Biosynthesis of Isofucosterol from [2-$^{13}C^2H_3$]Acetate and [1,2-$^{13}C_2$]Acetate in Tissue Cultures of *Physalis peruviana*—The Stereochemistry of the Hydride Shift from C-24 to C-25

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Migration of the hydrogen atom at C-24 of cycloartenol to C-25 from the *Re*-face of the double bond occurred in the biosynthesis of isofucosterol in cell cultures of *Physalis peruviana* fed [2-$^{13}C^2H_3$]acetate and [1,2-$^{13}C_2$]acetate. This was verified by ^{13}C n.m.r. spectroscopy.

Phytosterols have a methyl or an ethyl group at C-24.[1] Major sterols found in most higher plants are generally 24α-ethylsterols.[2] The 24α-ethylsterol side-chain is known to be formed from cycloartenol *via* 24-methylene (**2**) and 24-ethylidene-intermediates (**3**) by double transmethylations from *S*-adenosylmethionine (SAM).[3]

Nicotra *et al.*[4] have used *Pinus pinea* to study the stereochemistry of hydrogen migration from C-24 to C-25 in the biosynthesis of isofucosterol which has the 24-ethylidene side-chain, and reported that among the two diastereotopic methyl groups at C-25, the pro-*R* methyl group (C-26) was derived from C-2 of mevalonic acid (MVA). On the other hand, we recently demonstrated that the hydrogen atom at C-24 of cycloartenol (**1**) migrated stereospecifically to C-25 from the *Re*-face of the 24(25) double bond to form 24-methylene side-chain (**2**) in the biosynthesis of 24-methylenecycloartanol in tissue cultures of *Trichosanthes kirilowii*,[5] as shown in the Scheme. Consequently, the pro-*R* methyl group (C-26) and the pro-*S* methyl group (C-27) of intermediate (**2**) originate from C-6 and C-2 of MVA, respectively, as found for ergosterol in fungi by Arigoni[6] and by us.[5] These contrasting findings[4–6] suggested that stereochemical inversion at C-25 apparently occurs during the second methylation to the 24(28) double bond of intermediate (**2**) to form compound (**3**). To investigate this point we determined the stereochemistry of the hydrogen migration from C-24 to C-25 in the biosynthesis of isofucosterol in cell cultures of *Physalis peruviana* fed [2-$^{13}C^2H_3$]acetate and [1,2-$^{13}C_2$]acetate.

Results and Discussion

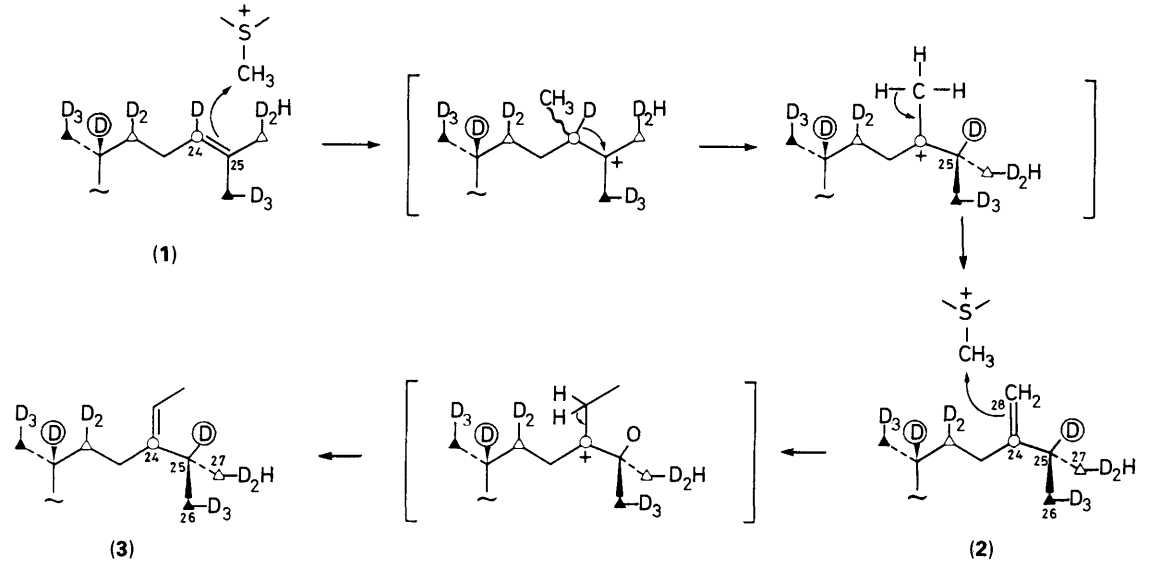
Sodium [2-$^{13}C^2H_3$]acetate (91.6 and 97.4 atom % of ^{13}C and ^2H, respectively) was added to 7-day-old suspension cultures of *Physalis peruviana* grown on Murashige–Skoog medium. After 18 days incubation, [$^{13}C^2H$]-labelled isofucosterol (**4**) was isolated from methanol extracts of the cells.

As shown in Figure 1, in the ^{13}C–{^1H} n.m.r. spectrum of labelled compound (**4**), ^{13}C signals of C-13 (δ_C 42.37), C-17 (δ_C 56.01), and C-24 (δ_C 145.82) were accompanied by singlet signals shifted due to a deuterium atom which migrated to the adjacent carbon (β-deuterium isotope shift; $^2\Delta\delta_{C(^2H)}$ −0.08, −0.11, and −0.03, respectively). The shifted signal due to the β-deuterium atom observed at C-24 suggests that the deuterium atom located at C-24 of cycloartenol (**1**)[7] migrated to C-25 in the biosynthesis of isofucosterol (**4**). When the hydrogen migration takes place stereospecifically, C-26 and C-27 should be labelled differently, one as $^{13}C^2H_2H$ and the other as $^{13}C^2H_3$, owing to the biosynthetic origin.

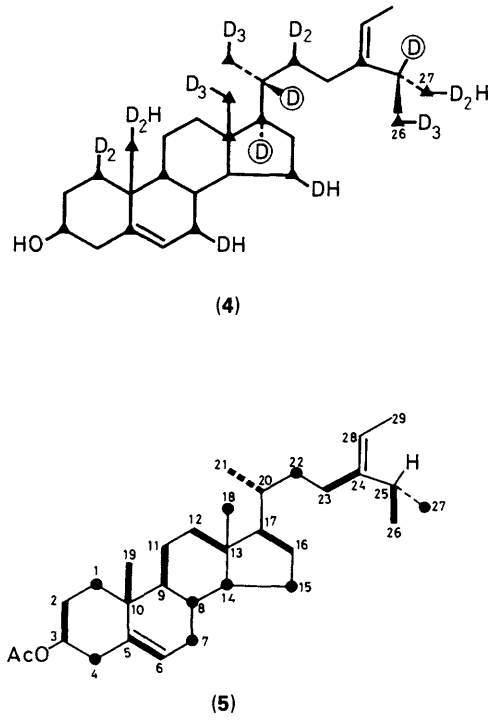
In the ^{13}C–{^1H}{^2H} n.m.r. spectrum of compound (**4**), the numbers of deuterium atoms directly attached to ^{13}C carbons were observed as shifted signals [α-deuterium isotope shift; $^1\Delta\delta_{C(^2H)}$], one deuterium atom on C-7 and C-15 [$^1\Delta\delta_{C(^2H)}$ −0.36], two deuterium atoms on C-22 [$^1\Delta\delta_{C(^2H_2)}$ −0.80], and three deuterium atoms on C-18 and C-21 [$^1\Delta\delta_{C(^2H_3)}$ −0.85 and −0.87, respectively], as shown in Table 1. The signals due to C-26 and C-27 resonate too proximately (δ_C 21.09 and δ_C 21.01) to allow us to differentiate the numbers of the deuterium atoms on these carbons.

In order to clarify the origins of the two methyl carbons, we fed sodium [1,2-$^{13}C_2$]acetate (90 atom % enriched for each carbon) diluted with unlabelled specimen to the cell cultures of the same plant and isolated [^{13}C]-labelled isofucosterol as the acetate (**5**). Among the eighteen enriched doublets in the ^{13}C n.m.r. spectrum of the isolated product (**5**), the signal at δ_C 21.09 was observed as an enriched doublet and the signal at δ_C 21.01 as an enriched singlet, as shown in Figure 2 and Table 2. These results indicate that the hydrogen migration takes place stereospecifically from C-24 to C-25.

Recently, we established the ^{13}C signal assignments of C-26 and C-27 of sitosterol and clionasterol with C-26 resonating at higher field than C-27 in both sterols.[9] On reduction using Adams' catalyst under deuterium gas, [^{13}C]isofucosteryl acetate (**5**) gave a mixture of [^{13}C,24,28-$^2H_2$]sitosteryl acetate (**6**) and [^{13}C,24,28-$^2H_2$]clionasteryl acetate (**7**) together with their non-deuteriated compounds (**8**) and (**9**) and also the 24-monodeuteriated analogue. The ^{13}C-labelling patterns of C-26 and C-27 of these compounds were analysed by means of the ^{13}C 'INADEQUATE' n.m.r. method.[10] As shown in Figure 3 and Table 2, ^{13}C–^{13}C coupled signals were clearly observed for the C-26 siganls of compounds (**8**) and (**9**) (δ_C 19.04, J_{CC} 35 Hz; and δ_C 18.98, J_{CC} 35 Hz, respectively) and were accompanied by the γ-deuterium isotope-shifted signals arising from compounds (**6**) and (**7**) (δ_C 19.01, J_{CC} 35 Hz; and δ_C 18.95, J_{CC} 35 Hz, respectively). On the other hand, C-27 signals of compounds (**8**) and (**9**) (δ_C 19.82 and 19.60, respectively) were observed as labelled singlets accompanied by γ-deuterium isotope-shifted signals arising from (**6**) and (**7**) (δ_C 19.79 and 19.57, respectively), as shown in Figure 3a. As we could observe no signals indicating the existence of a deuterium atom at C-25, these products have the same [^{13}C]-labelling patterns at C-26 and C-27 as the starting material (**5**). This led to the conclusion that [^{13}C]isofucosteryl acetate (**5**) has its C-26 (δ_C 21.09, J_{CC} 35 Hz) predominantly originating from C-6 of MVA, and its C-27



Scheme. Stereochemistry of deuterium migration from C-24 to C-25 in the biosynthesis of isofucosterol in tissue cultures of *Physalis peruviana* fed with [2-$^{13}C^{2}H_3$]acetate. △, ○, and ▲: Carbons originate from C-2, C-4, and C-6 of MVA, respectively



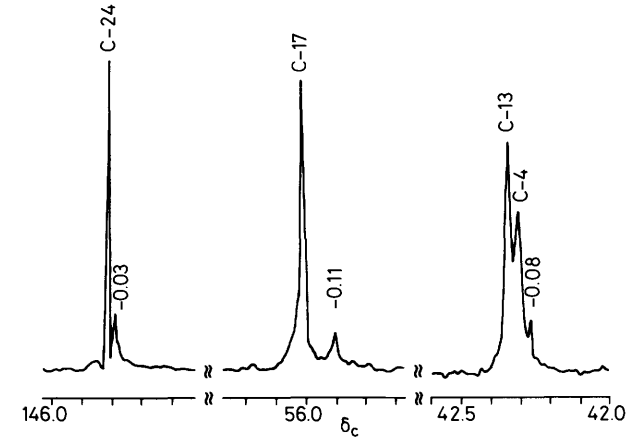


Figure 1. Expanded C-13, C-17, and C-24 regions of ^{13}C-{^{1}H} n.m.r. spectrum of [$^{13}C^{2}H$]isofucosterol (**4**) biosynthesized from [2-$^{13}C^{2}H_3$]-acetate in *P. peruviana* cells

(δ_C 21.01, singlet) predominantly arising from C-2 of MVA, as a result of hydride migration on the *Re*-face of the 24(25) double bond of cycloartenol (**1**).

The *Re*-face hydride shifts from C-24 to C-25 observed here for isofucosterol (**3**) and for 24-methylenecholesterol[11] in *P. peruviana* and also for 24-methylenecycloartanol (**2**) in *Trichosanthes kirilowii*[5] are the same. As shown in the Scheme, isofucosterol (**3**) is formed from 24-methylenecycloartanol (**2**) with retention of the deuterium atom at C-25. As we have previously shown, not only the 24α-ethylsterols[5] but also the 24β-ethylsterols[12] have the same biosynthetic origin for C-26 and C-27 as isofucosterol, although the mechanism of the hydrogen attack at C-25 is varied in higher plants. It is interesting that isofucosterol, biosynthesized in *Pinus pinea* reported by Nicotra *et al.*,[4] has the opposite biosynthetic stereochemistry at C-25 to that in *Physalis peruviana*. The reversed assignments of C-26 and C-27 of sitosterol and clionasterol in our previous reports[5,13] were due to the incorrect assumption that isofucosterol in the two different plants had the same stereochemistry at C-25.

Experimental

N.m.r. spectra were measured in [^{2}H]chloroform, using tetramethylsilane as internal standard (δ 0). ^{13}C-{^{1}H} N.m.r. spectra were recorded on a Varian XL-200 instrument at 50.309 MHz in the ^{1}H decoupling mode. Typical FT n.m.r. conditions were: spectral width (s.w.), 9 090.9 Hz; acquisition time (a.t.), 1.760 s; and pulse flip angle, 20°. The 'INADEQUATE' condition was optimized for J_{CC} 37 Hz (τ 0.007 s): 32 K data points were acquired with a frequency range of 3 200 Hz giving a digital resolution of 0.2 Hz/point. To suppress the singlet ^{13}C signals, an optimal 90° pulse was set for the sample and the steady-state condition was employed before data accumulation (number of transients, 29 K). ^{13}C-{^{1}H}{^{2}H} N.m.r. spectra were determined on a JEOL GX-400 instrument at 100.40 MHz under the following conditions: s.w., 24 038.5 Hz; a.t. 0.580 s; pulse delay, 3 s; and

Table 1. ^{13}C N.m.r. spectral data[a] for [$^{13}C^{2}H_3$]isofucosterol (**4**) biosynthesized from [2-$^{13}C^{2}H_3$]acetate in tissue cultures *P. peruviana*

Carbon	δ_C	$^1\Delta\delta_{C(^2H)}$	$^1\Delta\delta_{C(^2H_2)}$	Carbon	δ_C	$^1\Delta\delta_{C(^2H)}$	$^1\Delta\delta_{C(^2H_2)}$	$^1\Delta\delta_{C(^2H_3)}$
C-1	37.29	−0.36	*c*	C-16	28.24			
		−0.40		C-17	56.01	[−0.11]		
C-2	31.69			C-18	11.88	−0.28	−0.56	−0.85
C-3	71.78			C-19	19.40	−0.30	*c*	
C-4	42.30			C-20	36.15			
C-5	140.75			C-21	18.82	−0.28	−0.61	−0.87
C-6	121.66			C-22	35.96	−0.40	−0.80	
C-7	31.93	−0.36		C-23	27.92			
C-8	31.93			C-24	145.82	[−0.03]		
C-9	50.16			C-25	28.61			
C-10	36.52			C-26	21.09	−0.32	−0.60	−0.89
C-11	21.09			C-27	21.01	−0.32	−0.60	
C-12	39.81			C-28	116.43			
C-13	42.37	[−0.08]		C-29	12.76			
C-14	56.79							
C-15	24.32	−0.36						

[a] Chemical shifts and Δδ values are given in p.p.m. and $^2\Delta\delta_{C(^2H)}$ values are in square brackets. [b] The signal assignments except for C-26 and C-27 agreed with the data in ref. 8. [c] These signals were not observed due to overlapping with other signals.

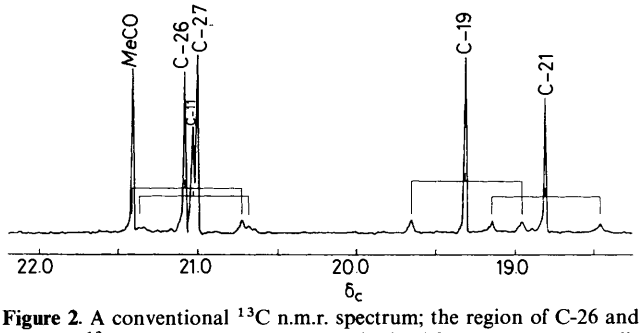
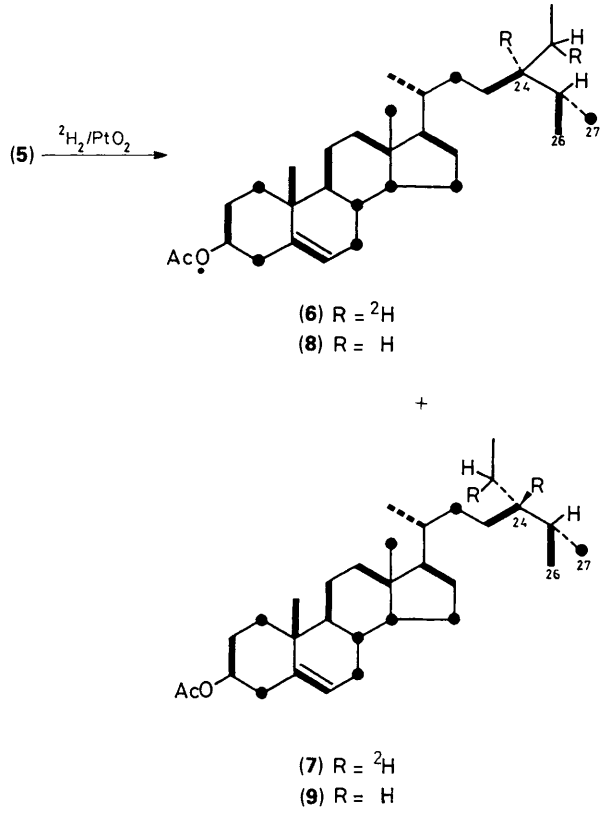


Figure 2. A conventional ^{13}C n.m.r. spectrum; the region of C-26 and C-27 of [^{13}C]isofucosteryl acetate (**5**) obtained from *P. peruviana* cells fed with [1,2-$^{13}C_2$]acetate



pulse flip angle, 45°. ^1H N.m.r. spectra were recorded on a Varian XL-200 instrument at 200.057 MHz under the following conditions: s.w., 2 137 Hz; a.t. 5 s; and pulse flip angle, 23°. The accuracies of δ_C and δ_H are ±0.02 and ±0.005 p.p.m., respectively, and J_{CC} and J_{HH} are ±1 and ±0.4 Hz, respectively. EI mass spectra were obtained on a Hitachi RMU-8GN spectrometer, and $[\alpha]_D$ was determined on a Hitachi Perkin-Elmer 141 instrument. M.p.s were measured on a Yanagimoto micro melting point apparatus and are uncorrected. High-performance liquid chromatography (h.p.l.c.) was performed with a Waters 600 multisolvent delivery system or a Knauer HPLC pump 64 equipped with a UVILOG-5IIIA u.v. detector at 210 nm and a YMC-pack SH343(s-15) ODS (25 cm × 20 mm i.d.) column eluted with methanol (6.0 ml/min). Induction and culture conditions of *Physalis peruviana* were given in our previous report.[5]

Feeding of Sodium [2-$^{13}C^{2}H_3$]*Acetate.*—To precultured cells of *P. peruviana* [at 25 °C for 7 days in Murashige–Skoog liquid medium (10.8 l) supplemented with 2,4-dichlorophenoxyacetic acid (10^{-6}M), calcium pantothenate (0.5 mg/l), vitamin C (0.5 mg/l), aspartate (3 mg/l), and yeast nitrogen base (0.5 g/l)] was added sodium [2-$^{13}C^{2}H_3$]acetate (233 mg/l, 91.6 and 97.4 atom % of ^{13}C and ^2H, respectively) through a membrane filter (0.22 μm). After 18 days of incubation, cells were collected (2.4 kg, fresh weight) and extracted with methanol (3 × 3 l) at room temperature. A mixture of sterols (260 mg) was isolated as described previously.[7] Repeated preparative h.p.l.c. of the sterol mixture gave [$^{13}C^{2}H$]isofucosterol (**4**) (34 mg), at 63 min, m.p. 136—137 °C, *m/z* 412 (M^+), together with 24-methylene-cholesterol (10.3 mg) at 54 min, and other minor sterols, stigmasterol (68 min), a mixture of 24-methylsterols (69 min), and sitosterol (77 min).

For (**4**), δ_H 0.683 (3 H, s, 18-H_3), 0.948 (3 H, d, *J* 7 Hz, 21-H_3), 0.977 (6 H, d, *J* 7.0 Hz, 26- and 27-H_3), 1.009 (3 H, s, 19-H_3), 1.590 (3 H, d, *J* 6.8 Hz, 29-H_3), 2.27 (1 H, A part of ABX, *J* 14 and 12 Hz, 4β-H), 2.29 (1 H, B part of ABX, *J* 14 and 5 Hz, 4α-H), 2.829 (1 H, septet, *J* 7.0 Hz, 25-H), 3.528 (1 H, m, 3-H), 5.110 (1 H, q, *J* 6.8 Hz, 28-H), and 5.359 (1 H, br d, *J* 5 Hz, 6-H).

Feeding of Sodium [1,2-$^{13}C_2$]*Acetate.*—To suspension cultures of *P. peruviana* incubated at 25 °C in modified

Table 2. ^{13}C N.m.r. spectral data[a] of [^{13}C]isofucosteryl acetate (**5**)[b] and a mixture of [^{13}C,24,28-$^{2}H_2$]sitosteryl acetate (**6**)[c] and [^{13}C,24,28-$^{2}H_2$]clionasteryl acetate (**7**)[c]

Carbon	(**5**) δ_C (J_{CC})	(**6**), (**7**), δ_C (J_{CC})	Carbon	(**5**) δ_C (J_{CC})	(**6**) δ_C (J_{CC})	(**7**) δ_C (J_{CC})
C-1	37.00(s)	37.00(s)	C-16	28.23(34)	28.24(34)	
C-2	27.78(36)	27.79(36)	C-17	55.99(34)	56.03(34)	56.00(34)
C-3	73.97(36)	73.97(36)	C-18	11.86(s)	11.86(s)	
C-4	38.12(s)	38.12(s)	C-19	19.31(35)	19.31(35)	
C-5	139.63(72)	139.61(72)	C-20	36.15(35)	36.15(35)	36.27(35)
C-6	122.59(72)	122.61(72)	C-21	18.80(35)	18.78(35)	18.83(35)
C-7	31.88(s)	31.90(s)	C-22	35.93(s)	33.94(s)	33.91(s)
C-8	31.88(s)	31.86(s)	C-23	27.90(42)	26.00(35)[d]	26.28(35)[d]
C-9	50.03(35)	50.03(35)	C-24	145.81(42)	Nd	Nd
C-10	36.59(35)	36.58(35)	C-25	28.62(35)	29.07(35)[d]	28.85(35)[d]
C-11	21.02(35)	21.03(35)	C-26	21.09(35)[e]	19.01(35)[f]	18.95(35)[f]
C-12	39.73(35)	39.72(35)	C-27	21.01(s)	19.79(s)[f]	19.57(s)[f]
C-13	42.34(35)	42.31(35)	C-28	116.44	Nd	Nd
C-14	56.68(s)	56.67(s)	C-29	12.76	*g*	12.20[h]
C-15	24.29(s)	24.29(s)	*Me*CO	21.43	21.42	
			Me*C*O	170.47	170.46	

[a] Chemical shifts are given in p.p.m. s indicates enriched singlet. Figures in J_{CC} are coupling constants of enriched doublet carbon; nd indicates that the signal was not determined because of collapse. [b] [^{13}C]Isofucosteryl acetate (**5**) was obtained from tissue cultures of *P. peruviana* fed [1,2-$^{13}C_2$]acetate. [c] Only the data of (**6**) and (**7**) obtained from (**5**) are shown in the table. The deuterium isotope shifts were determined as the chemical-shift difference between the signals of (**6**) and (**7**) and the signals of the non-deuteriated specimens (**8**) and (**9**), respectively, which were simultaneously obtained from (**5**). [d] $\Delta\delta_{C(^2H)}$ −0.09. [e] One peak of the doublet signal overlapped with the acetyl methyl signal. [f] $\Delta\delta_{C(^2H)}$ −0.03. [g] The signal was not determined due to overlapping with that of C-18. [h] $\Delta\delta_{C(^2H)}$ −0.12.

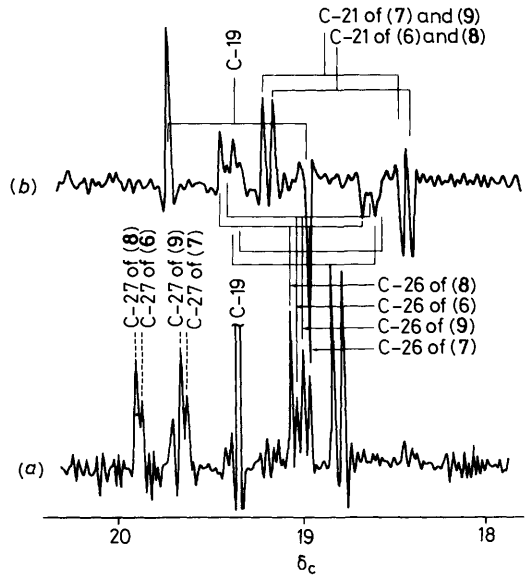


Figure 3. (*a*) Conventional and (*b*) the 'INADEQUATE' ^{13}C n.m.r. spectra; the region of C-26 and C-27 of a mixture of compounds (**6**)—(**9**) obtained from substrate (**5**)

Murashige–Skoog medium (7.2 l) described above, sodium [1,2-$^{13}C_2$]acetate (66.7 mg/l; 90 ^{13}C atom % enriched for each carbon) and unlabelled sodium acetate (133 mg/l) were added. After 4 weeks of incubation, cells (1.1 kg fresh weight) were collected and a mixture of phytosterols (170 mg) was isolated as reported previously.[5] Repeated preparative h.p.l.c. of the sterol mixture gave [^{13}C]isofucosterol (67 mg), m.p. 136—137 °C (from methanol), together with sitosterol (8.8 mg), stigmasterol (5.5 mg), and 24-methylenecholesterol (4.3 mg). [^{13}C]Isofucosterol (46.6 mg) was acetylated with acetic anhydride and pyridine to obtain [^{13}C]isofucosteryl acetate (**5**) (50 mg), m.p. 134.5—135.5 °C; $[\alpha]_D^{24}$ −40.8° (1.0 in $CHCl_3$) (lit.,[14] m.p. 130.5—131 °C; $[\alpha]_D$ −41.9°); *m/z* 394 (M^+ − 60); δ_H 0.680 (3 H, s, 18-H_3), 0.945 (3 H, d, *J* 7 Hz, 21-H_3), 0.974 (6 H, d, *J* 6.9 Hz, 26- and 27-H_3), 1.018 (3 H, s, 19-H_3), 1.586 (3 H, d, *J* 6.8 Hz, 29-H_3), 2.028 (3 H, s, Ac), 2.314 (2 H, br d, *J* 7.8 Hz, 4-H_2), 2.819 (1 H, septet, *J* 6.8 Hz, 25-H), 4.600 (1 H, m, 3-H), 5.094 (1 H, q, *J* 6.8 Hz, 28-H), and 5.370 (1 H, br d, *J* 5 Hz, 6-H).

Catalytic Deuteriation of [^{13}C]*Isofucosteryl Acetate* (**5**).—[^{13}C]Isofucosteryl acetate (**5**) (50 mg) was shaken in ethyl acetate (2 ml) with platinum(IV) oxide (8.4 mg) under deuterium gas for 30 min at room temperature. The reaction mixture was filtered and the filtrate was evaporated to obtain a crude product, which was crystallized from methanol–chloroform to afford a mixture of [^{13}C,24,28-$^{2}H_2$]-(**6**),[^{13}C]-(**8**), [^{13}C,24-^{2}H]sitosteryl acetate, [^{13}C,24,28-$^{2}H_2$]-(**7**),[^{13}C]-(**9**), and [^{13}C,24-^{2}H]clionasteryl acetate (46 mg), *m/z* 396 (71%, *M* − 60), 397 (100), and 398 (59).

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Received 21*st March* 1989; *Paper* 9/01217F