Biosynthesis of Sitosterol, Cycloartenol, and 24-Methylenecycloartanol in Tissue Cultures of Higher Plants and of Ergosterol in Yeast from [1,2-$^{13}C_2$]- and [2-$^{13}C^2H_3$]-Acetate and [5-$^{13}C^2H_2$]MVA†

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The [1,2]-methyl migrations postulated in the 'biogenetic isoprene rule' proposed by Ruzicka *et al.* have been verified by ^{13}C n.m.r. spectroscopy in the biosynthesis of cycloartenol (**10a**), 24-methylenecycloartanol (**11a**), and sitosterol (**12a**) using cultured cells of higher plants, *Rabdosia japonica*‡ and *Physalis peruviana*, and of ergosterol (**14a**) in yeast fed with [1,2-$^{13}C_2$]acetate. The [1,2]-hydride shifts from C-17 to C-20, and C-13 to C-17 have also been demonstrated in the biosynthesis of sitosterol (**12b**) in *R. japonica* and of ergosterol (**14b**) in yeast fed with [2-$^{13}C^2H_3$]acetate. The [1,2]-hydride shift from C-9 to C-8 has also been verified in 24-methylenecycloartanol (**11b**) fed [2-$^{13}C^2H_3$]acetate to tissue cultures of *Trichosanthes kirilowii* Maxim. var. *japonica*. In the side-chain formation of 24-methylenecycloartanol (**11b**) and ergosterol (**14b**), a [1,2]-hydride (deuteride) shift from C-24 to C-25 is observed. Conversely, no deuterium atom at C-24 or C-25 is observed in sitosterol (**12b**) formation. Both C-11 and C-12 of sitosterol (**12c**) labelled as ^{13}C-$^2H_2$ and ^{13}C-$^2H^1H$, biosynthesized from [5-$^{13}C^2H_2$]MVA in *R. japonica* suggest that squalene is released from an enzyme and the following oxidation does not distinguish a terminal double bond of one farnesyl moiety from the other to form epoxysqualenes (**8A**) and (**8B**).

Early studies in Bloch's laboratory showed that acetate is a precursor of cholesterol.[1] Since the 'biogenetic isoprene rule' was proposed by Ruzicka's group,[2] there have been many reports on the biosynthesis of cholesterol.[3] Sterols in plants differ from cholesterol in animals in having one or two additional carbon atoms at C-24 with either *R* or *S* chirality. Starting from acetate the formation of plant sterols follows the same pathway as that of cholesterol in mammals up to epoxysqualene (**8**).[4] The first cyclic product of (**8**) in higher plants is cycloartenol (**10**) instead of lanosterol (**13**) which is synthesized in mammals, yeast, and fungi *via* the cationic intermediate (**9**). In the formation of cycloartenol (**10**), the isoprene rule proposes two [1,2]-methyl migrations (from C-14 to C-13 and from C-8 to C-14) and three [1,2]-hydride shifts (from C-17 to C-20, from C-13 to C-17, and from C-9 to C-8) as shown in structure (**9**).[2] In the subsequent reactions required for phytosterol formation, the alkylation at C-24, demethylation at C-4 and C-14, and double bond formation in ring B leading to phytosterols have been mainly studied using radioisotopes as tracers.[4] The advantage of using stable isotopes which can be observed by n.m.r. spectroscopy is that chemical degradations are not required to analyse the labelling patterns.[5] Plant tissue culture techniques can achieve high incorporation of ^{13}C-labelled precursor, which is required for n.m.r. analysis because of relatively poor sensitivity and a high level of background natural ^{13}C.[6]

We report here experimental evidence for the methyl migrations and the hydride shifts proposed in the isoprene rule. Feeding of [5-$^{13}C^2H_2$]MVA together with [2-$^{13}C^2H_3$]acetate revealed the fate of all of the hydrogens comprising MVA (**2**), and demonstrated that sitosterol (**12**) is formed from both epoxysqualenes (**8A**) and (**8B**).

† Preliminary report: S. Seo, U. Sankawa, H. Seto, A. Uomori, Y. Yoshimura, Y. Ebizuka, H. Noguchi, and K. Takeda, *J. Chem. Soc., Chem. Commun.*, 1986, 1139.

‡ *Rabdosia japonica* was previously called *Isodon japonicus* Hara.

Results and Discussion

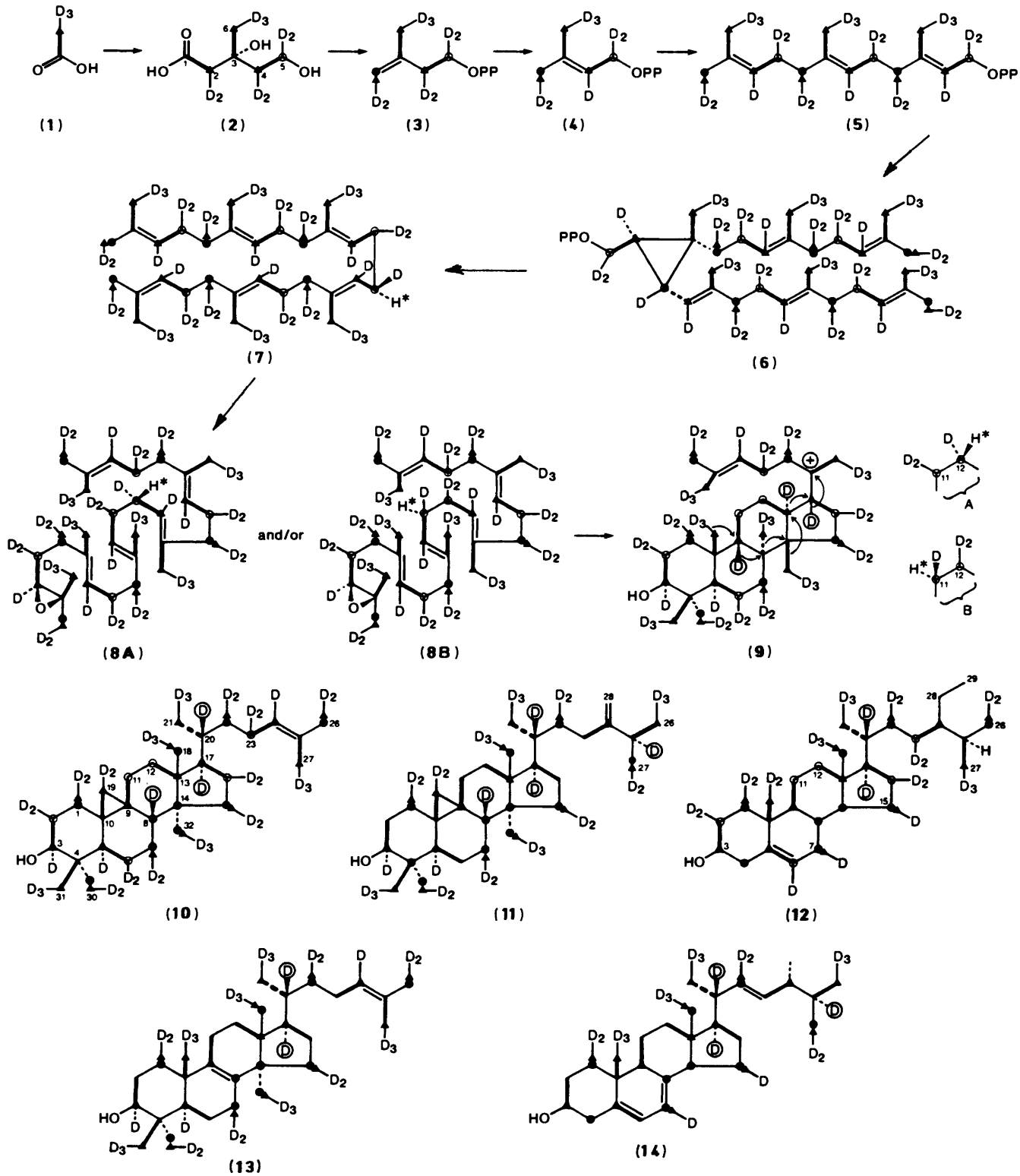
^{13}C *N.m.r. Signal Assignments.*—Cycloartenol (**10**) is the first cyclic product of epoxysqualene in higher plants.[7] As shown in Figure 1 and Table 1, all the ^{13}C n.m.r. signals of (**10**) were assigned by means of 2D INADEQUATE method.[8] The results agree with the recent assignments made by W. Kamisako *et al.*[9]

Sitosterol (**12**) is the most common and usually the main sterol in higher plants. It is known to decrease cholesterol absorption in mice.[10] The assignments of ^{13}C signals of (**12**) were made by comparison with those for cholesterol reported by Popják *et al.*[11]

^{13}C N.m.r. signal assignments for ergosterol (**14**) have been made by Wright.[12] We established the assignments of the C-13 and C-24 signals at δ_C 42.74 and 42.68, respectively, by means of the INEPT method.

[1,2]-*Methyl Migrations.*—The proposed incorporation patterns of [1,2-$^{13}C_2$]acetate (**1a**) into sterols are shown in the Scheme by thick lines (—) and closed circles (●) indicating intact acetate units and isolated ^{13}C atoms, respectively. The observed labelling patterns and ^{13}C-^{13}C coupling constants in the conventional proton decoupled ^{13}C n.m.r. (^{13}C-{^1H} n.m.r.) or INADEQUATE ^{13}C n.m.r. spectra of the acetate of cycloartenol (**10a**), 24-methylenecycloartanol (**11a**), sitosterol (**12a**), and ergosterol (**14a**) are shown in Table 1.

The acetate of cycloartenol (**10a**) and 24-methylenecycloartanol (**11a**) obtained from cultured cells of *P. peruviana* fed with [1,2-$^{13}C_2$]acetate showed ten singlets [eleven for (**11a**)] and twenty doublets in ^{13}C-{^1H} n.m.r. and INADEQUATE n.m.r. spectra. The singlet signals due to C-18 and C-32 clearly imply that the two [1,2]-methyl migrations, from C-14 to C-13 and from C-8 to C-14, take place in between the cationic intermediate (**9a**) and cycloartenol (**10a**), C-8 and C-14 appearing as singlets confirm the methyl migrations. The rest of the six singlets indicate that C-1, C-7, C-15, C-22, and C-27 in (**11a**), [C-26 in (**10a**)], and C-30 originate from C-2 of MVA.



Scheme. **a**: ● and — Carbons derived from [1,2-$^{13}C_2$]acetate; **b**: ▲ carbons derived from [2-$^{13}C^2H_3$]acetate; **c**: ○ carbons derived from [5-$^{13}C^2H_2$]MVA

The coupling constant of the cyclopropyl ring is small (J_{C-C} 10 or 12 Hz to C-19) as reported by Bradshaw *et al.*[13]

The methyl signals due to C-18 of sitosterol (**12a**) obtained from *R. japonica*[6] and ergosterol (**14a**) from yeast fed [1,2-$^{13}C_2$]acetate also appeared as a singlet together with the singlet due to C-14 agreeing with the methyl migration from C-14 to C-13.

[1,2]-*Hydride Shifts*.—The proposed labelling patterns from [2-$^{13}C^2H_3$]acetate (**1b**) into cycloartenol (**10b**), 24-methylene-

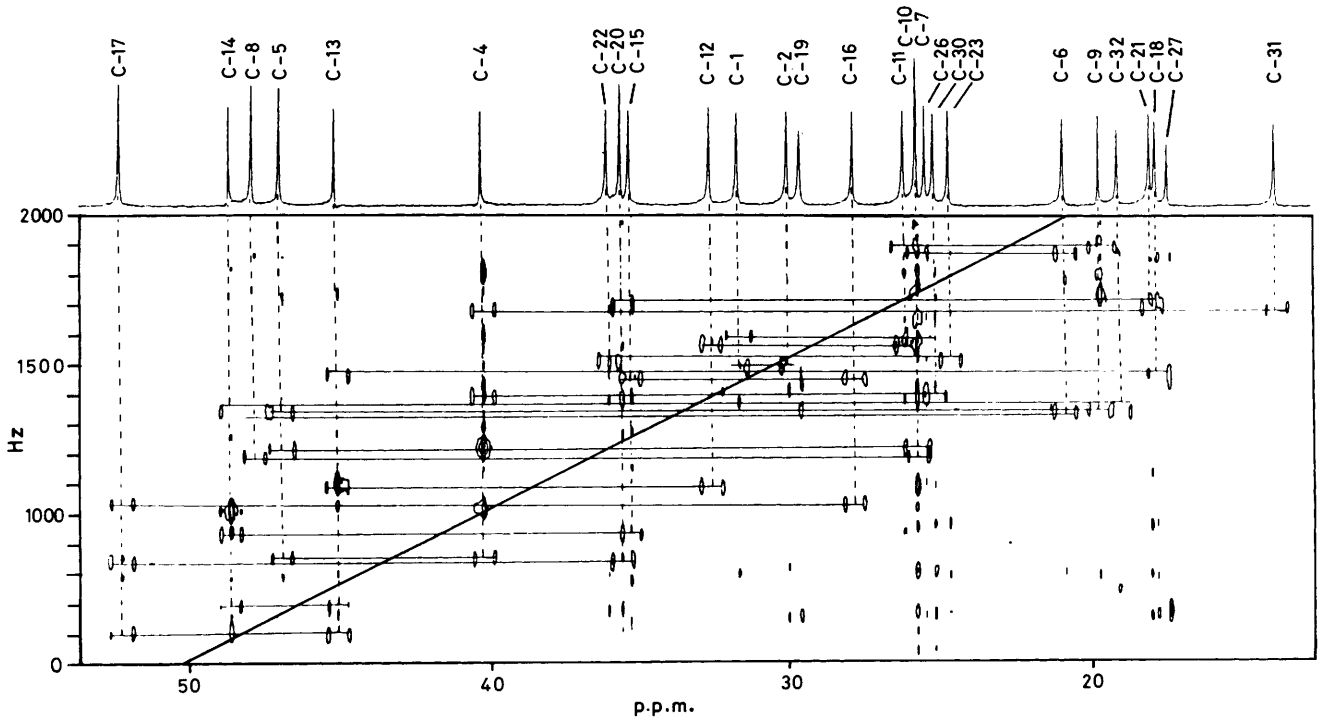


Figure 1. 2D INADEQUATE ^{13}C N.m.r. spectrum (50 MHz) of cycloartenol (**10**) with conventional ^{13}C spectrum at the top of the diagram. The C-2, C-4, C-23, C-26, and C-27 signals do not have full connectivities because the experiment did not cover the ^{13}C chemical shift range of the C-3, C-24, and C-25 signals. Connectivities of the C-9, C-10, and C-19 signals are not observed because of the J_{CC} values for cyclopropane ring (J_{CC} *ca.* 10 Hz) being smaller than the optimized value (J_{CC} 36 Hz) for maximum signal intensity

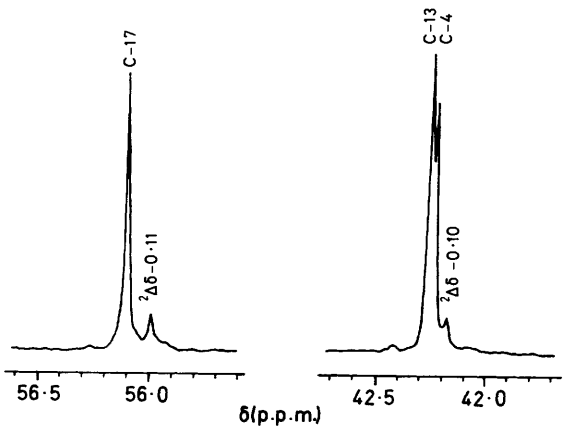


Figure 2. Expanded C-13 and C-17 regions of the ^{13}C-{^1H} n.m.r. spectrum (100 MHz) of sitosterol (**12b**) biosynthesized from [2-$^{13}C^2H_3$]acetate (**1b**) in tissue cultures of *R. japonica*

cycloartanol (**11b**), sitosterol (**12b**), ergosterol (**14b**), and its precursor lanosterol (**13b**) are shown in the Scheme. The carbon atom arising from C-2 of the acetate (**1b**), indicated by a triangle (▲), is incorporated into C-2, C-4, and C-6 of MVA (**2b**).

A carbon bonded directly to one deuterium atom or located next to a deuteriated carbon usually resonates upfield of the corresponding protonated species. The magnitude of the α-deuterium isotope shift ($^1\Delta\delta$) in ^{13}C n.m.r. spectroscopy is larger than that of the β-deuterium isotope shift ($^2\Delta\delta$) and the isotope shift shows additivity.[5,14]

Sodium [2-$^{13}C^2H_3$]acetate (**1b**) diluted with unlabelled sodium acetate was administered for four weeks to suspended cultures of *R. japonica*. A sterol mixture was obtained from the cells together with triterpenes such as ursolic acid and oleanolic acid, as reported in our previous paper.[15] Sitosterol (**12b**) was isolated from the mixture by means of reverse phase h.p.l.c. The ^{13}C- and ^2H-labelling patterns of (**12b**) were analysed by 100 MHz ^{13}C-{^1H}{^2H} and ^{13}C-{^1H} n.m.r. spectroscopy and compared with those of ergosterol (**14b**), which was obtained from yeast (*Saccharomyces cereviceae* IFO 1346) fed [2-$^{13}C^2H_3$]acetate (**1b**).

As shown in Table 2 and Figures 2 and 3, the signals due to C-13 [δ_C 42.28 for (**12b**) and 42.74 for (**14b**)] and C-17 [δ_C 56.12 for (**12b**) and 55.67 for (**14b**)] are accompanied by the β-deuterium isotopically shifted signals ($^2\Delta\delta$ −0.09 to −0.11). These findings indicate that the deuterium atoms located at C-13 and C-17 in an intermediate (**9b**) shift to one of the adjacent carbons, *i.e.*, C-12, C-14, or C-17 for C-13 and C-16 or C-20 for C-17. The C-16 atom of (**12c**) retains two deuterium atoms originating from [5-$^{13}C^2H_2$]MVA (**2c**) as mentioned later. Consequently, the β-deuterium atom of C-17 must be located at C-20.

As C-12 of (**12c**) also retains two deuterium atoms from [5-$^{13}C^2H_2$]MVA (**2c**), the β-deuterium atom of C-13 from [2-$^{13}C^2H_3$]acetate cannot be at C-12. If cycloartenol (**10**) or 24-methylenecycloartanol (**11**) labelled from [2-$^{13}C^2H_3$]acetate (**1b**) shows a β-deuterium isotopically shifted signal at C-13, the β-deuterium atom of C-13 cannot be located at C-14. 24-Methylenecycloartanol (**11b**) biosynthesized from [2-$^{13}C^2H_3$]-acetate (**1b**) was isolated from cultured cells of *Trichosanthes kirilowii* Maxim. var. *japonica*. As shown in Figure 4, the ^{13}C-{^1H} n.m.r. spectrum of (**11b**) showed that each signal of C-24 (δ_C 156.92), C-17 (δ_C 52.29), C-13 (δ_C 45.32), and C-9 (δ_C 20.02) is accompanied by a shifted signal due to a deuterium atom at the β-position of each carbon, $^2\Delta\delta$; −0.03, −0.11, −0.09, and −0.09, respectively. These findings clearly imply that the β-deuterium atom for C-13 must be located at C-17, because the other possible β-carbon, C-14, is occupied by a methyl group in this compound.

As one of the two adjacent carbons of C-9 is C-11, at which

Table 1. ^{13}C N.m.r. spectral data[a] of acetate of cycloartenol (**10a**), the acetate of 24-methylenecycloartanol (**11a**), sitosterol (**12a**), and ergosterol (**14a**) biosynthesized from the [1,2-$^{13}C_2$]acetate (**1a**)

	Acetate of (**10a**)		(**11a**)		(**12a**)		(**14a**)	
Carbon	δ_C	(J_{CC})	δ_C	(J_{CC})	δ_C	(J_{CC})	δ_C	(J_{CC})
1	31.63	(s)	31.98	(s)	37.30	(s)	38.33	(s)
2	26.82	(38)	30.41	(37)	31.69	(37)	31.75	(38)
3	80.70	(38)	78.85	(37)	71.78	(37)	69.98	(38)
4	39.46	(36)	40.50	(36)	42.25	(s)	40.54	(s)
5	47.21	(36)	47.13	(35)	140.77	(72)	139.32	(71)
6	20.95	(36)	21.13	(35)	121.66	(72)	119.09	(71)
7	25.84	(s)	26.02	(s)	31.94	(s)	115.87	(s)
8	47.83	(s)	48.00	(s)	31.94	(s)	140.67	(s)
9	20.18	(43)	20.02	(43)	50.19	(35)	46.21	(36)
10	26.00	(10)	26.10	(12)	36.52	(35)	37.00	(34)
11	26.54	(43)	26.50	(43)	21.11	(35)	21.14	(36)
12	32.89	(34)	32.92	(35)	39.82	(35)	39.06	(37)
13	45.31	(34)	45.32	(35)	42.28	(35)	42.74	(37)
14	48.84	(s)	48.83	(s)	56.81	(s)	54.42	(s)
15	35.56	(s)	35.58	(s)	24.32	(s)	22.99	(s)
16	28.15	(34)	28.17	(33)	28.25	(34)	28.19	(34)
17	52.29	(34)	52.29	(33)	56.12	(34)	55.67	(34)
18	17.98	(s)	18.04	(s)	11.87	(s)	12.06	(s)
19	29.78	(10)	29.90	(12)	19.40	(35)	16.27	(34)
20	35.91	(34)	36.13	(35)	36.17	(35)	40.24	(35)
21	18.25	(34)	18.32	(35)	18.81	(35)	21.07	(35)
22	36.38	(s)	35.02	(s)	34.00	(s)	135.03	(s)
23	24.98	(44)	31.33	(41)	26.19	(35)	131.51	(43)
24	125.29	(44)	156.92	(41)	45.89	(35)	42.68	(43)
25	130.89	(42)	33.82	(35)	29.24	(36)	33.04	(36)
26	25.72	(s)	22.01	(35)	19.81	(s)	19.89	(36)
27	17.64	(42)	21.88	(s)	19.09	(36)	19.61	(s)
28			105.94		23.12		17.57	
29					12.01			
30	25.43	(s)	25.45	(s)				
31	15.15	(36)	14.01	(36)				
32	19.30	(s)	19.33	(s)				
$COCH_3$	21.33							
$COCH_3$	170.54							

[a] Chemical shift is given by δ_C, s indicates a singly labelled carbon, and $^1J_{CC}$ (in Hz) indicates a doubly labelled carbon. Compounds (**10a**) and (**11a**) were obtained from a *P. peruviana* callus, (**12a**) from an *R. japonica* callus, and (**14a**) from yeast.

sitosterol (**12c**) retains two deuterium atoms from [5-^{13}C-$^2H_2$]MVA (**2c**) as described later, the deuterium atom at C-9 in an intermediate (**9b**) shifts to C-8, then the cyclopropyl ring system is formed between C-19 and C-9. Unfortunately, the number of deuterium atoms at C-19 of (**12b**) could not be observed owing to signal overlap. The C-19 atom of ergosterol (**14b**) retains three deuterium atoms consistent with the intermediacy of lanosterol (**13b**).

The ^{13}C-{^1H}{^2H} n.m.r. spectrum of (**11b**) demonstrated that all carbon atoms originating from C-2, C-4, and C-6 of MVA retain the deuterium atoms, $^2H_2$, $^2H_1$, and $^2H_3$, respectively, and only C-19 which arose from C-6 of MVA loses one deuterium atom and retains two (Table 2).

The biosynthetic process of sitosterol (**12b**) and ergosterol (**14b**) from cycloartenol (**10b**) and lanosterol (**13b**), respectively, includes demethylations at C-4 and C-14 and double bond formation in the B-ring. Some deuterium atoms are lost during these processes. The absence of a deuterium atom at C-3 in (**12b**) and (**14b**) suggests a 3-oxo intermediate during the removal of the two methyl groups at C-4.[3,4] One of the deuterium atoms at C-7 in (**12b**) was displaced by a protium atom. This observation confirms that the 7α-hydrogen is introduced by NADH-dependent reduction of the 7(8) double bond in the intermediate.[3,4] The equatorial orientation of the deuterium at C-7 is also indicated by a small α-deuterium isotope shift ($^1\Delta\delta$ −0.37).[16] Actually, C-1 of (**12b**) and (**14b**) showed two shifted signals indicating two labelling patterns at C-1 with one α-deuterium atom oriented equatorially and axially. We also reported two shifted signals for a carbon with one α-deuterium atom in oleanolic acid and ursolic acid.[15] Displacement of one of the deuterium atoms at C-15 by a protium atom in (**12b**) and (**14b**) agrees with 14(15) double bond formation during the demethylation at C-14 followed by hydrogenation to 14α-H and 15β-H.[3,4]

For the side-chain formation of stigmasterol, reduction of a 24-ethyldesmosterol-type intermediate has been proposed.[17] This was confirmed by the absence of deuterium atom observed at C-24 or C-25 of sitosterol (**12b**). Conversely, the β-deuterium shifted signal was observed on the C-24 of (**11b**) and (**14b**) confirming that the first methylation takes place accompanied by a [1,2]-hydride shift from C-24 to C-25.[18] The C-26 (*pro-R* methyl group at C-25) and C-27 (*pro-S* methyl group at C-25) of (**14b**) retain three and two deuterium atoms, respectively.[19]

Stereochemistry at C-11 *and* C-12 *of Sitosterol* (**12c**).—As mentioned above, we studied the fate of hydrogens attached to C-2, C-4, and C-6 of MVA originating from C-2 of acetate in sterol biosynthesis. In the next step, the fate of hydrogens attached to C-5 of MVA was studied by feeding it with [5-$^{13}C^2H_2$]MVA (**2c**),[20] which was prepared from ^{13}C-labelled

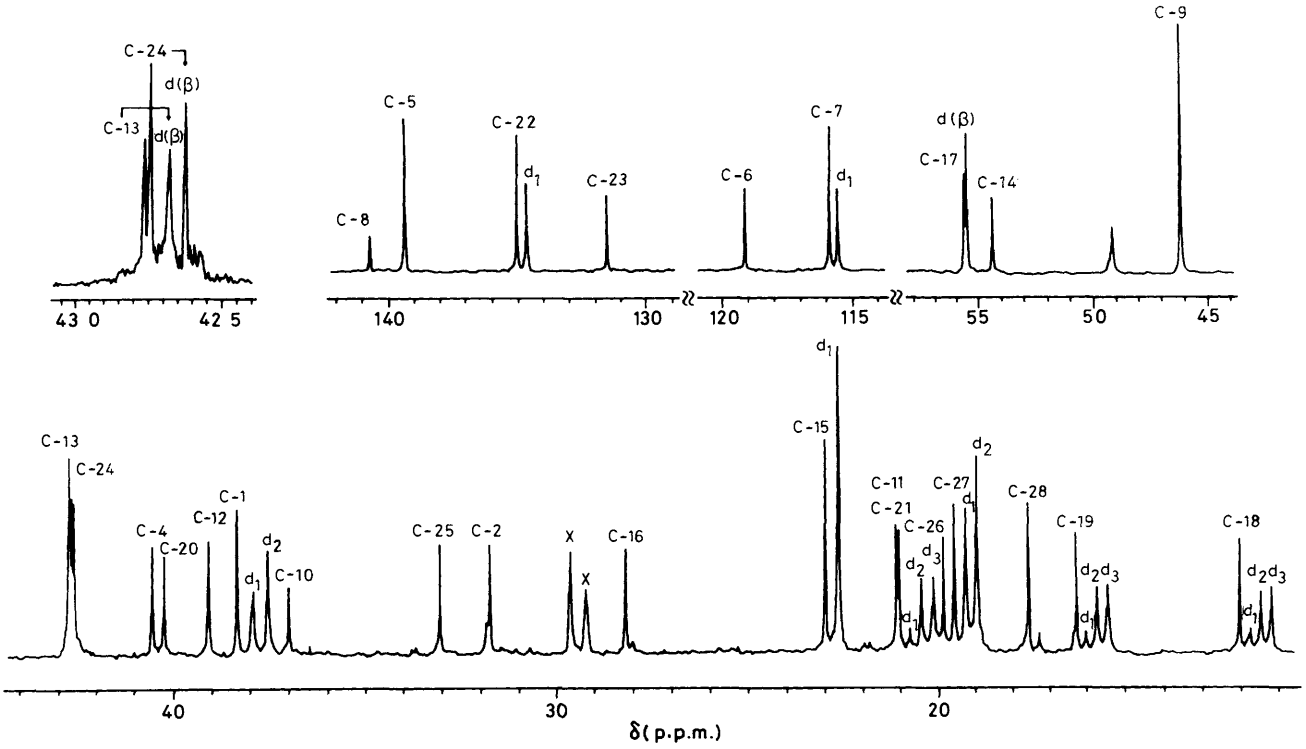


Figure 3. ^{13}C-{^{1}H}{^{2}H} N.m.r. spectrum (100 MHz) of ergosterol (**14b**) biosynthesized from [2-$^{13}C^{2}H_3$]acetate (**1b**) in yeast. The signal due to C-3 (δ_C 69.98) is not included. d_1, d_2, and d_3 Refer to signals shifted by one, two, and three α-deuterium atoms, respectively. d(β) Refers to a β-deuterium isotope shifted signal

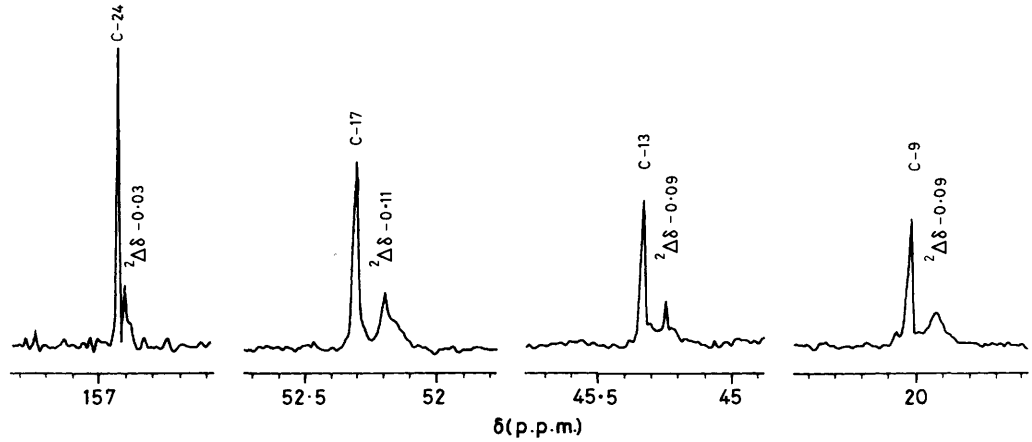


Figure 4. Expanded C-24, C-17, C-13, and C-9 regions of the ^{13}C-{^{1}H} n.m.r. spectrum (100 MHz) of 24-methylenecycloartanol (**11b**) biosynthesized from [2-$^{13}C^{2}H_3$]acetate (**1b**) in tissue cultures of *T. kirilowii* Maxim. var. *japonica*. The region of the C-24 signal has been edited with different weighting factors from the others

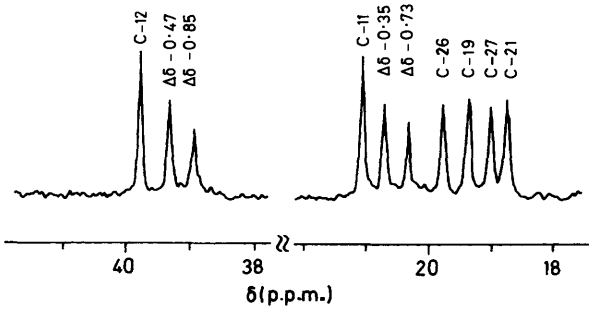


Figure 5. Expanded C-12 and C-11 regions of the ^{13}C-{^{1}H}{^{2}H} n.m.r. spectrum (100 MHz) of sitosterol (**12c**) biosynthesized from [5-$^{13}C^{2}H_2$]MVA (**2c**) in tissue cultures of *R. japonica*

barium carbonate and lithium aluminium deuteride.[15] Sitosterol (**12c**) was isolated from cultured cells of *R. japonica* fed [5-$^{13}C^{2}H_2$]MVA together with ursolic acid and oleanolic acid as reported in our previous paper.[15] As shown in Figure 5 and Table 2, the ^{13}C-{^{1}H}{^{2}H} n.m.r. spectrum of (**12c**) showed six carbons, C-2, C-6, C-11, C-12, C-16, and C-23, labelled from [5-$^{13}C^{2}H_2$]MVA. There are three singlets for C-11 at δ_C 21.11 [$^{13}C(11)$-$^{1}H_2$], 20.76 [$^{1}\Delta\delta$ −0.35 due to $^{13}C(11)$-$^{2}H^{1}H$], and 20.38 [$^{1}\Delta\delta$ −0.73 due to $^{13}C(11)$-$^{2}H_2$]. Also C-12 has three singlets at δ_C 39.82 [$^{13}C(12)$-$^{1}H_2$], 39.35 [$^{1}\Delta\delta$ −0.47 due to $^{13}C(12)$-$^{2}H^{1}H$], and 38.97 [$^{1}\Delta\delta$ −0.85 due to C(12)-$^{2}H_2$]. These data indicate that both carbons, C-11 and C-12 of sitosterol (**12c**) are labelled in two ways, ^{13}C-$^{2}H^{1}H$ and ^{13}C-$^{2}H_2$, as observed in oleanolic acid and ursolic acid biosynthesized from [5-$^{13}C^{2}H_2$]MVA.[15] There is a remarkable difference in the

Table 2. ^{13}C-^{2}H Labelling patterns[a] of 24-methylenecycloartanol (**11b**), sitosterol (**12b**), and ergosterol (**14b**) biosynthesized from [2-$^{13}C^{2}H_3$]acetate (**1b**) and sitosterol (**12c**) in square brackets from [5-$^{13}C^{2}H_2$]MVA (**2c**)

	(**11b**)				(**12b**), [(**12c**)]				(**14b**)			
		$^{1}\Delta\delta_{C(^{2}H)}$				$^{1}\Delta\delta_{C(^{2}H)}$				$^{1}\Delta\delta_{C(^{2}H)}$		
Carbon	δ_C	d_1	d_2	d_3	δ_C	d_1	d_2	d_3	δ_C	d_1	d_2	d_3
C-1	31.98	−0.33 −0.44	−0.77		37.30	−0.37 −0.41	*c*		38.33	−0.37 −0.42	−0.80	
C-2[b]	30.41				31.69		[−0.80]		31.75			
C-3	78.85	−0.54			71.78				69.98			
C-4	40.50				42.25[d]				40.54			
C-5	47.13	−0.64			140.77[f]				139.32			
C-6[b]	21.13				121.66	[−0.38]			119.09			
C-7	26.02	−0.41	*c*		31.94	−0.37			115.87	−0.29		
C-8	48.00				31.94				140.67			
C-9	20.02	(−0.09)[e]			50.19				46.21			
C-10	26.10				36.52				37.00			
C-11[b]	26.50				21.11	[−0.35]	[−0.73]		21.14			
C-12[b]	32.92				39.82	[−0.47]	[−0.85]		39.06			
C-13	45.32	(−0.09)[e]			42.28[d]	(−0.10)[e]			42.74[d]	(−0.09)[e]		
C-14	48.83				56.81				54.42			
C-15	35.58	−0.39	−0.77		24.32	−0.34			22.99	−0.34		
C-16[b]	28.17				28.25		[−0.74]		28.19			
C-17	52.29	(−0.11)[e]			56.12	(−0.11)[e]			55.67	(−0.10)[e]		
C-18	18.04	−0.31	−0.60	−0.85	11.87	−0.29	−0.57	−0.86	12.06	−0.28	−0.57	−0.85
C-19	29.90	−0.44	−0.86		19.40	*c*	*c*		16.27	−0.27	−0.56	−0.83
C-20	36.13				36.17				40.24			
C-21	18.32	*c*	*c*	*c*	18.81	−0.33	−0.61	−0.91	21.07	−0.30	−0.61	−0.91
C-22	35.02	−0.42	−0.80		34.00	−0.42	−0.81		135.03	−0.37		
C-23[b]	31.33				26.19		[−0.79]		131.51			
C-24	156.92	(−0.03)[e]			45.89				42.68[d]	(−0.12)[e]		
C-25	33.82				29.24				33.04			
C-26	22.01	−0.31	−0.60	*c*	19.81	−0.31	*c*		19.89	−0.28	−0.60	−0.90
C-27	21.88	−0.31	−0.61		19.09	*c*	*c*	*c*	19.61	−0.32	−0.61	
C-28	105.94				23.12				17.57			
C-29					12.01							
C-30	25.45	−0.31	−0.61									
C-31	14.01	−0.29	−0.57	−0.88								
C-32	19.33	−0.29	−0.58	−0.93								

[a] ^{13}C N.m.r. spectra were recorded on a JEOL GX-400 spectrometer in the ^{1}H and ^{2}H decoupling [^{2}H]chloroform (δ_C 77.000). d_1, d_2, and d_3 Refer to one, two, and three deuterium atoms, respectively, attached to the carbon. Compound (**11b**) was obtained from *T. kirilowii*, (**12b**) and (**12c**) were obtained from *R. japonica*, and (**14b**) was obtained from yeast. [b] Carbons originating from [5-$^{13}C^{2}H_2$]MVA. [c] These signals were not observed due to overlap with other signals. [d] These signals were assigned by INEPT. [e] These are $^{2}\Delta\delta_{C(^{2}H)}$ values. [f] This value in our preliminary report, S. Seo *et al.*, *J. Chem. Soc., Chem. Commun.*, 1986, 1139, has been corrected.

resonances between sitosterol and the triterpenes. Although the signals due to C-11 of the triterpenes also showed two singlets with corresponding α-deuterium isotope shifts, each singlet was accompanied by a doublet coupled to ^{13}C-12, which was simultaneously incorporated into the same triterpene molecule because of less dilution of labelled MVA.[15] Conversely, sitosterol (**12c**) showed only two singlets with α-deuterium isotope shifts. This fact may indicate that the MVA pool for sitosterol synthesis having a high dilution value is independent of that for the triterpenes with a lower dilution value. Presqualene (**6c**) is formed with loss of the 1-*pro-S* hydrogen atom of one of the two farnesyl moieties starting from [5-$^{13}C^{2}H_2$]MVA (**2c**) *via* IPP (**3c**) and DMAPP (**4c**). Presqualene (**6c**) is then reduced by NADPH to give squalene (**7c**) as found in rats.[3] Squalene (**7c**) seems to be released from the enzyme and loses the presqualene geometry, and then is oxidized to afford epoxysqualenes (**8Ac**) and (**8Bc**) being labelled from [5-$^{13}C^{2}H_2$]MVA as shown in the Scheme. Cyclization of epoxysqualenes (**8Ac**) and (**8Bc**) and the following modifications afford sitosterol (**12c**) with both C-11 and C-12 labelled as ^{13}C-$^{2}H_2$ and $^{13}C^{2}H^{1}H$ [(**12Ac**) and (**12Bc**)] *via* (**10c**) and (**11c**). The signal for C-23 as well as those for C-2 and C-16 accompanied by α-deuterium isotopically shifted signals ($^{1}\Delta\delta$ −0.74 to −0.80) corresponds to labelling with two deuterium atoms without protium exchange after MVA. Consequently, sitosterol is not primarily formed by reduction of the 22(23) double bond of stigmasterol.

Experimental

N.m.r. spectra (^{1}H,^{13}C) were obtained in [^{2}H]chloroform using tetramethylsilane as an internal standard (δ_C and δ_H 0 p.p.m.). ^{13}C-{^{1}H} N.m.r. spectra were recorded on a Varian XL-400 n.m.r. instrument at 100.579 MHz in the ^{1}H-decoupling mode. Typical Fourier transformation (F.t.) n.m.r. conditions were: spectral width (SW) 14 993 Hz; acquisition time (AT) 2.002 s; and pulse flip angle 28°.

2D INADEQUATE ^{13}C N.m.r.[8] spectra of cycloartenol (**10**) were obtained on a Varian XL-200 n.m.r. spectrometer at 50.309 MHz by 2D F.t. of 64 free induction decads (f.i.d.s) with 2 K data points. Zero-filling was used to finally yield a data matrix of 2 048 × 1 024 points. Other F.t. conditions were as follows: SW 2 200 Hz; AT 0.465 s; pulse delay time (PD) 4.135 s; and number of transients 768. 1D INADEQUATE ^{13}C N.m.r.

spectra[9] were obtained on a Varian XL-400 instrument under the following conditions: SW 17 668 Hz; AT 1.699 s; PD 5 s; τ 0.00581 s; and number of transients 60 928. ^{13}C-{^{1}H}{^{2}H} Spectra were determined on a JEOL GX-400 n.m.r. instrument at 100 MHz under the following conditions: SW 20 000 Hz; AT 0.819 s; PD 1 s; pulse width 5 μs; and number of transients 2 749. ^{1}H N.m.r. spectra were recorded on a Varian XL-200 n.m.r. instrument at 200.057 MHz, under the following conditions: SW 2 137 Hz; AT 5 s; and pulse flip angle 37°.

Tissue Cultures of Physalis peruviana.—The aerial part of *Physalis peruviana* cultivated in a greenhouse was sterilized successively using running tap water, 70% ethanol, and 10% sodium hypochlorite and sterilized water. The process was carried out on a clean a bench as possible. Small explants (*ca.* 0.5 cm) from stems and leaves were plated on slants containing Murashige-Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 10^{-4}—10^{-6}M) and kinetin (0—10 p.p.m), and incubated at 25 °C. The callus induced at 2,4-D (10^{-4}M) and kinetin (0.2 p.p.m.) after 4 weeks was sub-cultured every 4 to 6 weeks on the same medium supplemented with 2,4-D (10^{-6}M), kinetin (0 p.p.m.), calcium pantothenate (500 μg l^{-1}), nicotinic acid (500 μg l^{-1}), vitamin C (500 μg l^{-1}), aspartate (3 mg l^{-1}), and yeast nitrogen base (500 mg l^{-1}, DIFCO). About 8 weeks before the feeding experiments, the callus was transferred onto the liquid medium contaning the same nutrition and placed in a fresh medium every 4 weeks.

Feeding of Sodium [1,2-$^{13}C_2$]*Acetate* (**1a**) *to the* P. peruviana *Callus.*—The precultured cells were incubated at 25 °C in the modified Murashige-Skoog liquid medium (9.4 l) described above supplemented with sodium [1,2-$^{13}C_2$]acetate (90 ^{13}C atom% enriched for each carbon; 1.160 g) and unlabelled sodium acetate (2.320 g). After 4 weeks, cultured cells (2.5 kg fresh weight) were collected and extracted with methanol (3 × 2 l) at room temperature. The hexane soluble part (13.7 g) of the methanol extract was chromatographed on silica gel repeatedly eluted with hexane, chloroform, and ethyl acetate, which gave a mixture of phytosterol (590 mg) and a triterpene fraction (143 mg). The latter was acetylated with acetic anhydride and pyridine and then chromatographed on silica gel. The triterpene acetate fraction (27.4 mg) was separated by h.p.l.c. [Nucleosil 7-ODS 250 × 10 mm i.d., eluted with acetonitrile–methanol–chloroform (800:200:6) at 2 ml min^{-1}, and detected using a differential refractometer R401 (Waters)] to obtain the acetate of cycloartenol (**10a**) (11 mg) and the acetate of 24-methylenecycloartanol (**11a**) (2.21 mg). Cycloartenyl acetate (11 mg) was hydrolysed with 1% potassium hydroxide in methanol (3 ml) and gave cycloartenol (**10a**) (8.5 mg), *m/z* 426 (M^+); $\delta_H(CDCl_3)$ 0.333 and 0.560 (2 H, AB system, *J* 4.5 Hz, 19-H), 0.809 (3 H, s, 31-H), 0.883 (3 H, d, *J* 6.2 Hz, 21-H), 0.891 (3 H, s, 32-H), 0.964 (3 H, s, 18-H), 0.966 (3 H, s, 30-H), 1.605 (3 H, br s, 27-H or 26-H), 1.683 (3 H, br s, 26-H or 27-H), 3.280 (1 H, dd, *J* 10.5 and 4.5 Hz, 3-H), and 5.110 (1 H, t, *J* 7 Hz, 24-H); $\delta_C(CDCl_3)$ 13.95 (31), 17.57 (27), 17.97 (18), 18.16 (21), 19.23 (32), 19.92 (9), 21.05 (6), 24.84 (23), 25.34 (30), 25.63 (26), 25.92 (7), 25.96 (10), 26.38 (11), 28.03 (16), 29.79 (19), 30.26 (2), 31.84 (1), 32.77 (12), 35.44 (15), 35.74 (20), 36.21 (22), 40.32 (4), 45.09 (13), 46.92 (5), 47.79 (8), 48.59 (14), 52.07 (17), 78.51 (3), 124.74 (24), and 130.35 (25). 24-Methylenecycloartanyl acetate (2.2 mg) was hydrolysed with 1% potassium hydroxide in methanol (1 ml) to give 24-methylenecycloartanol (**11a**) (1.8 mg), *m/z* 440 (M^+); $\delta_H(CDCl_3)$ 0.335 and 0.560 (2 H, AB system, *J* 4.5 Hz, 19-H), 0.811 (3 H, s, 31-H), 0.897 (3 H, d, *J* 6.3 Hz, 21-H), 0.899 (3 H, s, 32-H), 0.968 (6 H, s, 18-H and 30-H), 1.026 (3 H, d, *J* 6.8 Hz, 26-H or 27-H), 1.031 (3 H, d, *J* 6.9 Hz, 27-H or 26-H), 3.29 (1 H, m, 3-H), 4.670 (1 H, br s, 28-H), and 4.720 (1 H, br s, 28-H).

Feeding of Sodium [1,2-$^{13}C_2$]*Acetate* (**1a**) *to Yeast.*—Yeast (*Saccharomyces cereviceae* IFO 1346) was incubated at 28 °C and 140 r.p.m. for 48 h in a synthetic medium (2 l) containing sodium [1,2-$^{13}C_2$]acetate (150 mg l^{-1}), unlabelled sodium acetate (300 mg l^{-1}), glucose (20.0 g l^{-1}), yeast nitrogen base (13.4 g l^{-1}), and L-asparagine monohydrate (3.0 mg l^{-1}). The cells (39.5 g) were collected by centrifugation and refluxed with 15% potassium hydroxide in ethanol (200 ml) under nitrogen. The filtrate was diluted with water (300 ml) and extracted with ether (3 × 400 ml). The extracts were washed with water, dried, and evaporated to give a crude extract which was crystallized from methanol–chloroform to afford ergosterol (**14a**) (154 mg); $\delta_H(CDCl_3)$ 0.629 (3 H, s, 18-H), 0.823 (3 H, d, *J* 6.8 Hz, 26-H or 27-H), 0.838 (3 H, d, *J* 6.8 Hz, 27-H or 26-H), 0.917 (3 H, d, *J* 6.9 Hz, 28-H), 0.944 (3 H, s, 19-H), 1.035 (3 H, d, *J* 6.5 Hz, 21-H), 3.640 (1 H, m, 3-H), 5.17—5.21 (2 H, m, 22-H and 23-H), 5.39 (1 H, dt, *J* 5.5 and 2.6 Hz, 7-H), and 5.57 (1 H, dd, *J* 6 and 2.5 Hz, 6-H). The purity was more than 96% when a sample was analysed by g.l.c. (1% OV-1 on Gas chrome Q 1.5 m × 4 mm i.d.; column temp. 250 °C).

Feeding of Sodium [2-$^{13}C^2H_3$]*Acetate* (**1b**) *to Yeast.*—Yeast was incubated in the same medium (1 l) to which was added sodium [2-$^{13}C^2H_3$]acetate (150 mg) and unlabelled acetate (300 mg). Ergosterol (**14b**) (100 mg) was isolated by the procedure described above.

Feeding of Sodium [2-$^{13}C^2H_3$]*Acetate* (**1b**) *to the* R. japonica *Callus.*—The phytosterol mixture (248 mg) biosynthesized from sodium [2-$^{13}C^2H_3$]acetate obtained in our previous report was used.[15] Preparative h.p.l.c. [Develosil ODS 250 × 20 mm i.d. eluting with methanol (7 ml min^{-1}) and monitoring at 208 nm] of the sterol mixture gave 4 peaks at 61 min [sitosterol (130 mg)], 58 min [stigmasterol (24 mg)], 55 min [24-methyl sterol mixture (18 mg)], and 48 min [unidentified material (13 mg)].

Tissue Cultures of Trichosanthes kirilowii *Maxim. var.* japonica.—Young plants (2 months old) of *T. kirilowii* were sterilized and explants were plated on Linsmaier-Skoog medium supplemented with 2,4-D (0—10^{-4}M) and kinetin (0—2.0 p.p.m.) as described for *P. peruviana*. The callus formed with 2,4-D (10^{-5}M) and kinetin (0.2 p.p.m.) was subcultured every 4 weeks in the same medium with 2,4-D (10^{-6}M) and kinetin (0.02 p.p.m.). Eight weeks before feeding, the callus was transferred to liquid medium and subcultured every 4 weeks.

Feeding of Sodium [2-$^{13}C^2H_3$]*Acetate* (**1b**) *to the* T. kirilowii *Maxim. var.* japonica *Callus.*—Sodium [2-$^{13}C^2H_3$]acetate (130 mg l^{-1}) was added to the *T. kirilowii* callus grown on the medium (7.8 l) mentioned above for 16 days. After 12 days incubation, the cells were collected and extracted with hot methanol (4 × 2 l). The methanol extracts (86.8 g) were partitioned between water and butanol, and the butanol-soluble fraction (6.1 g) was chromatographed on silica gel. The fraction (1.38 g) eluted with hexane was further chromatographed on silica gel, eluting with hexane–chloroform–ethyl acetate (3:1:1) to give a sterol mixture (240 mg), a 4-demethyltriterpene fraction (44 mg), and a triterpene fraction (24 mg). The triterpene fraction was separated by h.p.l.c. (YMC-pack SH-343 S-15 ODS 250 × 20 mm i.d., eluting with methanol at 6 ml min^{-1}, detecting at 205 nm) to afford cycloartenol (**10b**) (1.8 mg) and 24-methylenecycloartanol (**11b**) (6.6 mg). They were identified by mass spectrometry and ^{1}H and ^{13}C n.m.r. spectroscopy.

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