Biosynthesis of Ursolic Acid in Cell Cultures of *Perilla frutescens* Britt. var. *acuta* Kuda: Mechanism of D- and E-ring Formation

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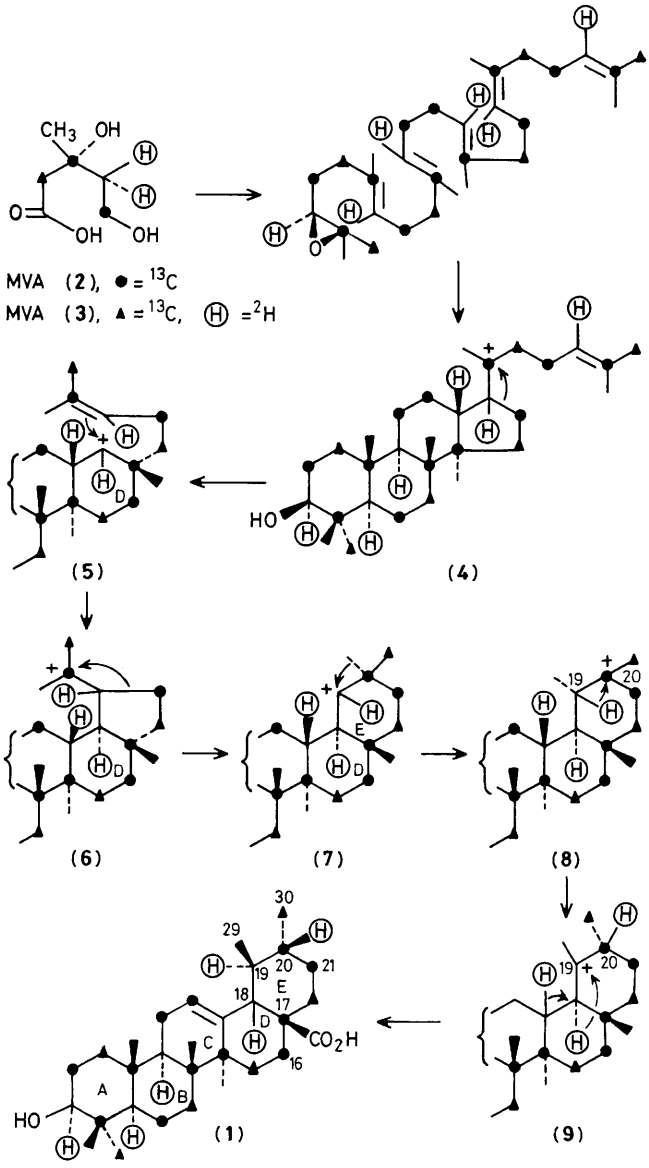
^{13}C N.m.r. analysis of ursolic acid produced by cultivation of *Perilla frutescens* Britt. var. *acuta* Kudo in the presence of [3,5-$^{13}C_2$]- and [4-$^{2}H_2$,2-^{13}C]-mevalonolactone verified two backbone rearrangements and a 1,2-hydride shift with regard to formation of the D- and E-ring during biosynthesis of ursolic acid.

Ursolic acid (**1**) together with oleanolic acid is widely distributed throughout the plant kingdom. Ruzicka, Eschenmoser, Jeger, and Arigoni postulated a 'biogenetic isoprene rule' for the biosynthesis of terpenoids including oleanene- and ursene-type triterpenes.[1] The hypothesis for these pentacyclic triterpenes involves a series of Wagner–Meerwein rearrangements during the formation of the D- and E-ring systems from the cyclization intermediate (**4**) of 2,3-oxidosqualene (Scheme 1). It has been demonstrated that the ^{13}C-labelling patterns, in the proton noise decoupled $^{13}C\{^1H\}$ n.m.r. spectra of oleanene- and ursene-type triterpenes biosynthesized from [4-^{13}C]mevalonolactone (MVA) and [1,2-$^{13}C_2$]acetate respectively, were consistent with Ruzicka's hypothesis.[2,3] Hydride shifts, in the biosynthesis of the oleanene-type triterpene β-amyrin, have previously been verified[4] but this has not been demonstrated in the case of ursene-type triterpenes owing to the difficulty involved in chemically degrading these molecules. In the present paper, we report more definitive evidence for the mechanism of D- and E-ring formation involving backbone rearrangements and hydride-shifts in the biosynthesis of ursolic acid.

Cell cultures of *Perilla frutescens* Britt. var. *acuta* Kudo were grown in Linsmaier–Skoog liquid medium. [3,5-$^{13}C_2$]-MVA (**2**) was prepared from [3-^{13}C]-1,1-dimethoxybutan-3-one and ethyl [1-^{13}C]acetate.[5] A solution of (**2**) 0.3 g (*ca.* 90% ^{13}C) in 50% ethanol (15 ml) was distributed among 15 bottles containing cell cultures of *P. frutescens* (100 ml × 15). Cells were harvested after two weeks and extracted with methanol. Ursolic acid was obtained by preparative t.l.c. as described previously.[3] The $^{13}C\{^1H\}$ n.m.r. spectrum of its methyl ester was then compared to enriched and unenriched samples. The resulting spectrum, overlapping with the naturally abundant ^{13}C spectrum, clearly showed that the signals of eight carbon atoms, C-2, -4, -6, -8, -10, -11, -12, and -14 were singlets and enriched about twofold, while the signals of four carbon atoms, C-16, -17, -20, and -21 were doublets, being those derived from both the C-3 and C-5 of MVA.† The ^{13}C-double labelling patterns, in particular the appearance of these doublet signals, arising from ^{13}C–^{13}C couplings, proved that the biosynthesis of the D- and E-ring systems proceeds along the route (**4**)→(**5**)→(**6**)→(**7**) postulated earlier.

Staunton[6] previously reported an application of deuterium-isotope shifts in ^{13}C n.m.r. spectra for detecting and quantifying the biosynthetic incorporation of deuterium, and Simpson *et al.*[7] recently showed that β-isotope shifts are additive and moreover are dependent on the stereospecificity of labelling.

We applied this method to a study of the hydride shift with regard to formation of the E-ring in the biosynthesis of ursolic acid. [4-$^{2}H_2$,2-^{13}C]MVA, (**3**) (*ca.* 90% ^{13}C; 90% ^{2}H) prepared from [2-^{2}H]-1,1-dimethoxybutan-3-one[8] and ethyl [2-^{13}C]acetate, was distributed among 10 bottles containing cultures of *P. frutescens* and ursolic acid was then isolated in the same manner. The resulting $^{13}C\{^1H\}$ n.m.r. spectrum of its methyl ester clearly showed that the signals of five carbon atoms, C-1, -8, -15, -22, and -23 were enriched about twofold, while the signal of the C-30 methyl group (δ 21.187) derived from the C-2 of MVA was not enriched but accompanied by an



Scheme 1

† $^{13}C\{^1H\}$ N.m.r. data (50.10 MHz, [$^2H_5$]pyridine): δ_C 28.12 (s, C-2), 40.06 (s, C-4), 18.85 (s, C-6), 39.41 (s, C-8), 37.34(s, C-10), 23.67 (s, C-11), 125.71 (s, C-12), 42.57 (s, C-14), 24.96 [d, C-16, $^1J(^{13}C–^{13}C)$ 35 Hz], 48.11 [d, C-17, $^1J(^{13}C–^{13}C)$ 35 Hz], 39.41 [d, C-20, $^1J(^{13}C–^{13}C)$ 35 Hz], 31.11 [d, C-21, $^1J(^{13}C–^{13}C)$ 35 Hz].

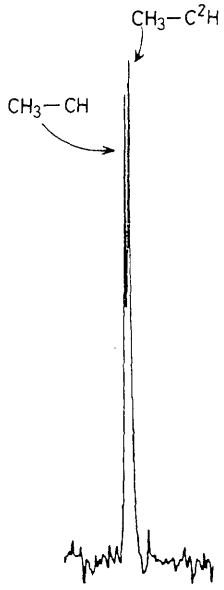


Figure 1. Signal of C-30 methyl group from 50.10 MHz $^{13}C\{^1H\}$ n.m.r. spectrum of methyl ursolate derived from [4-$^2H_2$,2-^{13}C]mevalonolactone (in $CDCl_3$).

isotopically shifted signal (δ − 0.123 p.p.m.) owing to the presence of an axial deuterium on the adjacent carbon atom, C-20 (Figure 1). Moreover, the high incorporation level of the deuterium indicates that the hydride-shift occurs in the same MVA molecule incorporated, a fact which therefore shows that the deuterium on C-20 had migrated from the C-19 derived from the C-4 of MVA as shown in Scheme 1 (**8**)→(**9**). These results confirm Ruzickas 'biogenetic isoprene rule' hypothesis as applied to the formation of the D- and E-ring during the biosynthesis of ursolic acid.

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