The Role of Leucine in Isoprenoid Metabolism. Incorporation of [3-^{13}C]Leucine and of [2-^{3}H,4-^{14}C]-β,β-Dimethylacrylic Acid into Phytosterols by Tissue Cultures of *Andrographis paniculata*

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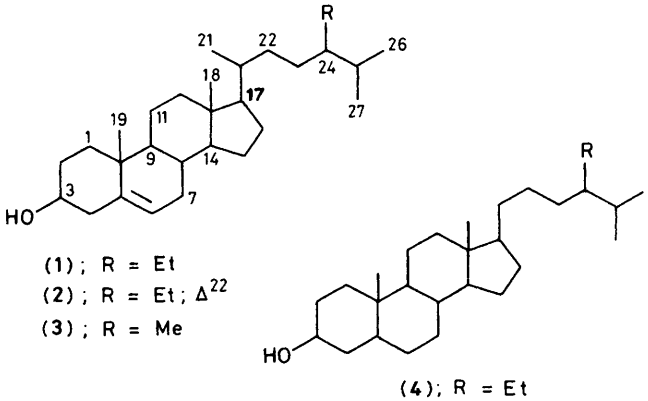
[3-^{13}C]Leucine is incorporated into phytosterols by tissue cultures of *Andrographis paniculata* by breakdown to acetyl-CoA and its subsequent incorporation *via* (3*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and mevalonic acid; [2-^{3}H,4-^{14}C]-β,β-dimethylacrylic acid also is not incorporated intact.

In 1944 Bloch reported[1] that rats incorporate uniformly deuteriated leucine into cholesterol *via* prior breakdown to acetate. By contrast, later experiments indicated[2] that β,β-dimethylacrylate may be incorporated into cholesterol intact. Extensive studies during the 1950's established that the catabolic pathway for leucine in bacteria[3] and the anabolic pathway to terpenoids and steroids in mammals[4] cross at (3*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). There is thus a rational mechanism for the incorporation of leucine into terpenoids without prior degradation.

We have studied the incorporation of leucine in representative plant and microbial systems into terpenoids and steroids[5] and here report on the fate of [3-^{13}C]leucine when it is incorporated into phytosterols by tissue cultures of *Andrographis paniculata*. The question to be resolved is whether leucine is incorporated directly or *via* prior breakdown to acetate.

Andrographis cultures biosynthesise a simple mixture of β-sitosterol (**1**), stigmasterol (**2**), and campesterol (**3**) (typically 55 : 10 : 35 by g.l.c. of the acetates on 1% OV-1 at 250 °C). Incorporation of [3-^{13}C]leucine intact *via* HMG-CoA will label C-3, 5, 9, 13, 17, and 24. On the other hand, degradation of leucine to [2-^{13}C]acetate and re-incorporation will label C-1, 3, 5, 7, 9, 13, 15, 17, 18, 19, 21, 22, 24, 26, and 27. We have shown that the labelling patterns of phytosterols isolated after administration of either [2-^{13}C]acetate or [3-^{13}C]leucine are essentially the same.

For analysis by ^{13}C n.m.r. spectroscopy, the phytosterol mixture was hydrogenated to stigmastanol (**4**) and campestanol (**5**) (65 : 35). The ^{13}C n.m.r. spectra of the saturated sterols were readily assigned by reference to the published spectrum of cholestanol (**6**),[6] and checked by the labelling pattern from [2-^{13}C]acetate. As expected, there were only very minor differences for the carbon resonances of the tetracyclic nucleus and the attached centres (C-18 to C-21) between stigmastanol, campestanol, and cholestanol. The values for the side chain carbon atoms (C-22 to C-26) are in accord with predictions using the Lindeman–Adams rule.[7] The spectrum of the stigmastanol–campestanol mixture enriched from [2-^{13}C]acetate also provides a clear distinction between C-18 (δ 12.1) and C-19 (δ 12.3), that supports the reassigned values for cholestanol.[6,8—10] Thus, because of the high degree of enrichment, the signal at δ 12.1 is a triplet (singlet + doublet from coupling with labelled C-13), while the signal at δ 12.3 is a singlet.



(**1**); R = Et
(**2**); R = Et; Δ^{22}
(**3**); R = Me

(**4**); R = Et
(**5**); R = Me
(**6**); R = H

[2-^{13}C]Sodium acetate (800 mg) was distributed between 80 flasks (100 ml each) of seven-day-old *Andrographis* suspen-

Table 1

	δ (CDCl$_3$)[c]				% Increase	
	A	B	C	D	E	F
	Peaks common to stigmastanol and campestanol					
C-3[a]	71.4	71.4	71.3	71.2	253	19
C-14	56.5	56.5	56.5	56.5	−29	2
C-9[a]	54.4	54.3	54.3	54.4	205	25
C-5[a]	44.8	44.8	44.8	45.0	246	19
C-13[a]	42.6	42.6	42.6	42.6	207	24
C-12	40.0	40.0	40.0	40.1	0	0
C-4	38.2	38.2	38.2	38.3	−1	2
C-1[a]	37.0	37.0	37.0	37.1	280	23
C-8	35.5	35.5	35.5	35.6	3	3
C-10		35.4	35.4	35.5		
C-7[a]	32.1	32.1	32.1	32.1	209	21
C-2	31.5	31.5	31.5	31.6	1	0
C-6	28.7	28.7	28.7	28.8	−3	3
C-16	28.3	28.2	28.2	28.3	−1	−9
C-15[a]	24.2	24.2	24.2	24.3	249	15
C-11	21.3	21.2	21.2	21.4	1	0
C-19[a]	12.3	12.3	12.3	12.4	297	14
C-18[a]	12.1	12.1	12.1	12.1	348	13
	Peaks unique to stimastanol or campestanol					
C-17[a]	56.2[b]	56.2	56.2	56.4	245[b]	12[b]
C-24[a]	45.8	45.8	38.8	39.6	280	15
C-20	36.2	36.1	35.9	35.8	0	0
C-22[a]	33.9	33.9	33.7	36.2	201	9
C-25	29.1	29.1	32.4	28.0	4	−8
C-23	26.0	26.0	30.3	23.9	−8	0
C-28	23.0	23.0	15.3	—	5	5
C-27[a]	19.8	19.8	20.2	22.8	291	7
C-26[a]	19.0	19.0	18.2	22.6	286	11
C-21[a]	18.7	18.7	18.6	18.7	265	10
C-29	11.9	11.9	—	—	6	4

A Stigmastanol–campestanol mixture on which % increase values in E and F are based. B Stigmastanol (this work). C Campestanol (this work). D Cholestanol (ref. 6). E From [2-^{13}C]sodium acetate. F From [3-^{13}C]leucine. [a] Carbon atoms expected to be labelled from [2-^{13}C]acetate. [b] Only the stigmastanol peaks were used to calculate the values of E and F; intensities of campestanol peaks were low and hence unreliable for calculation of % increase. [c] Reference CDCl$_3$ at δ 77.0.

sion cultures.[11] The phytosterols (24 mg) were isolated after 16 days by ethyl acetate extraction of the freeze-dried tissues and preparative layer chromatography (p.l.c.) ($CHCl_3$–MeOH; 95 : 5). Hydrogenation (PtO_2–AcOH; 20 °C; 760 mm) afforded a mixture of ^{13}C-labelled stigmastanol and campestanol. Enrichments (Table 1) were calculated from the ^{13}C n.m.r. spectrum with reference to the natural abundance spectrum of a mixture of stigmastanol and campestanol of the same composition.

In a second experiment, [3-^{13}C]leucine[12] (800 mg) was incubated with *Andrographis* cultures and worked up as above to afford a mixture of stigmastanol and campestanol (16.4 mg; 60 : 40). ^{13}C Enrichments were obtained as before and are listed in Table 1.

Although the level of ^{13}C-enrichment from [3-^{13}C]leucine is only about 1/15 that from [2-^{13}C]acetate, the figures nevertheless clearly show that the same carbon atoms are enriched in the two experiments. It follows that, within experimental limits, *Andrographis* cultures incorporate leucine into phytosterols predominantly *via* breakdown to acetate, as happens when leucine is incorporated into cholesterol in rats.

In 1954 Bloch fed [3-^{14}C]-β,β-dimethylacrylic acid to rats,[2] and concluded that it labelled the cholesterol formed in their livers without prior breakdown to acetate or acetoacetate. To examine the incorporation of β,β-dimethylacrylic acid into plant sterols, we have synthesised [2-^{3}H,4-^{14}C]-β,β-dimethylacrylic acid from methyl tetrolate, $(^{14}CH_3)_2CuLi$, and $^{3}H_2O$.[13] [2-^{3}H]-β,β-Dimethylacrylic acid was prepared similarly and mixed with the doubly-labelled material to produce a $^{3}H/^{14}C$ ratio of 7.1. This mixture (100 mg, m.p. 66—68 °C, recrystallised from MeOH–H_2O) was fed to *Andrographis* suspension cultures as for leucine and sodium acetate above, and the plant sterols harvested as before. In two separate experiments the plant sterols (isolated by p.l.c. and crystallised from EtOH) had $^{3}H/^{14}C$ ratios 1.04 and 1.74 and $I_{spec\ (^{14}C)}$ 1.7 and 0.9%, respectively. Paniculide,[5] isolated in the same experiment had a $^{3}H/^{14}C$ ratio of 1.5 and 1.3 respectively. Clearly, in contrast to Bloch's experiments in rats, β,β-dimethylacrylic acid can have been incorporated intact into phytosterols to only a minor extent. Incorporation *via* acetoacetate and acetyl-CoA would readily accommodate the observed results.

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