Direct Evidence for Three 1,2-Hydride Migrations in the Biosynthesis of Cycloartenol from [2-$^{13}C^2H_3$]Acetate in Tissue Cultures of *Physalis peruviana*

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[$^{13}C^2H$]-Labelled cycloartenol (**2**) was isolated from cultured cells of *Physalis peruviana* fed with [2-$^{13}C^2H_3$]acetate. The distribution of the deuterium atoms was determined by ^{13}C-{^1H}{^2H} n.m.r. spectroscopy. The 1,2-hydride migrations (20-H from C-17, 17-H from C-13, and 8-H from C-9) were verified in the biosynthesis of cycloartenol (**2**).

In the course of our studies on the biosynthesis of triterpenes[1,2] and sterols,[3,4] we have shown the usefulness of ^{13}C n.m.r. spectroscopy especially for examining carbon–carbon rearrangements and hydrogen migrations. Recently, we studied by ^{13}C-{^1H}{^2H} n.m.r. spectroscopy the biosynthesis of phytosterols from [2-$^{13}C^2H_3$] acetate and observed two deuterium atoms at C-19 of 22-dihydrochondrillasterol and chondrillasterol as evidence that these 24β-ethylsterols were biosynthesized *via* cycloartenol in higher plants.[5]

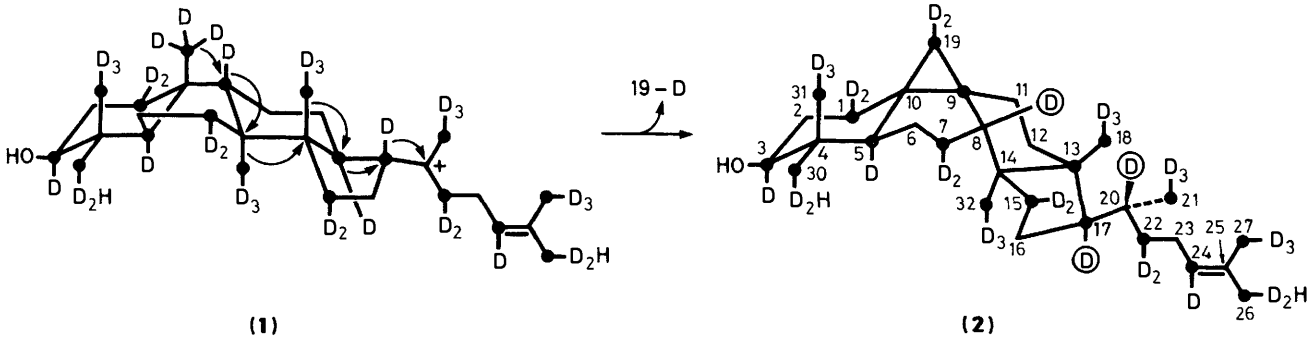
Cycloartenol, isolated in 1953,[6] is generally accepted as the first cyclic intermediate for phytosterol biosynthesis in photosynthetic organisms[7–13] in contrast to vertebrates and fungi, where the first cyclization product is lanosterol. Formation of cycloartenol from oxidosqualene should involve two methyl migrations and three hydride migrations as shown in the intermediate (**1**),[14–20] according to the 'biogenetic isoprene rule'[21] proposed for lanosterol biosynthesis. The two methyl migrations (from C-14 to C-13 and from C-8 to C-14) have been demonstrated in tissue cultures of *Physalis peruviana*[4] and *Gardenia jasmoides*.[22] Only one hydrogen migration (9-H to C-8) has been demonstrated in potatoes.[23] We now report the direct evidence for all the three hydrogen migrations in the biosynthesis of cycloartenol (**2**) in the tissue cultures of *P. peruviana*.

Results and Discussion

Sodium [2-$^{13}C^2H_3$]acetate (233 mg/l, 91.6 and 97.4 atom% of ^{13}C and ^2H, respectively) was administered to 7-day-old suspension cultures of *Physalis peruviana* grown in Murashige-Skoog liquid medium. After 18 days incubation, [$^{13}C^2H$]-labelled cycloartenol (**2**) was isolated from methanolic extracts of the cells. ^{13}C-{^1H}{^2H} N.m.r. spectra of (**2**) were analysed based on our ^{13}C signal assignments,[4] which have been established using the 2D-INADEQUATE method. From [2-$^{13}C^2H_3$]acetate we expected to have 35 deuterium atoms, of which 27 were clearly observed as shown in the Table. Of these 27, 24 deuterium atoms were attached directly to the ^{13}C atoms since they were observed as coupled signals in the ^{13}C-{^1H} n.m.r. spectrum and confirmed as singlet signals shifted by α-deuterium isotope effects ($^1\Delta\delta_{C(D)}$) in the ^{13}C-{^1H}{^2H} spectrum. The three remaining deuterium atoms due to C-17 (δ_C 52.28), C-13 (δ_C 45.27), and C-9 (δ_C 20.00), which were accompanied by β deuterium shifted signals ($^2\Delta\delta_{C(D)}$ −0.11, −0.09, and −0.10, respectively), were confirmed as singlet signals in the ^{13}C-{^1H} n.m.r. spectrum as shown in the Figure. These facts indicate that the deuterium atoms at C-17, C-13, and C-9 in intermediate (**1**) migrate to C-20, C-17, and C-8, respectively, and agree with the results obtained with 24-methylenecycloartenol biosynthesized from [2-$^{13}C^2H_3$] acetate in tissue cultures of *Trichosanthes kirilowii*[4] and sitosterol in tissue cultures of *Rabdosia japonica*.[4] A deuterium atom was clearly observed at C-24. This is the atom which has been demonstrated to migrate to C-25 in the biosynthesis of 24-methylenecycloartanol,[4] to migrate back from C-25 to C-24 in the biosynthesis of 24β-ethylsterols,[5] and to be eliminated in the biosynthesis of 24α-ethylsterols.[4]

Experimental

^{13}C-{^1H}{^2H} and ^{13}C-{^1H} N.m.r. spectra were recorded on a JEOL GX-400 instrument at 100.4 MHz in [^2H]chloroform with tetramethylsilane as an internal reference. The typical F.T. n.m.r. conditions for ^{13}C-{^1H}{^2H} n.m.r. were: spectral width (s.w.), 15 503.9 Hz; acquisition time (a.t.) 0.600 s; pulse width, 4 μs; pulse delay, 2.2 s; and number of transients, 2 000, and for



(**1**) (**2**)

●; Carbons derived from C–2 of [2-$^{13}C^2H_3$]acetate

Table. ^{13}C-{^{1}H}{^{2}H} N.m.r. data of cycloartenol (**2**) from [2-$^{13}C^{2}H_3$] acetate in tissue cultures of *Physalis peruviana*

	δ_C	$^{2}\Delta\delta_{C(D)}$[a]	$^{1}\Delta\delta_{C(D)}$[b] D_1	D_2		δ_C	$^{2}\Delta\delta_{C(D)}$[a]	$^{1}\Delta\delta_{C(D)}$[b] D_1	D_2	D_3
C-1	31.97		−0.43	−0.82	C-17	52.28	−0.11			
C-2[c]	30.38				C-18	18.04		−0.29	−0.59	−0.86
C-3	78.51		−0.52		C-19	29.90		−0.47	−0.88	
C-4[c]	40.48				C-20[c]	35.88				
C-5	47.11		−0.60		C-21	18.28		−0.30	−0.60[d]	−0.88[d]
C-6[c]	21.13				C-22	36.35		−0.47[d]	−0.85[d]	
C-7	26.02		−0.40	−0.83[d]	C-23	24.94				
C-8[c]	47.98				C-24	125.23		−0.37		
C-9	20.00	−0.10			C-25[c]	130.86				
C-10[c]	26.06				C-26	25.73		−0.29[d]	−0.59[d]	
C-11[c]	26.48				C-27	17.64		−0.31	−0.55[d]	−0.80
C-12[c]	32.89				C-28	—				
C-13	45.27	−0.09			C-29	—				
C-14	48.78				C-30	25.44		−0.31	−0.63	
C-15	35.58		−0.39	−0.78	C-31	14.01		−0.31	−0.57	−0.88
C-16[c]	28.14				C-32	19.31		−0.30	−0.59	−0.89

[a] β-Deuterium isotope shift. [b] α-Deuterium isotope shift. [c] The carbons were not labelled with [2-$^{13}C^{2}H_3$] acetate. [d] These signals overlapped with other signals.

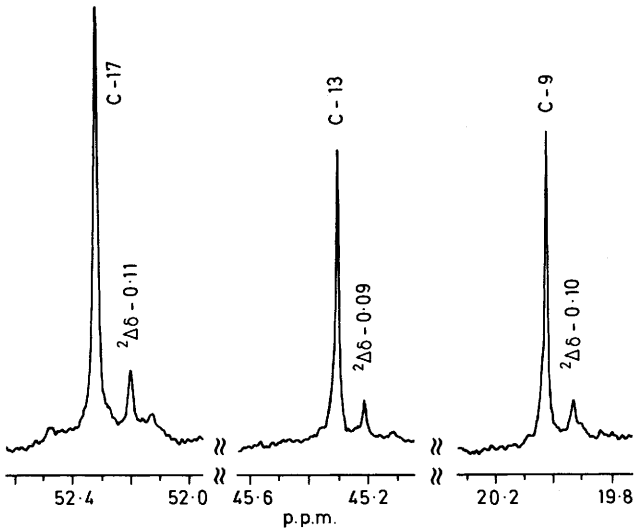


Figure. C-9, C-13, and C-17 Regions of the ^{13}C-{^{1}H} n.m.r. spectrum (100 MHz) of cycloartenol (**2**) biosynthesized from [2-$^{13}C^{2}H_3$]-acetate in tissue cultures of *P. peruviana*.

^{13}C-{^{1}H} n.m.r.: s.w., 15 503.9 Hz; a.t., 0.968 s; pulse flip angle, 35°; and number of transients, 3 000. E.i. mass spectra were obtained on a Hitachi RMU-8GN spectrometer and $[\alpha]_D$ was measured on a Hitachi Perkin-Elmer 141 instrument. M.p.s were determined on a Yanagimoto micro melting point apparatus and are uncorrected. H.p.l.c. was performed with a Waters 600 multisolvent delivery system with a UVILOG-5IIIA u.v. detector at 210 nm.

Feeding of Sodium [2-$^{13}C^{2}H_3$]*Acetate*.—Induction and cultured conditions of *Physalis peruviana* callus were given in our previous report.[4] Cells of *P. peruviana* were precultured 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), nicotinic acid (0.5 mg/l), vitamin C (0.5 mg/l), aspartate (3 mg/l), and yeast nitrogen base (0.5 g/l, DIFCO). Sodium [2-$^{13}C^{2}H_3$] acetate (2.520 g, 91.6 and 97.4 atom% of ^{13}C and ^{2}H, respectively) was added through a membrane filter (0.22 μm). After 18 days incubation, cells were collected (2.4 kg fresh weight) and extracted with methanol (3 × 3 l) at room temperature. The butanol-soluble part (9.0 g) of the methanolic extracts (42 g) was chromatographed on silica gel (100 g, deactivated with 10 g of water) eluted successively with hexane (1.0 l), chloroform (2.0 l), acetone (200 ml), and then methanol (1.0 l). The chloroform fraction (1.66 g) was chromatographed on silica gel (Lobar B) eluted with hexane–chloroform–ethyl acetate (3:1:1) to give a fraction containing sterols and triterpenes (400 mg). It was acetylated with acetic anhydride and pyridine followed by chromatography on silica gel (Lobar B) eluted with hexane–chloroform–ethyl acetate (15:1:1) to give an acetate mixture (355 mg). The acetate mixture (355 mg) was refluxed in 1% methanolic potassium hydroxide (70 ml) for 1 h, diluted with water (100 ml), and extracted with ethyl acetate (2 × 300 ml). The extracts were washed with water, dried (Na_2SO_4), and evaporated to dryness to give a crude mixture of sterols and cycloartenol (300 mg). This mixture was chromatographed on silica gel (Lobar B) eluted with hexane–chloroform–ethyl acetate (5:1:1) to afford a mixture of sterols (260 mg) and crude cycloartenol (13.5 mg). The crude cycloartenol was purified by h.p.l.c. [YMC-pack SH-343 (S-15) ODS, 25 cm × 20 mm i.d.; eluted with methanol (6 ml/min)] to obtain cycloartenol (**2**) (8.0 mg), m.p. 116—117 °C (from MeOH), $[\alpha]_D^{24}$ + 46° (0.30 in $CHCl_3$) (lit.,[6] m.p. 115 °C, $[\alpha]_D$ 54°), *m/z* 426 (M^+).

References

1 S. Seo, Y. Tomita, and K. Tori, *J. Am. Chem. Soc.*, 1981, **103**, 2075.
2 S. Seo, Y. Yoshimura, A. Uomori, H. Noguchi, Y. Ebizuka, U. Sankawa, H. Seto, and K. Takeda, *J. Am. Chem. Soc.*, 1988, **110**, 1740.
3 S. Seo, A. Uomori, Y. Yoshimura, and K. Tori, *J. Chem. Soc., Perkin Trans. 1*, 1984, 869.
4 (*a*) S. Seo, U. Sankawa, H. Seto, A. Uomori, Y. Yoshimura, Y. Ebizuka, H. Noguchi, and K. Takeda, *J. Chem. Soc., Chem. Commun.*, 1986, 1139; (*b*) S. Seo, A. Uomori, Y. Yoshimura, K. Takeda, H. Seto, Y. Ebizuka, H. Noguchi, and U. Sankawa, *J. Chem. Soc., Perkin Trans. 1*, 1988, 2407.
5 S. Seo, A. Uomori, Y. Yoshimura, U. Sankawa, Y. Ebizuka, H. Seto, and K. Takeda, *J. Chem. Soc., Chem. Commun.*, 1987, 1876.
6 H. R. Bentley, J. A. Henry, D. S. Irvine, and F. S. Spring, *J. Chem. Soc.*, 1953, 3673.

7 U. Eppenberger, L. Hirth, and G. Ourisson, *Eur. J. Biochem.*, 1969, **8**, 180.
8 J. R. Lenton, J. Hall, A. R. H. Smiss, E. L. Ghisalberti, H. H. Rees, L. J. Goad and T. W. Goodwin, *Arch. Biochem. Biophys.*, 1971, **143**, 664.
9 G. F. Gibbons, L. J. Goad, T. W. Goodwin, and W. R. Nes, *J. Biol. Chem.*, 1971, **246**, 3967.
10 L. J. Altman, C. Y. Han, A. Bertolino, G. Handy, D. Laungani, W. Muller, S. Schwartz, D. Shanker, W. H. de Wolf, and F. Yang, *J. Am. Chem. Soc.*, 1978, **100**, 3235.
11 H. H. Rees, L. J. Goad, and T. W. Goodwin, *Tetrahedron Lett.*, 1968, 723.
12 E. Caspi and J. Sliwowski, *J. Am. Chem. Soc.*, 1975, **97**, 5032.
13 M. J. E. Hewlins, J. D. Ehrhardt, L. Hirth, and G. Ourisson, *Eur. J. Biochem.*, 1969, **8**, 184.
14 P. Benveniste, L. Hirth, and G. Ourisson, *Phytochemistry*, 1966, **5**, 45.
15 G. Ponsinet and G. Ourisson, *Phytochemistry*, 1967, **6**, 1235.
16 R. J. Kemp, A. S. A. Hammam, L. J. Goad, and T. W. Goodwin, *Phytochemistry*, 1968, **7**, 447.
17 R. Heintz and P. Benveniste, *Phytochemistry*, 1970, **9**, 1499.
18 F. F. Knapp and H. J. Nicholas, *Phytochemistry*, 1971, **10**, 85.
19 L. J. Goad and T. W. Goodwin, *Biochem. J.*, 1966, **99**, 735.
20 L. J. Goad and T. W. Goodwin, *Eur. J. Biochem.*, 1967, **1**, 357.
21 A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, 1955, **38**, 1890; L. Ruzicka, *Proc. Chem. Soc.*, 1959, 341.
22 W. Kamisako, C. Honda, K. Suwa, and K. Isoi, *Magn. Reson. Chem.*, 1987, **25**, 683.
23 H. H. Rees, L. J. Goad, and T. W. Goodwin, *Biochem. J.*, 1968, **107**, 417.

Received 22*nd April* 1988; *Paper* 8/01581C