirred at room temperature for 3 h. The mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with satd $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure to give a ca. 1:1 mixture of Δ^{24}- and Δ^{25}-olefins (365 mg). A solution of the olefinic mixture in THF (10 mL) and conc HCl (1 mL) was stirred at room temperature for 3 h. The reaction mixture was diluted with satd $NaHCO_3$ and extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was separated by reversed-phase HPLC [methanol, 9.9 mL/min, UV detection (215 nm), typical retention times for the Δ^{24}- and Δ^{25}-olefins, 35.0 and 38.0 min, respectively] and crystallization of the desired Δ^{25}-olefin from methanol furnished [26,27-$^2H_6$]**8** (148 mg, 60% from the C-25 alcohol) as white needles, mp 138 °C. ^{1}H NMR δ: 0.68 (s, H_3-18), 0.96 (d, J = 6.6 Hz, H_3-21), 1.01 (s, H_3-19), 1.61 (s, H_3-28), 3.54 (m, H-3), 5.35 (br s, H-6). ^{13}C NMR δ: 11.85 (C-18), 18.45 (C-28), 18.79 (C-21), 19.39 (C-19), 21.08 (C-11), 24.32 (C-15), 28.18 (C-16), 31.05 (C-23), 31.68 (C-2), 31.91 (C-7), 31.91 (C-8), 34.33 (C-20), 35.96 (C-22), 36.51 (C-10), 37.25 (C-1), 39.75 (C-12), 42.31 (C-4), 42.34 (C-13), 50.13 (C-9), 55.83 (C-17), 56.74 (C-14), 71.81 (C-3), 121.71 (C-6), 123.13 (C-24), 128.42 (C-25), 140.75 (C-5). ^{13}C-{^{1}H}{^{2}H} NMR δ: 19.06, 19.62 (C-26, C-27); HREIMS found m/z: 404.3917 (M^+), calcd for $C_{28}H_{40}{}^2H_6O$: 404.3919.

4.2. Feeding experiments

Cell cultures of *O. sativa* were maintained as described previously.[1]

To cultured cells of *O. sativa* (2 weeks, four 500 mL-flasks, each containing 350 mL N6 medium (SIGMA, Chu (N_6) basal salt mix) supplemented with sucrose 30 g/L, proline 2.8 g/L, casein hydrolysate 300 mg/L, and 2,4-D 2 mg/L) was added evenly a solution of each substrate (50 mg) in acetone (1 mL) and Tween 80 (1.5 mL) through a membrane filter. Incubation was continued for another 2 weeks and the cells were collected by filtration. The CHC^{13}-MeOH extract was chromatographed on silica gel to give the sterol fraction as described previously.[1] 24-Methylsterol was separated by HPLC [methanol, 9.9 mL/min, detection with UV (210 nm), typical retention time, 45 min]. Sterol analysis of the 24-methylsterols was performed with Shim pack CLC-ODS column (15 cm × 6 mm i.d.) using methanol (1.0 mL/min) as solvent. The separated 24-methylcholesterols (typical amount, 8 mg) were analyzed by ^{13}C NMR (Fig. 1), ^{13}C-{^{1}H}{^{2}H} NMR (Fig. 3) and GC–MS (Fig. 2).

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