Biosynthesis of the 24-Methylcholesterols Dihydrobrassicasterol and Campesterol in Cultured Cells of *Amsonia elliptica*: Incorporation of [1,2-$^{13}C_2$]-Acetate and [2-^{13}C,$^{2}H_3$]Acetate

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Feeding experiments with [1,2-$^{13}C_2$]acetate and [2-^{13}C,$^{2}H_3$]acetate in suspension cultures of *A. elliptica* demonstrate that campesterol has C-26 (the *pro-R* methyl group at C-25) and C-27 (the *pro-S* methyl group at C-25) arising from C-6 and C-2 of mevalonate (MVA), respectively. In contrast, dihydrobrassicasterol has C-26 and C-27 derived from C-2 and C-6 of MVA, respectively. In both 24-methylcholesterols, the methyl group derived from C-2 and C-6 of MVA retained two and three deuterium atoms, respectively. Furthermore, no signal due to a deuterium atom at C-24 or C-25 originating from [2-^{13}C,$^{2}H_3$]acetate was found for campesterol and dihydrobrassicasterol. These data suggest that both campesterol and dihydrobrassicasterol are biosynthesized by regio-facial specific reduction of the 24(25)-double bond of the 24-methyl-24(25)-ene side-chain which is formed by double-bond isomerization of $\Delta^{24(28)}$-sterol.

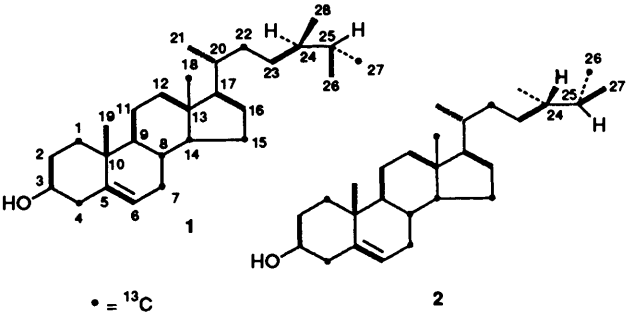
Sterols have important functions as architectural components of membranes and have been suggested to play an essential role in the regulation of cell growth.[1] Cholesterol in animals and ergosterol in fungi are formed *via* lanosterol, while phytosterols are formed *via* cycloartenol which is a 9,19-cyclic derivative. Both intermediates have a 24(25)-double bond in the side-chain. The double bond is reduced to form cholesterol in animals or alkylated at C-24 and hydrogenated at C-25 to form 24-alkylsterols in fungi, algae and higher plants. Common 24-ethylsterols in vascular plants are sitosterol and stigmasterol, which have the 24α-configuration. A 24-methylsterol in vascular plants is a mixture of campesterol (24α) and dihydrobrassicasterol (24β). Sterols with the 24β-configuration generally appear in algae, fungi and three families of tracheophytes (Cucurbitaceae,[2] Verbenaceae[3] and Crassulaceae[4]). We and others have studied the stereospecificity of the hydrogen addition at C-25 and have demonstrated that 24α- and 24β-ethylsterols,[5–7] ergosterol,[7–9] campesterol,[9] 24-methylenesterols[9–10] and isofucosterol[11] have the same biosynthetic stereochemistry at C-25. These sterols have C-26 (the *pro-R* methyl group at C-25) and C-27 (the *pro-S* methyl group at C-25) arising from C-6 and C-2 of MVA, respectively. The opposite stereochemistry at C-25 was shown to exist in cholesterol,[12,13] steroidal sapogenin[14] and dihydrobrassicasterol.[9] The stereoselective hydrogen attack at C-25 is reported to operate *via* three mechanisms: (i) reduction of the 24(25)-double bond leading to cholesterol[12,13] and 24α-ethylsterols,[7,15] (ii) reduction of the 25(26)-double bond to form 24β-ethylsterols[6] and (iii) hydrogen migration from C-24 to C-25 to form ergosterol,[7,8] 24-methylenesterols[10] and isofucosterol.[11] Nes and Le[16] recently demonstrated, using *Gibberella fujikuroi* that two alternative biosynthetic pathways operate in the formation of 24β-methylsterol end-products. One is for brassicasterol which is formed by hydrogen migration followed by reduction of the 24(28)-double bond similar to ergosterol synthesis and the other is for dihydrobrassicasterol which is synthesized by $\Delta^{24(25)}$-reduction.

Recently, we reported that both campesterol and dihydrobrassicasterol are formed *via* an intermediate with a 24-methylene side-chain in *A. elliptica* suspension cultures.[17] The present study was undertaken to clarify the mechanism of 24α- and 24β-methylsterol side-chain formation in higher plants by using the same suspension cultures incubated with [1,2-$^{13}C_2$]acetate and [2-^{13}C,$^{2}H_3$]acetate.

Results and Discussion

In our early study using cultured cells of *Physalis peruviana*,[9] the biosynthetic chirality at C-25 of dihydrobrassicasterol was the opposite of that of campesterol. The results were obtained with a 24-epimeric mixture. Recently, Patterson[18] reported the separation of this mixture and we have also recently isolated campesterol and dihydrobrassicasterol.[17]

The cultured cells of *A. elliptica*, which produce campesterol and dihydrobrassicasterol as the major sterols, were incubated with sodium [1,2-$^{13}C_2$]acetate. After 4 weeks, [^{13}C]-labelled campesterol (**1**) and [^{13}C]-labelled dihydrobrassicasterol (**2**)



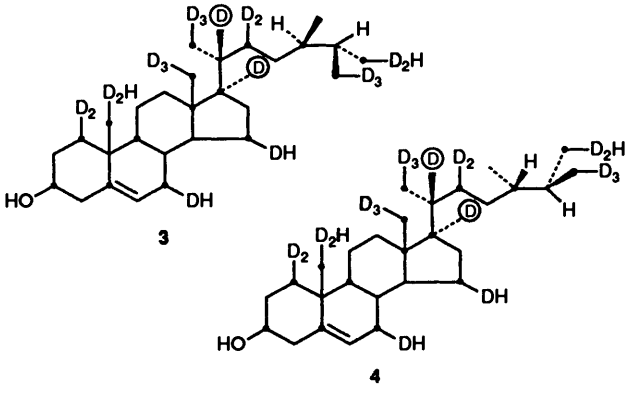
were isolated from the cells by preparative-scale recycling HPLC. The labelling patterns were examined by ^{13}C-{^{1}H} NMR spectroscopy. The ^{13}C signal assignments of C-26 (the *pro-R*) and C-27 (the *pro-S*) of the campesterol **1** and the dihydrobrassicasterol **2** were previously reported[9] by correlation with the assignment of [27-^{13}C]ergosterol synthesized by Arigoni[8] and the assignments were confirmed by Colombo *et al.*[19] who prepared (25*S*)-[27-^{2}H]campesterol and (25*R*)-[26-^{2}H]dihydrobrassicasterol.

As shown in Table 1, all carbons except two, C-26 and C-27, of both 24-epimeric 24-methylsterols have the same labelling

Table 1 ^{13}C NMR spectral data of labelled campesterol **1** and labelled dihydrobrassicasterol **2** biosynthesized with [1,2-$^{13}C_2$]acetate in *A. elliptica*

	Campesterol **1**			Dihydrobrassicasterol **2**		
Carbon	δ_C	J_{CC}[a]	(J_{CC})[b]	δ_C	J_{CC}	(J_{CC})
C-1	37.27	s[c]	(34)	37.29	s	(34)
C-2	31.68	37		31.70	36	
C-3	71.81	37		71.84	36	
C-4	42.32	s		42.34	s	
C-5	140.77	72		140.79	72	
C-6	121.71	72		121.74	72	
C-7	31.92	s		31.94	s	
C-8	31.92	s		31.94	s	
C-9	50.15	35		50.17	35	
C-10	36.51	35		36.54	35	
C-11	21.10	35		21.12	35	
C-12	39.80	35		39.80	35	
C-13	42.32	35		42.34	35	
C-14	56.79	s	(33)	56.79	s	(33)
C-15	24.31	s	(33)	24.33	s	(33)
C-16	28.25	34		28.22	34	
C-17	56.13	34		56.02	34	
C-18	11.88	s	(35)	11.89	s	(35)
C-19	19.41	35		19.43	35	
C-20	35.89	35		36.22	35	
C-21	18.72	35		18.93	35	
C-22	33.72	s	(34)	33.75	s	(34)
C-23	30.30	35		30.61	35	
C-24	38.85	35		39.10	35	
C-25	32.43	35		31.49	35	
C-26	20.22	35		17.63	s	(35)
C-27	18.27	s	(35)	20.55	35	
C-28	15.39			15.48		

[a] Intra-unit coupling constant of doublet in Hz. [b] Inter-unit coupling constant of doublet in Hz. [c] Singlet.



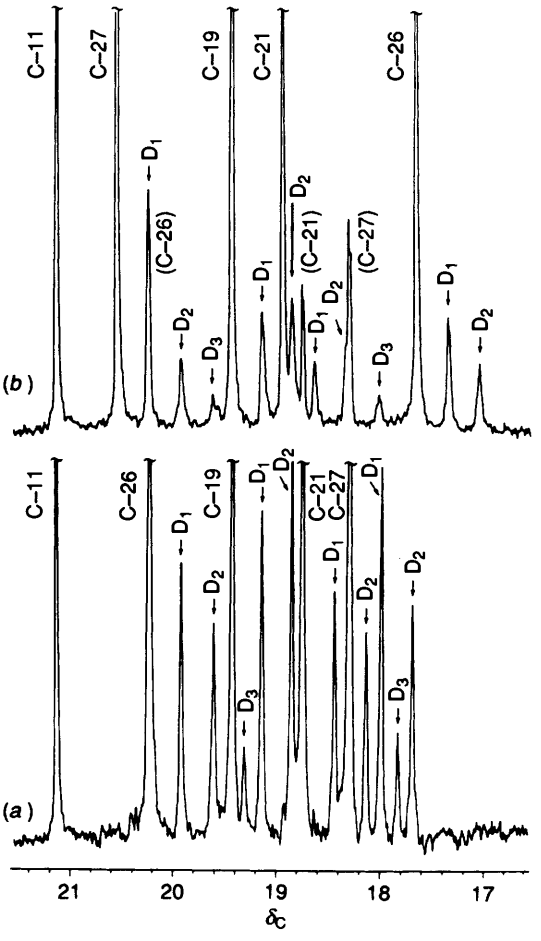


Fig. 1 The C-26 and C-27 region of ^{13}C-{^1H}{^2H} NMR spectra of (*a*) the campesterol **3** and (*b*) the dihydrobrassicasterol **4** biosynthesized from [2-^{13}C,$^2H_3$]acetate in cultured cells of *A. elliptica*. Signals due to campesterol contaminant in compound **4** are shown in parentheses.

patterns. Eight pairs of doublets, C-2/C-3, C-5/C-6, C-9/C-11, C-10/C-19, C-12/C-13, C-16/C-17, C-20/C-21 and C-23/C-24, and eight singly labelled carbons, C-1, C-4, C-7, C-8, C-14, C-15, C-18 and C-22, were observed. Some of the singly labelled carbons were accompanied by a doublet due to inter-unit C–C coupling of the acetate unit but the intensity was weak. The campesterol **1** showed a doublet C-26 (δ_C 20.22, d, J 35 Hz) coupled to C-25 (δ_C 32.43, d, J 35 Hz) and singly labelled C-27 (δ_C 18.27). In the case of the dihydrobrassicasterol **2**, a coupling was observed between C-27 (δ_C 20.55, d, J 35 Hz) and C-25 (δ_C 31.49, d, J 35 Hz), and C-26 (δ_C 17.63) was observed as a singlet. The singly labelled and doubly labelled methyl group is believed to originate from C-2 and C-6 of MVA, respectively. These results agree with our previous results with *P. peruviana*.[9]

In order to study the mechanism of the hydrogen addition at C-25 with opposite stereoselectivity between 24α- and 24β-methylcholesterols, the suspension cultures of *A. elliptica* were incubated with sodium [2-^{13}C,$^2H_3$]acetate, which was added in two portions every 14 days. The cells were harvested at 2 weeks after the last addition of acetate, and a mixture of [^{13}C,^2H]-labelled 24-methylcholesterols was isolated. The ^{13}C-{^1H}{^2H} NMR spectrum of the mixture showed good incorporation of [2-^{13}C,$^2H_3$]acetate. The mixture was subjected to recycling reversed-phase HPLC, and [^{13}C,^2H]-labelled campesterol (**3**) was isolated. As the deuteriated compound seemed to show a leading effect on HPLC,[17] the isolated [^{13}C,^2H]-labelled dihydrobrassicasterol showed weaker deuterium-labelling signals in the ^{13}C-{^1H}{^2H} NMR spectrum than expected from that of the mixture. We then obtained [^{13}C,^2H]-labelled dihydrobrassicasterol (**4**) containing the leading part of compound **4**, which was contaminated by a small amount of campesterol. The ^{13}C-{^1H}{^2H} NMR spectrum of each sterol is shown in Fig. 1(*a*) and (*b*), and the results are summarized in Table 2. The campesterol molecules in Fig. 1(*b*) which contaminate the dihydrobrassicasterol **4** were not labelled with deuterium atoms; C-21 (δ_C 18.72) showed no signals indicating deuterium atoms which are observed in Fig. 1(*a*). The C-26 of the campesterol **3** and the C-27 of the dihydrobrassicasterol **4**, which arise from C-6 of MVA, obviously retained three deuterium atoms. The C-26 of the dihydrobrassicasterol **4** and the C-27 of the campesterol **3** were clearly labelled as $^{13}C^2H_2{}^1H$, in agreement with the origin of C-2 of MVA. The C-24 resonances of both sterols **3** and **4** are shown in Fig. 2. Neither the α-deuterium isotope-shifted signal nor the β-deuterium isotope-shifted signal was observed at C-24, indicating loss of the 24-^2H from intermediate **5**. The β-deuterium isotope shifted signal at C-24 due to the stereo-

Table 2 ^{13}C NMR spectral data of labelled campesterol **3** and labelled dihydrobrassicasterol **4** biosynthesized with [2-^{13}C,$^{2}H_3$]acetate in *A. elliptica*[a]

	Campesterol **3**				Dihydrobrassicasterol **4**			
	$^{13}C^{2}H_n$	$^{1}\Delta\delta_{C,^{2}H_1}$	$^{1}\Delta\delta_{C,^{2}H_2}$	$^{1}\Delta\delta_{C,^{2}H_3}$	$^{13}C^{2}H_n$	$^{1}\Delta\delta_{C,^{2}H_1}$	$^{1}\Delta\delta_{C,^{2}H_2}$	$^{1}\Delta\delta_{C,^{2}H_3}$
C-1	$^{13}C^{2}H_2$	−0.36	−0.78		$^{13}C^{2}H_2$	−0.37	−0.77	
		−0.43				−0.43		
C-7	$^{13}C^{2}H_1{}^{1}H_1$	−0.37			$^{13}C^{2}H_1{}^{1}H_1$	−0.37		
C-13	$^{13}C(\beta\text{-}^{2}H)$	−0.09[b,c]			$^{13}C(\beta\text{-}^{2}H)$	−0.09[b,c]		
C-15	$^{13}C^{2}H_1{}^{1}H_1$	−0.34			$^{13}C^{2}H_1{}^{1}H_1$	−0.34		
C-17	$^{13}C(\beta\text{-}^{2}H)$	−0.11[b,c]			$^{13}C(\beta\text{-}^{2}H)$	−0.11[b,c]		
C-18	$^{13}C^{2}H_3$	−0.29[c]	−0.57[c]	−0.86[c]	$^{13}C^{2}H_3$	−0.29[c]	−0.58[c]	−0.86[c]
C-19	$^{13}C^{2}H_2{}^{1}H_1$	−0.29	−0.58		$^{13}C^{2}H_2{}^{1}H_1$	−0.30	−0.59	
C-21	$^{13}C^{2}H_3$	−0.30	−0.60	−0.91	$^{13}C^{2}H_3$	−0.31	−0.64	−0.93
C-22	$^{13}C^{2}H_2$	−0.41	−0.83		$^{13}C^{2}H_2$	−0.38	−0.82	
		−0.42				−0.44		
C-26	$^{13}C^{2}H_3$	−0.31	−0.62	−0.93	$^{13}C^{2}H_2{}^{1}H_1$	−0.30	−0.61	
C-27	$^{13}C^{2}H_2{}^{1}H_1$	−0.30	−0.61		$^{13}C^{2}H_3$	−0.31	−0.63	−0.94

[a] Carbons labelled with deuterium atom(s) are shown; other carbons were not labelled with ^{2}H. [b] β-Deuterium isotope shift ($^{2}\Delta\delta_{C,^{2}H}$). [c] Inter-unit coupling (J_{CC} 35 Hz) was observed.

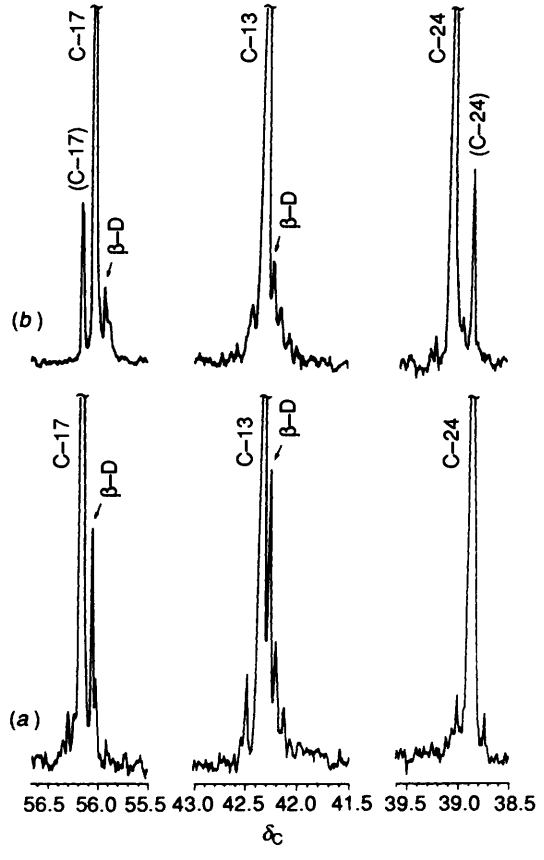


Fig. 2 The C-24, C-17 and C-13 regions of ^{13}C-{^{1}H}{^{2}H} NMR spectra of (*a*) the campesterol **3** and (*b*) the dihydrobrassicasterol **4** biosynthesized from [2-^{13}C,$^{2}H_3$]acetate in cultured cells of *A. elliptica*. Signals due to campesterol contaminant in compound **4** are shown in parentheses.

specific deuterium migration Ⓓ from C-24 to C-25 (**5** ⟶ **6**) was previously observed[7,10,11] in the biosynthesis of ergosterol, 24-methylenecholesterol, 24-methylenecycloartanol and isofucosterol. The α-deuterium isotope-shifted signal at C-24, which indicated overall retention of the 24-^{2}H in intermediate **5**, was observed in the biosynthesis of 24β-ethylsterols.[6] For comparison, Fig. 2 also shows the β-deuterium isotope-shifted signals of C-13 and C-17 of substrates **3** and **4** which have deuterium atoms Ⓓ on the adjacent carbons, where two deuterium migrations, from C-13 to C-17 and from C-17 to C-20, take place during cyclization of 2,3-epoxydihydrosqualene.

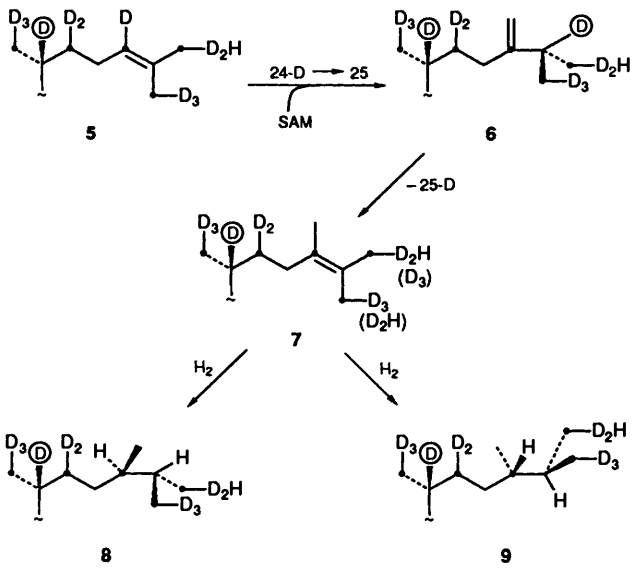
These findings suggest that the main biosynthetic pathway (**5** ⟶ **6** ⟶ **7** ⟶ **8** and **9**) for campesterol and dihydrobrassicasterol in *A. elliptica* involves 24-methylene side-chain (**6**), migration of the double bond, and reduction of the 24-methyl-24(25)-ene side-chain (**7**). The findings also supported our previous results[17] which indicated the intermediate nature of the 24-methylenecholesterol side-chain (structure **6**). This mechanism is similar to the biosynthesis of campesterol in *Spinacea* and *Medicago* species[20] and of dihydrobrassicasterol in *Gibberella fujikuroi*.[16] 24-Methyldesmosterol isolated from *Physalis peruviana* callus[21] and other plants[22] seems to be an intermediate in the biosynthesis of 24α- and 24β-methylcholesterol in higher plants. Other mechanisms, which are not major ones in *A. elliptica*, include reduction of the 24(28)-double bond of compound **6** for ergosterol[7] and brassicasterol[16] formation, reduction of the 25(26)-double bond of cyclolaudenol-type side-chains, and reduction of the 23(24)-double bond, which has been observed in *Zea mays*.[23] Although previous results were obtained as a mixture of 24α- and 24β-methylsterols, separate intermediates in the formation of epimeric 24-methylsterols were suggested to occur in tracheophytes.[24–26] Other mechanisms may operate in the formation of 24β-methylsterol in different organisms.

C-19 retained only two deuterium atoms, indicating loss of a deuterium atom from the methyl group. This is evidence that both 24-methylcholesterols were formed *via* the 9,19-cyclic intermediate cycloartenol, while ergosterol[7] and cholesterol,[13] which are formed *via* lanosterol, were demonstrated previously to have their C-19 retain three deuterium atoms originating from [2-^{13}C,$^{2}H_3$]acetate.

We therefore concluded that both 24-methylcholesterols are formed *via* cycloartenol, a 24-methylene intermediate, and a 24-methyl-24(25)-ene intermediate. Direction of reduction of the 24(25)-double bond seems to lead to opposite stereochemistry at C-24 and C-25 of the campesterol **1** and the dihydrobrassicasterol **2**.

Experimental

Sodium [2-^{13}C,$^{2}H_3$]acetate (91.6 and 97.4 atom-% of ^{13}C and ^{2}H, respectively) was purchased from Cambridge Isotope Laboratories (UK) and sodium [1,2-$^{13}C_2$]acetate from Amersham (UK). NMR spectra (^{13}C,^{1}H) were obtained in [^{2}H]chloroform with tetramethylsilane as internal standard (δ_C 0 and δ_H 0). ^{13}C-{^{1}H} NMR spectra were recorded on a Varian XL-400 instrument at 100.577 MHz. Typical FT NMR con-



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ditions were: spectral width (s.w.), 17 006.8 Hz; acquisition time (a.t.), 0.882 s; and pulse flip angle, 10°. $^{13}C\{^{1}H\}\{^{2}H\}$ NMR spectra were determined on a JEOL GSX-500 instrument at 125.65 MHz under the following conditions: s.w. 18 867.9 Hz; a.t., 0.868 s (1.309 s); pulse delay (p.d.) 2.0 s; and pulse width (p.w.), 5.0 μs. ^{1}H NMR spectra were obtained with a Varian VXR-200 or XL-200 instrument at 200.057 MHz under the following conditions: s.w. 3200 Hz; a.t., 2.9 s; and p.w., 12 μs. *J*-Values are given in Hz.

Preparative HPLC was performed with LC-908 recycling preparative HPLC equipment (Japan Anal. Ind., Tokyo, Japan) with a JAi UV detector R-301 and JAi RI detector RI-3HC at 210 nm using (system A) a YMC-pack ODS SH 343 (s-15) column (250 × 20 mm i.d.) eluted with methanol (9 cm^3 min^{-1}), and (system B) a Develosil ODS T-7 (s-7) column (250 × 20 mm i.d.) eluted with methanol (9 cm^3 min^{-1}).

Feeding of Sodium [1,2-$^{13}C_2$]*Acetate*.—Tissue cultures of *Amsonia elliptica* (Thunb.) Roem. et Schult. were prepared as described previously.[17] The callus on the agar medium was transferred onto Linsmaier–Skoog liquid medium supplemented with 2,4-dichlorophenoxyacetic acid (1 × 10^{-5} mol dm^{-3}) and kinetin (0.02 ppm). After 4 weeks of incubation, the cells were transferred into fresh medium (8.4 dm^3) to which sodium [1,2-$^{13}C_2$]acetate (588 mg) and non-labelled acetate (1176 mg) were added. The mixture was incubated at 25 °C for 4 weeks on a rotary shaker in the dark. The cells (450 g fresh weight) were collected and extracted with methanol (2 dm^3) and a hot dichloromethane–methanol mixture (1:1; 2 dm^3). The extracts were combined (21 g) and partitioned between water (300 cm^3) and butan-1-ol (300 cm^3), and the butanol-soluble fraction (6.2 g) was chromatographed on a silica-gel column and eluted successively with dichloromethane (500 cm^3), acetone (100 cm^3), and methanol (1 dm^3). The fraction eluted with dichloromethane (0.5 g) was further chromatographed on silica gel (Merck, Lobar B), eluting with a mixture of hexane–ethyl acetate–chloroform (4:1:1) to obtain a sterol mixture (79 mg). HPLC (system A) of the sterol mixture gave a mixture of (24*R*)- and (24*S*)-24-methylcholesterol (32.6 mg) together with stigmasterol (4.8 mg), sitosterol (10.2 mg), a mixture of 24-methylcholestanols (6.5 mg), 24-methylenecholesterol (3.3 mg) and 24-ethylcholestanol (2.1 mg). These were characterized by ^{1}H and ^{13}C NMR spectra. The mixture of 24-methylcholesterols was further subjected to HPLC (system B) and gave [^{13}C]campesterol **1** (20 mg) and [^{13}C]dihydrobrassicasterol **2** (11.5 mg); δ_H for **1**: 0.679 (3 H, s, 18-H_3), 0.770 (3 H, d, *J* 6.0, 28-H_3), 0.802 (3 H, d, *J* 6.6, 27-H_3), 0.850 (3 H, d, *J* 6.8, 26-H_3), 0.910 (3 H, d, *J* 6.4, 21-H_3), 1.009 (3 H, s, 19-H_3), 3.52 (1 H, m, 3-H) and 5.35 (1 H, br d, *J* 5.0, 6-H); δ_H for **2**: 0.679 (3 H, s, 18-H_3), 0.779 (3 H, d, *J* 6.6, 28- or 26-H_3), 0.785 (3 H, d, *J* 6.6, 26- or 28-H_3), 0.858 (3 H, d, *J* 6.8, 27-H_3) 0.922 (3 H, d, *J* 6.2, 21-H_3), 1.012 (3 H, s, 19-H_3), 3.52 (1 H, m, 3-H) and 5.36 (1 H, br d, *J* 5.0, 6-H).

Feeding of Sodium [2-^{13}C,$^{2}H_3$]*Acetate*.—A mixture of sodium [2-^{13}C,$^{2}H_3$]acetate (810 mg) and non-labelled sodium acetate (1620 mg) was dissolved in water and the solution was added to suspension cultures of *A. elliptica* grown in Linsmaier–Skoog medium (9 dm^3) in two portions every 2 weeks. The suspension was incubated for a total of 4 weeks at 25 °C in the dark on a rotary shaker. The cells (632 g fresh weight) were extracted as described above. A sterol mixture (136.4 mg) was obtained by partition and silica-gel chromatography. HPLC (system A) of the sterol mixture gave a 24-methylcholesterol mixture (62 mg), which was submitted to HPLC (system B) to give [^{13}C,^{2}H]campesterol **3** (34 mg) and [^{13}C,^{2}H]dihydrobrassicasterol **4** (23 mg).

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