On the Role of Leucine in Terpenoid Metabolism†

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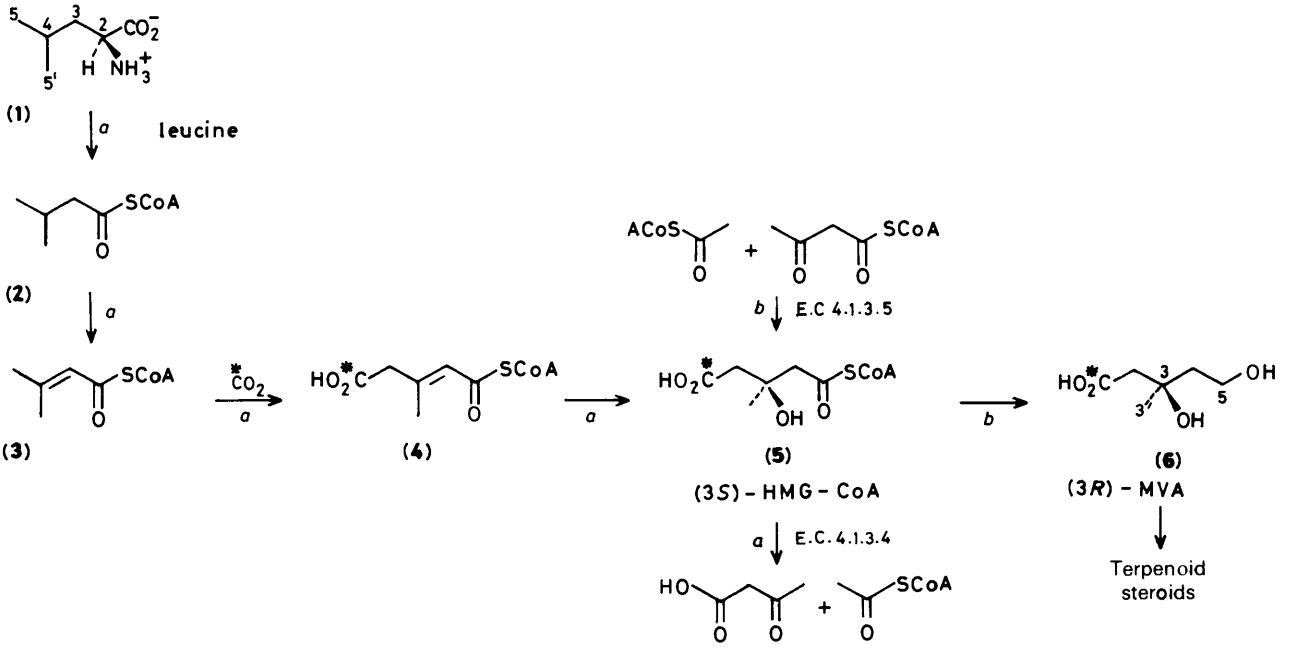
Incorporation of [2-^{13}C]-, [3-^{13}C]-, (4*R*)-[5-^{13}C]- and (4*S*)-[5-^{13}C]-leucines into paniculide by tissue cultures of *Andrographis paniculata* shows that (*a*) (3*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from leucine breakdown *is not* incorporated direct into mevalonic acid (MVA) but that (*b*) both the acetyl-CoA and acetoacetate produced by leucine breakdown *via* HMG-CoA *are* subsequently incorporated into HMG-CoA and MVA.

The possible participation of the amino acid leucine in the biosynthesis of isoprenoids has long intrigued chemists and biochemists. The catabolic pathway for leucine in animals was established in the early 1950s, largely by the work of M. J. Coon and his collaborators.[1–3] It was subsequently shown that the *same* (3*S*)-antipode of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (**5**) lies both on the catabolic pathway of leucine[4,5] (Scheme 1, *a*) and on the anabolic pathway from acetate to (3*R*)-mevalonic acid (MVA) (**6**)[6,7] (Scheme 1, *b*).



Scheme 1.

Numerous biosynthetic studies in mammals, insects, micro-organisms and plants support the view that Nature synthesizes terpenoids and steroids from acetate *via* mevalonic acid. However, the intersection of the terpenoid anabolic and leucine catabolic pathways at HMG-CoA raises the intriguing question of whether the carbon skeleton of leucine might not enter isoprenoids intact *via* HMG-CoA [apart from the loss of C-1 in the conversion of (**1**) to (**2**)], thus affording an alternative, if less important, pathway to terpenoids and steroids. Indeed, the question is not merely of theoretical interest but could have significant medical consequences. Thus, atherosclerosis, a major cause of coronary heart disease in humans, is attributed to high levels of plasma cholesterol. As more than half the body's cholesterol is made by *de novo* synthesis, the possibility that leucine (readily available from a normal diet) might be a source of cholesterol, must be a matter for serious concern.

The literature has for the past 40 years contained scattered references to relevant work. Thus Bloch in 1944,[8] well before MVA was isolated from distillers' solubles and identified as the key intermediate in terpenoid biosynthesis,[9,10] showed that deuteriated leucine and isovaleric acid [see (**2**), Scheme 1] are incorporated by rats into cholesterol *via* acetate. Bloch, Clark and Harary later showed[11] that acids corresponding to (**3**), (**4**) and (**5**) (Scheme 1) are converted in rats into cholesterol without, apparently, prior breakdown to acetate or acetoacetate, while Brady and Gurin found[12] that isovalerate and acetoacetate were similarly incorporated into cholesterol intact. There have been more recent attempts to detect the incorporation of leucine into terpenoids, including gibberrellins,[13] hop resins,[14] ipomeamarone,[15] cantharidin,[16] and the indole alkaloid vindoline.[17] Suga and his colleagues have studied[18,19]

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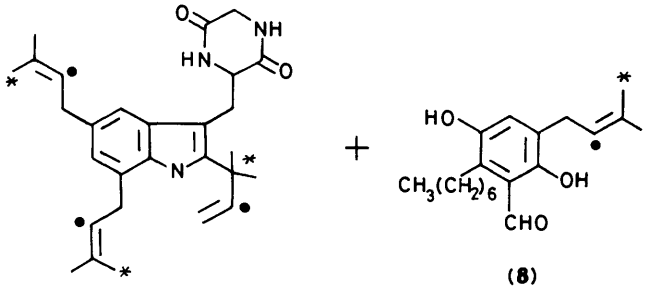
the incorporation in different plants of (*S*)-[U-^{14}C]leucine into linalool and geraniol and into squalene and β-amyrin.[20]

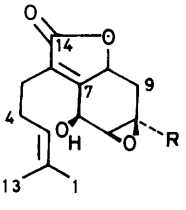
Two studies that precede the work reported here deserve serious attention. The first concerns incorporation of leucine into mevalonic acid and β-carotene by the fungus *Phyomyces blakesleeanus*. Goodwin[21] and Chichester[22] independently reported in 1952 that *P. blakesleeanus* incorporates $^{14}CO_2$ into β-carotene *but only if leucine is present in the medium.* A more recent study by Lowry and Chichester[23] demonstrated that 88% of the radioactivity thus incorporated is located at C-1 of MVA. Since C-1 is lost in the conversion of MVA into isoprenoids, only 12% of label, located elsewhere in the MVA molecule, can get through to β-carotene. These results and earlier experiments by the Chichester group[24] which investigated the fate of leucines labelled with ^{14}C at C-1, C-2, C-3, C-4, and C-5 upon conversion into β-carotene, are readily explained in terms of the transformations indicated in Scheme 1. Thus $^{14}CO_2$ probably carboxylates β,β-dimethylacrylyl-CoA (**3**) as in the course of leucine catabolism in mammals and thus labels C-1 of HMG-CoA (**5**) and MVA (**6**) successively. If this were the only source of MVA, the β-carotene would be totally unlabelled (see above). The 12% of MVA molecules labelled at positions other than C-1 (predictably at C-3/C-3′ and C-5, although this was not established) could arise from cleavage of HMG-CoA to acetyl-CoA and acetoacetate, followed by reassembly. Chichester's earlier results with specifically labelled leucines can be accommodated on the same basis: C-1 of leucine is not incorporated; C-2 and C-3 are weakly incorporated since they become acetyl-CoA in equilibrium with a large unlabelled pool, while C-4 and C-5 are strongly incorporated since the acetoacetate formed from them presumably encounters a smaller pool. It is particularly significant that *P. blakesleeanus* seems to transform HMG-CoA formed from leucine degradation *to a major extent* (88%) directly into MVA. Unfortunately, however, the evidence for this is indirect.

Clearly, in order to establish beyond doubt the mode of incorporation of leucine into isoprenoids, it is necessary to incorporate specifically ^{13}C-labelled leucines into isoprenoids and determine the labelling pattern by ^{13}C n.m.r. spectroscopy. This approach has been used in only one instance apart from the work here reported. *Aspergillus amstelodami* produces two metabolites, echinuline (**7**) and flavoglaucine (**8**), substances of mixed biogenesis which contain, respectively, three and one meroterpenoid (C_5) units. Fuganti and his colleagues have studied[25,26] the incorporation of (4*R*)*- *and* (4*S*)*-[5-^{13}C]leucines into echinuline and flavoglaucine and found that a major mode of incorporation, although not the only mode, is in accordance with cleavage of the intermediate HMG-CoA to acetoacetate (C-4, C-5, and C-5′) and acetyl-CoA (C-2 and C-3), and intact incorporation of the acetoacetate fragment. Their experiments do not provide evidence as to the fate of C-2 and C-3, nor does the evidence exclude the possible direct incorporation of HMG-CoA to a significant extent. We have investigated the *general* problem of the incorporation of leucine into isoprenoids in a range of biological systems, with regard to two possible pathways indicated in Scheme 1, *a* or *b*, and bearing in mind that either or both pathways may function in any one system and that the balance may vary as between different systems.



(**7**)

(**8**)



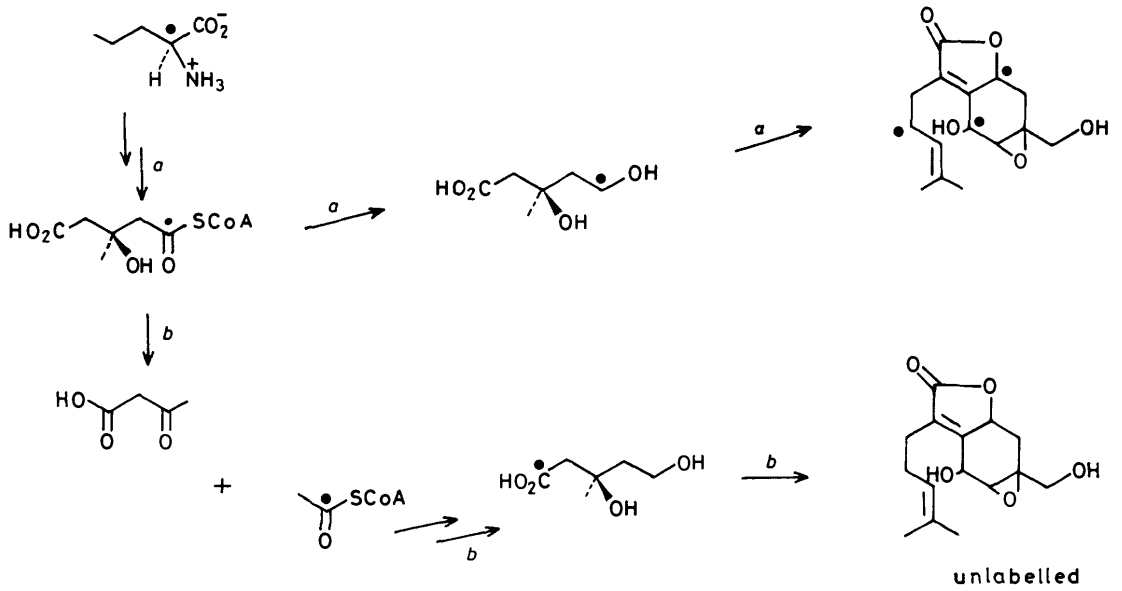
(**9**) R = CH_2OH

(**10**) R = CH_3

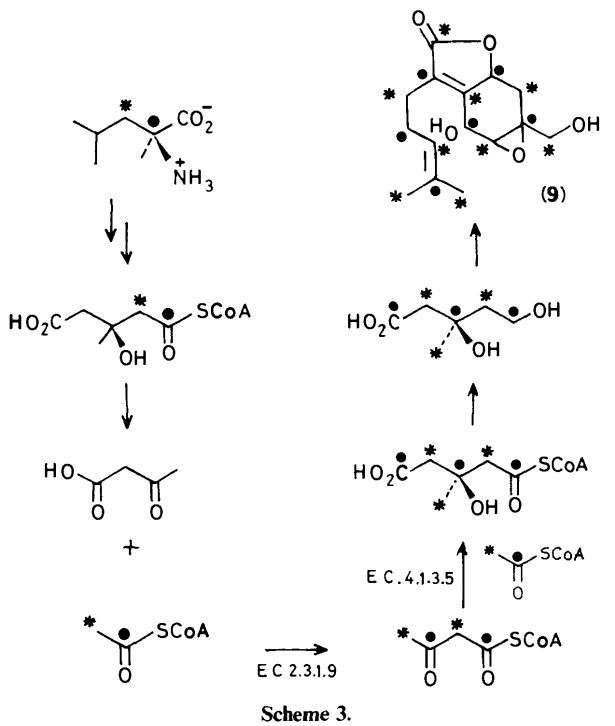
We wished to establish the fate of *each* carbon atom of leucine when the amino acid is incorporated into an isoprenoid. The method of choice must be to incorporate leucines specifically labelled with ^{13}C and to locate the labelled carbon atoms in the product by ^{13}C n.m.r. For this, a system that efficiently incorporates the labelled precursor into the product is required and our best system to date has been a callus culture of *Andrographis paniculata.* This culture produces a family of sesquiterpenoid lactones whose chemistry and biosynthesis we have studied in detail.[27–34] The major lactones are paniculide B (paniculide) (**9**), and paniculide A (**10**) and we discovered some time ago that *Andrographis* suspension cultures incorporate [U-^{14}C]leucine into paniculide rather well [I_{spec} 15—20%]. This therefore appeared to be an ideal system for investigating *how* leucine is incorporated.

Our first experiments[35,36] showed quite clearly that *leucine is not incorporated intact via* HMG-CoA. Thus, leucine labelled at C-2 will label C-1 of HMG-CoA and, if this is incorporated intact, will further label C-5 of MVA and hence C-4, C-8, and C-12 of paniculide (Scheme 2a). (2*R*,*S*)-[2-^{13}C]Leucine was prepared[35] by a modification of the method of Pichat[37] for [2-^{14}C]leucine. When this was administered to *Andrographis* suspension cultures together with (2*S*)-[U-^{14}C]leucine, neither the paniculide isolated (I_{spec} 15.5%) nor the derived crystalline diacetate showed any peak enhancement whatsoever in the ^{13}C n.m.r. spectrum. Had direct incorporation occurred, the peak enhancement for each of three enriched peaks, calculated from [U-^{14}C]leucine incorporation, would have been *ca.* 50%. In a repeat experiment, radioactivity was incorporated [I_{spec} 28.3%] only into paniculide A (**10**). However, in this case also, neither the ^{13}C n.m.r. spectrum nor the mass spectrum showed any evidence of ^{13}C incorporation. *Clearly, therefore, in these experiments, HMG-CoA from leucine breakdown did not enter paniculide direct.* But how is the label from C-2 of leucine totally lost in the product? According to the leucine catabolic pathway (Scheme 1, *a*), C-2 of leucine becomes C-1 of acetyl-CoA (catalysed by EC 4.1.3.4) and when this condenses with *unlabelled* acetoacetyl-CoA (catalysed by EC 4.1.3.5) (Scheme 1, *b*), only C-1 of MVA is labelled and this is lost as $^{13}CO_2$ on formation of isopentenylpyrophosphate (Scheme 2, *b*).

In order to support the conclusions drawn from the above experiment, (2*R*,*S*)-[3-^{13}C]- and (2*RS*)-[3-^{14}C]-leucines were synthesized[38] and administered to *Andrographis* cultures. According to the results with [2-^{13}C]leucine, C-3 of leucine should become C-2 of acetyl-CoA and then C-2 of MVA which should label C-3, C-7, and C-11 of paniculide. The ^{13}C spectrum of the paniculide formed (Fig. 1B) indeed showed enhancement at C-3, C-7, and C-11, but there were six *additional* enriched peaks at C-1, C-5, C-9, C-13, C-14, and C-15. Moreover the intensities were comparable over the nine enriched peaks, and this must mean that the [3-^{13}C]*leucine formed [2-^{13}C]*acetyl-



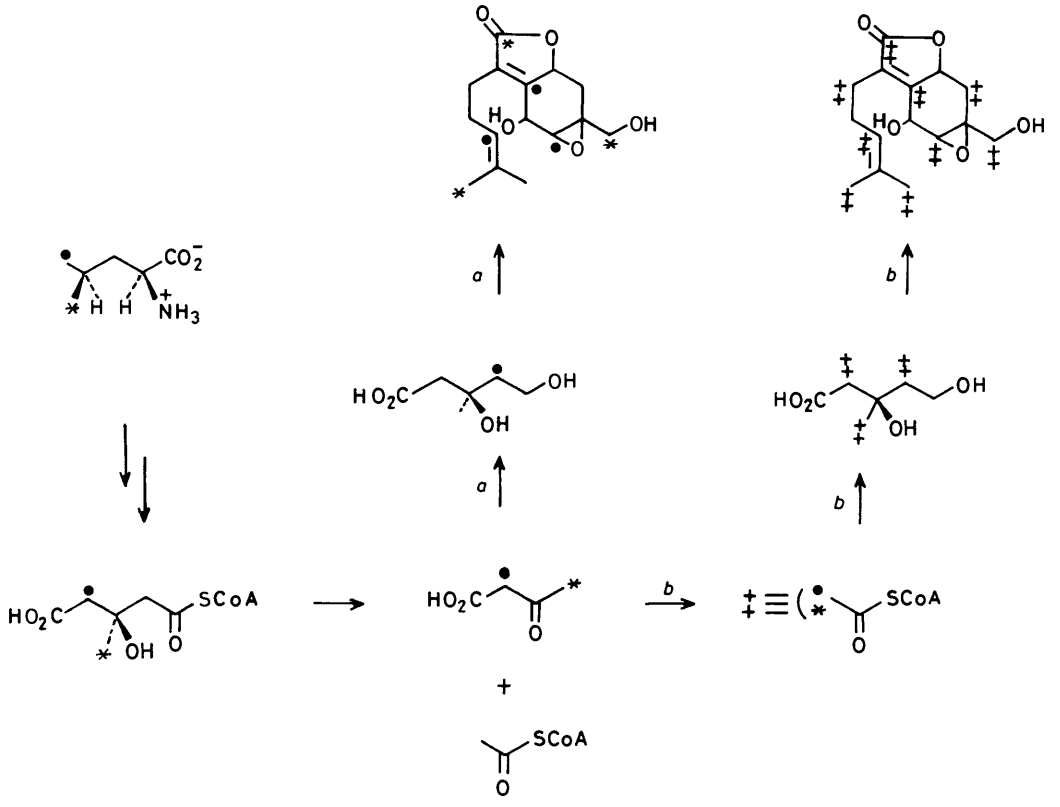
Scheme 2.



Scheme 3.

CoA which condenses with itself (EC 2.3.1.9) and then with a third equivalent (EC 4.1.3.5) to label MVA at C-2, C-3′, and C-4 (Scheme 3). When [2-^{13}C]leucine was metabolized by cultures contemporaneous with those used in the above experiment and the product harvested under the same conditions, the complementary result was obtained (see Scheme 3). Thus *six* positions were enriched (C-1 was lost from each molecule of triply labelled MVA), namely C-2, C-4, C-6, C-8, C-10, and C-12, as expected (Fig. 1C). The apparent discrepancy between this and the earlier experiment in which *no* label was incorporated from [2-^{13}C]leucine, can be explained if the condensing enzyme, acetyl-CoA acetyltransferase (EC 2.3.1.9), was functioning at a time when *labelled* acetyl-CoA was available in the later but not in the earlier experiment. The two sets of experiments were in fact separated by some three years so that the cultures, as well as the precise feeding conditions (see Experimental section), may have differed significantly.

The experiments described so far reveal the fate of C-2 and C-3 of leucine that become, respectively, C-1 and C-2 of acetyl-CoA. What of C-4, C-5, and C-5′ of leucine that furnish three of the carbon atoms of acetoacetate? Is the latter incorporated at all and if so, is it incorporated intact? To answer these questions we labelled the two methyl groups attached to C-4 of leucine individually and specifically with ^{13}C. These methyl groups are diastereotopic and hence distinguishable by an enzyme. It has recently been established[39a] that the isovaleryl-CoA dehydrogenase [the enzyme that converts (**2**) into (**3**), Scheme 1] from rat liver exhibits *anti*-stereochemistry. (4*S*)-[5-^{13}C]Leucine (·) would analogously label C-3, C-7, and C-11 of paniculide and (4*R*)-[5-^{13}C]leucine (*) would label C-13, C-14, and C-15 (Scheme 4, *a*). The two isotopomers were synthesized essentially according to the route used by Fuganti in his studies with echinulin.[25] Since this has been reported only in outline and several of the steps turned out not to be trivial, full details of our procedures are given in the Experimental Section (see p. 2432).[39b] Each isotopomer was separately incubated with the *Andrographis* suspension culture and the paniculide harvested and purified as before. The ^{13}C n.m.r. spectra of the two specimens of paniculide thus produced were at first sight alarming: they were apparently indistinguishable not only from each other but also from the spectrum of paniculide biosynthesized from [3-^{13}C]leucine (Fig. 2A, *cf.* Fig. 1B). The *major* pathway must therefore correspond to cleavage of *either* [2-^{13}C]· {from (4*S*)-[5-^{13}C]leucine} or [4-^{13}C]*acetoacetate {from (4*R*)-[5-^{13}C]leucine} to [2-^{13}C]-acetyl-CoA and elaboration as before (Scheme 4, *b*). However, it was possible to detect the *additional* labelling arising from pathway *a* in Scheme 4, as follows. Subtraction of the natural abundance spectrum from the labelled spectrum was increased until all but three of the labelled peaks were nulled (this also produced negative 'natural abundance' peaks). Careful scrutiny of the new difference spectra (Fig. 2, B and C) then revealed about 15% of additional enrichment, in each case, of the three peaks that should be labelled (Scheme 4, *a*) following direct incorporation of acetoacetate. In summary, our experiments demonstrate that, in the system examined, (*a*) leucine *is not* incorporated into paniculide directly *via* HMG-CoA; (*b*) leucine *is* incorporated *via* the two end products of the leucine catabolic pathway, *both*



Scheme 4.

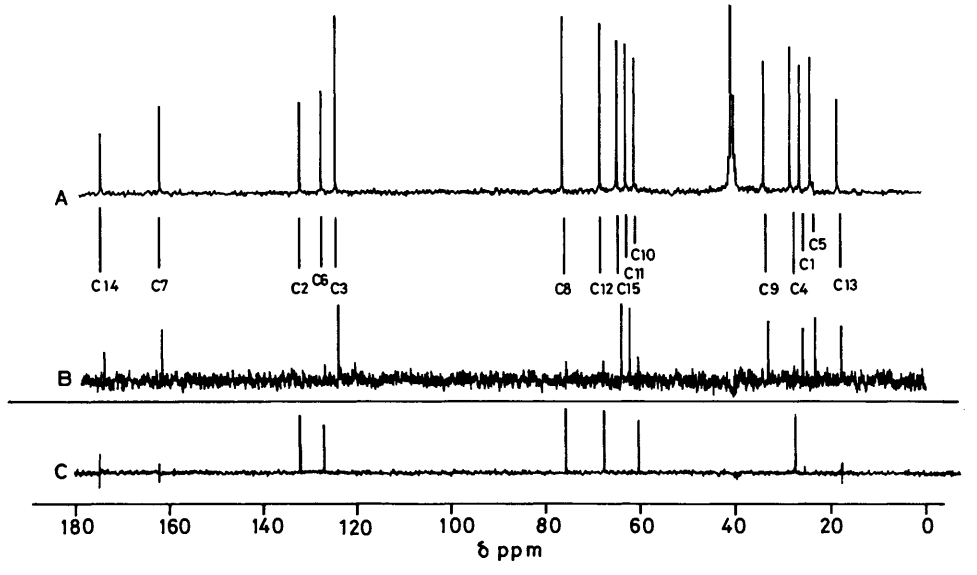


Figure 1. (A) Natural abundance spectrum of paniculide in $(CD_3)_2SO$ at 90.56 MHz. (B) ^{13}C N.m.r. difference spectrum of paniculide labelled from [3-^{13}C]leucine minus paniculide at natural abundance in $(CD_3)_2SO$ at 90.56 MHz. (C) ^{13}C N.m.r. difference spectrum of paniculide labelled from [2-^{13}C]leucine minus paniculide at natural abundance in $(CD_3)_2SO$ at 50 MHz

of which are incorporated, thus establishing for the first time the fate of each carbon atom of leucine when it is incorporated into a terpenoid. Since the configurations of the (4*R*)- and (4*S*)-[5-^{13}C]leucines are established by their synthesis, the labelling patterns of paniculides biosynthesized from them is consistent with the *anti*-stereochemistry of the isovaleryl-CoA dehydrogenase reaction in a higher plant. This turns out to be analogous to the stereochemistry recently demonstrated for the enzyme from rat liver mitochondria [39a] and, also indirectly, for the fungal dehydrogenase from *Aspergillus* spp.[25]

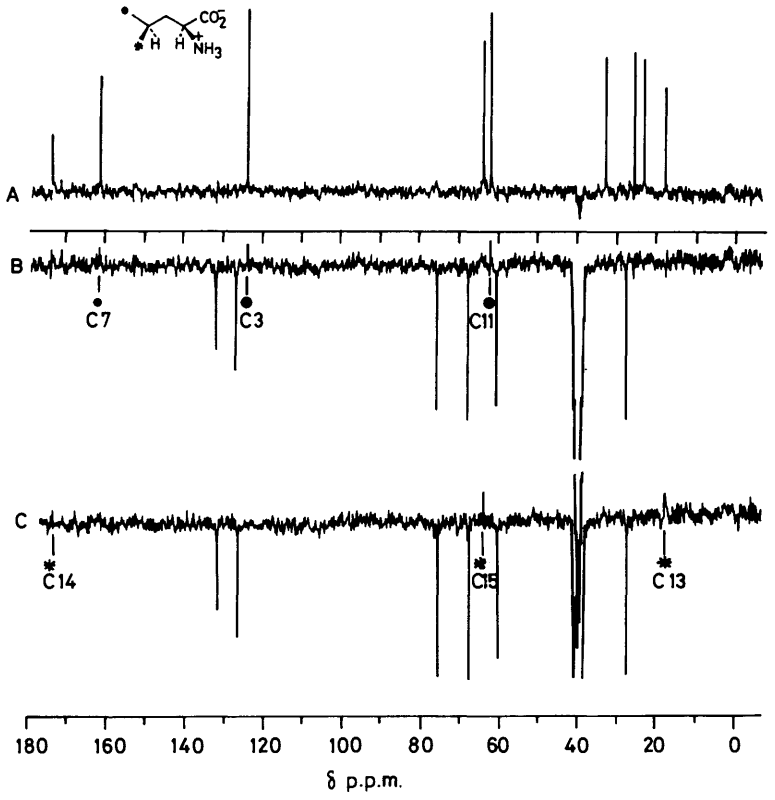


Figure 2. Conditions as for Fig. 1(C). (A) ^{13}C N.m.r. difference spectrum showing the labelling pattern of paniculide derived from (4*R*)-[5-^{13}C] leucine (*). Paniculide derived from (4*S*)-[5-^{13}C]leucine (') gave superficially the same result. (*B*) Difference spectrum showing peaks labelled *via* [2-^{13}C]acetoacetate. (C) Difference spectrum showing peaks labelled *via* [4-^{13}C]acetoacetate

Experimental

^1H N.m.r. spectra were obtained on either a Perkin-Elmer R32 (90 MHz) or a Bruker WP200SY (200 MHz) spectrometer, in the latter case employing a deuterium lock system, setting chloroform ($CHCl_3$) at δ 7.25 as internal standard. Proton broadband-decoupled ^{13}C n.m.r. spectra were recorded at 50 MHz in $CDCl_3$, setting the reference $CDCl_3$ signal at δ 77.0. Mass spectra were obtained on a VG/Kratos MS12 or MS 9025 (high resolution) spectrometer.

Procedures for establishing and maintaining stock tissue cultures of *Andrographis paniculata* have been described.[40] Hypocotyl isolates were grown as callus cultures on a modified White's medium,[41] solidified with 0.7% agar (Oxoid No. 3) and containing Fe–EDTA as an iron source. Callus cultures were grown on agar 21 days after subculture and then inoculated into liquid medium (composition as solid medium without agar; 0.5 g fresh callus/100 ml medium) contained in 250 ml modified Erlenmeyer flasks having four vertical glass ridges running up the inside of each flask to aid agitation. Flasks were kept on a horizontal shaker (140 rev min^{-1}) for the stated length of time at 25 ± 1 °C with illumination (approx. 3 200 lx).

Synthesis of (2RS)-(2-^{13}C]*Leucine*.[35,37]—Vacuum line techniques described by Pichat[37] were used. A solution of Grignard reagent was prepared by treating 1-bromo-2-methylpropane (2.74 g, 20 mmol) with magnesium turnings (490 mg, 20 mmol) in dry ether (40 ml). The flask containing this solution was transferred to the vacuum line and frozen in liquid nitrogen. Carbon dioxide (5 mmol), which was generated by reaction of concentrated sulphuric acid with ^{13}C labelled barium carbonate (0.992 g, 5 mmol, 87.6 atom % ^{13}C), was then distilled into the flask containing the Grignard reagent. The reaction flask was sealed off from the rest of the vacuum line and allowed to warm up to −25 °C. Vigorous stirring was started as soon as possible. Any carbon dioxide remaining in the vacuum line was condensed by freezing the reaction mixture in liquid nitrogen once more. The mixture was stirred for a further 10 min, dilute sulphuric acid (6M, 20 ml) was slowly added with cooling followed by silver sulphate (5 g), and then the reaction mixture was distilled at atmospheric pressure. The ether, which distilled first, was discarded and the product was entrained in the aqueous distillate. The [1-^{13}C]3-methylbutanoic acid was titrated with 1M sodium hydroxide as it distilled, using phenolphthalein as indicator. After evaporation of water under reduced pressure, the sodium salt was dried completely by heating *in vacuo* at 100 °C overnight.

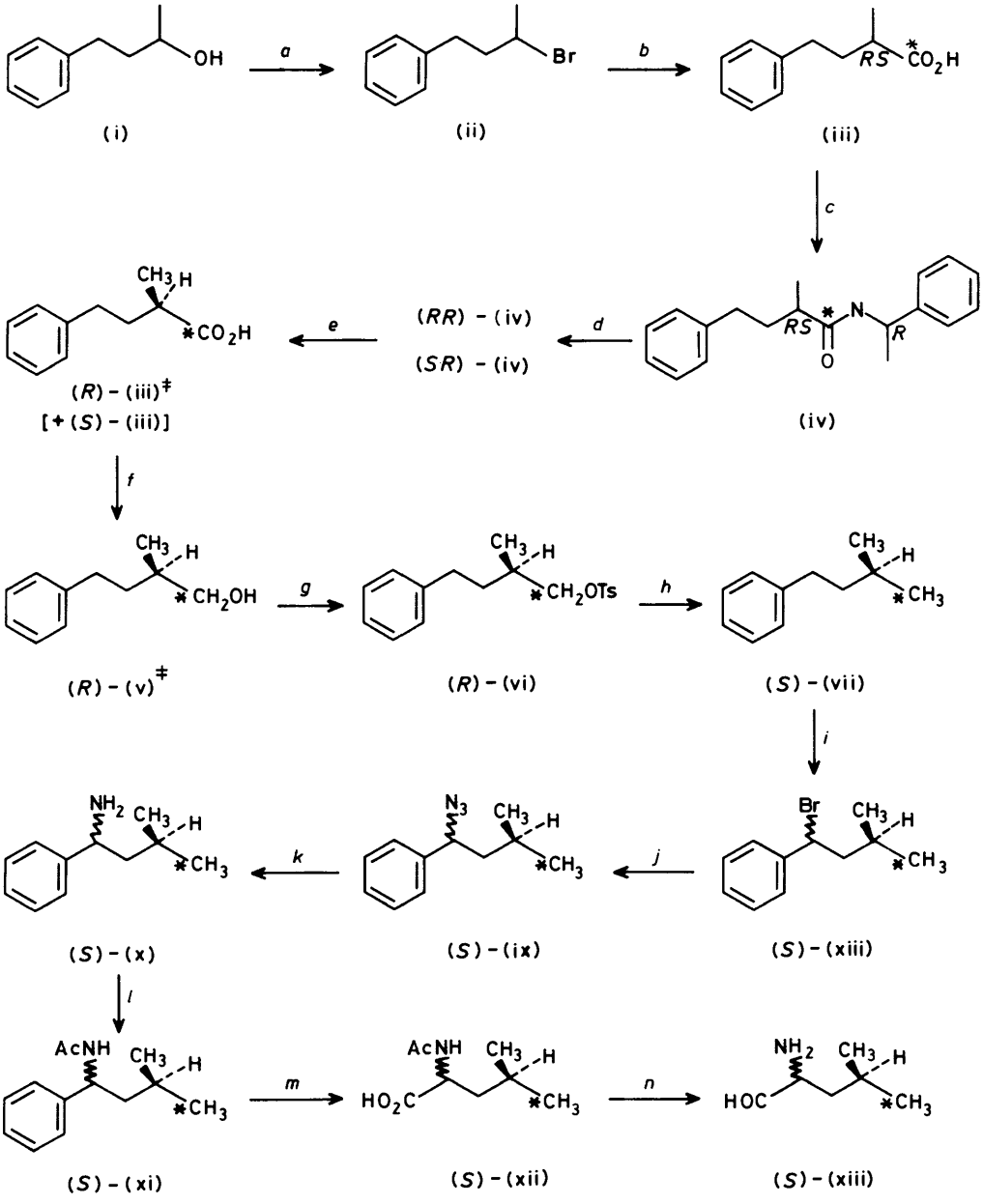
Dry ether (50 ml) was distilled into the flask containing the dry salt at the same time as dry hydrogen chloride (6 mmol), (generated from sodium chloride and concentrated sulphuric acid). The mixture was stirred overnight at room temperature, after which the excess of hydrogen chloride was removed by distillation *in vacuo* of part of the ether. The reaction flask was then purged with nitrogen before adding carbonyl bisimidazole (1.0 g, *ca.* 6 mmol). The reaction mixture was stirred at room temperature for 2 h and refluxed for a further 30 min, after which the imidazole derivative was reduced at 0 °C by careful addition of lithium aluminium hydride (95 mg, 2.5 mmol). Small aliquots of the reaction mixture were removed during reduction to be monitored by analytical g.l.c. on 1% SE 30 at 50 °C. Addition of lithium aluminium hydride was stopped as soon as the first traces of alcohol could be detected. The aluminium salts were decomposed by addition of dilute sulphuric acid (5 ml).

The reaction mixture was re-cooled to 0 °C then saturated with ammonia. Potassium cyanide (390 mg, 6 mmol), ammonium chloride (321 mg, 6 mmol) and a saturated solution of ammonia in methanol (12 ml) were then added and the mixture stirred at room temperature for 48 h. Ether was removed under reduced pressure and the residue acidified with concentrated

hydrochloric acid (40 ml). The reaction mixture was left to stand overnight before being refluxed for 8 h; it was then evaporated to dryness, taken up in a small volume of water and treated with charcoal. This aqueous solution was applied to a column (35 cm × 2.5 cm) of Dowex 50 W8 cation exchange resin in the H^+ form and the column eluted with 0.3M hydrochloric acid. The presence of amino acid in the eluted fractions was determined by the ninhydrin test. Fractions containing the product (2*RS*)-[2-^{13}C]leucine were combined and evaporated to dryness. The residue was dissolved in the minimum quantity of water and applied to a similar column of Dowex 50 in H^+ form, then eluted with 0.3M hydrochloric acid (50 ml) and washed with distilled water until no trace of chloride ion could be detected (silver nitrate). The free amino acid was eluted with 2.0M ammonium hydroxide, yielding (2*RS*)-[2-^{13}C]leucine (282 mg, 43% yield based on barium carbonate).

The ^{13}C n.m.r. spectrum of this material was recorded in D_2O/NH_4OH/dioxane; δ_C 55.1 (C-2), 44.3 (C-3, d, *J* 35 Hz); 25.2 (C-4), 23.2 and 22.1 (C-5 and C-5′), C-1 not observed. The spectrum was similar to that of unlabelled leucine, except that the peak at δ_C 55.1 was greatly enhanced. The δ_C value agrees well with the literature value[42] for the α carbon of leucine (δ 54.4 ppm) which was obtained in neutral solution. The ^{13}C content was *ca.* 70 atom %.

Synthesis of (2RS,4R)- *and* (2RS,4S)-[5-^{13}C]*Leucine*.—This synthesis follows the route reported in outline by Fuganti.[25]



Scheme 5. Synthesis of (2*RS*,4*S*)- and (2*RS*,4*R*)-[5-^{13}C]leucines[25]

Reagents: a, Me_3SiCl, LiBr; *b*, Mg–Et_2O, $*CO_2$, 6M HCl; *c*, $SOCl_2$ (*R*)-α-methylbenzylamine, CH_2Cl_2, Et_3N; *d*, silica gel chromatography; *e*, N_2O_4, CCl_4, NaOAc, heat, 6M HCl; *f*, $LiAlH_4$; *g*, TosCl, pyridine; *h*, $LiAlH_4$; *i*, NBS, $(PhCOO)_2$, CCl_4; *j*, NaN_3, $(CH_3)_2CO$, H_2O; *k*, $LiAlH_3$; *l*, CH_3COCl, Et_3N, CH_2Cl_2; *m*, $RuCl_3 \cdot 3H_2O$–$NaIO_4$, CCl_4, CH_3CN, H_2O; *n*, 6M HCl.

‡ Only one-series is shown

2-Bromo-4-phenylbutane (ii).—4-Phenylbutan-2-ol (24.0 g, 0.17 mol) in anhydrous acetonitrile (25 ml) was added to a stirred suspension of lithium bromide (41 g, 0.47 mol), trimethylsilyl chloride (64.8 g, 0.60 mol) in acetonitrile (115 ml).[43] The reaction was refluxed under argon during 86 h (t.l.c. monitoring), *ca.* 2/3 solvent evaporated under reduced pressure and the residue diluted with ice-water and extracted with ether (3 × 100 ml). The ether was washed with satd. aqueous sodium hydrogen carbonate (2 × 50 ml), brine (2 × 50 ml) and dried ($MgSO_4$). Evaporation of the solvent under reduced pressure afforded the oily 2-bromo-4-phenylbutane (ii) (31.36 g, 95%); δ 1.67 (3 H, d, J = 7 Hz, CH_3), 1.9—2.2 (2 H, m, CH_2), 2.6—2.9 (2 H, m, CH_2Ph), 3.9—4.25 (1 H, m, CHBr), and 7.2 (5 H, m, ArH).

(RS)-[1-^{13}C]-2-*Methyl-4-phenylbutanoic Acid* (iii).—A Grignard reagent prepared from 2-bromo-4-phenylbutane (10.65 g, 50 mmol) and Mg (1.2 g, 50 mmol) in ether (120 ml) was carbonated with $^{13}CO_2$ [generated from $Ba^{13}CO_3$ (90 atoms ^{13}C, Amersham International p.l.c.), 5.0 g, 25—38 mmol] according to the procedure previously described for [1-^{13}C]-isobutyric acid,[38] except that the prepared reagent was stirred at 20 °C for 6 h before being decomposed with 6M HCl. The acid was separated by extraction into 4M NaOH, acidification with conc. HCl and re-extraction into ether. The combined and dried ether extracts on removal of solvent under reduced pressure afforded the acid (iii) (2.73 g, 61%) as a colourless oil, δ 1.20 (3 H, dd, J_{HH} 7.5 Hz, $^3J_{CH}$ 6 Hz, CH_3), 1.5—2.3 (1 H, m, CH_2), 2.67 (2 H, t, J 8 Hz, CH_2Ph), 2.46 (1 H, q, J 7.5 Hz, CH), and 7.2 (5 H, m, ArH).

Diastereoisomeric Amides (*iv*) *from* (RS)-[1-^{13}C]-2-*Methyl-4-phenylbutanoic Acid and* (R)-(+)-α-*Methylbenzylamine*.—The above acid (6.93 g, 34.4 mmol) was converted into the acid chloride with thionyl chloride (20 ml) (dropwise addition at 20 °C, followed by reflux under argon for 2 h). The acid chloride, obtained by removal of the excess of $SOCl_2$ (Buchi), was dissolved in dry CH_2Cl_2 (30 ml) and cooled to 0 °C under argon; to this was added Et_3N (15 ml) in CH_2Cl_2 (10 ml) and then dropwise (*R*)-(+)-α-methylbenzylamine (15 g, 125 mmol). The mixture was stirred at 0 °C for 30 min and stored at 0 °C overnight. Iced water (50 ml) was added, the amides extracted into ether (5 × 50 ml), the ether washed successively with 2M HCl, water and satd. aqueous sodium hydrogen carbonate, and dried ($MgSO_4$). Solvent evaporation afforded the *amide mixture* (iv) (9.35 g, 85%).

This was separated as follows. The mixture was adsorbed on silica gel (10 g, 60—120 mesh), dried *in vacuo*, and added to a chromatographic column (32 cm × 3 cm) containing silica gel (HF 254, 81 g) packed under N_2 pressure (10 p.s.i.) in hexane. Elution with 5, 10 and 50% EtAc–hexane successively afforded first the (RR)-*amide* (1.34 g), followed by mixed (*RR*)- and (*SR*)-amide (5.05 g) and then (SR)-*amide* (1.4 g).

The (*RR*)-amide, (*RR*)-(iv), crystallised from ethyl acetate–hexane, had m.p. 93—95 °C, δ 1.10 (3 H, dd, J_{HH} 7 Hz, $^3J_{CH}$ 6 Hz, CH_3CHCO), 1.48 (3 H, d, J 7 Hz, CH_3CHNH), 1.55—2.0 (2 H, m, CH_2), 2.12 (1 H, m, CHCO), 2.60 (2 H, t, J 7 Hz, $ArCH_2$), 5.18 (1 H, m, CHNH), 5.6 (1 H, br s, NH), 7.15 (5 H, m, ArH), and 7.3 (5 H, s, ArH); *m/z* (relative intensity) 282 (3), 178 (89), 120 (4), 105 (100), 91 (39), and 74 (29) (Found: C, 81.25; H, 8.4; N, 5.0. M^+, 282.1819. $C_{19}H_{23}NO$ requires C, 80.85; H, 8.15; N, 4.95% $^{12}C_{18}{}^{13}CH_{23}NO$ requires 282.1813).

The (*SR*)-amide, (*SR*)-(iv), crystallized from ethyl acetate–hexane, had m.p. 98—100 °C, δ 1.15 (3 H, dd, J_{HH} 7 Hz, $^3J_{CH}$ 6 Hz, CH_3CHCO), 1.45 (3 H, d, J 7 Hz, CH_3CHNH), 1.60—1.95 (2 H, m, CH_2), 1.98 (1 H, m, CHCO), 2.52 (2 H, t, J 7 Hz, $ArCH_2$), 5.28 (1 H, m, CHNH), 5.6 (1 H, br s, NH), 7.2 (5 H, ArH), and 7.3 (5 H, ArH); *m/z* 282 (4), 120 (4), 178 (96), 105 (100), 91 (46), and 74 (38) (Found: C, 80.5; H, 8.3; N, 4.95. M^+, 282.1818).

Regeneration of the Optically Pure Acids (R)-(*iii*) *and* (S)-(*iii*) *from the Diastereoisomeric Amides*.[44]—*Anhydrous sodium acetate* (2.46 g 30 mmol) was added to N_2O_4(2M) in CCl_4 (20 ml; prepared by passing NO_2 gas into CCl_4 at 0 °C and standardising iodometrically) at −60 °C. The mixture was warmed to 0 °C and then the (*RR*)-amide (1.4 g, 5 mmol) in CCl_4 (10 ml) was added dropwise with stirring. After a further 2 h at 0 °C, the reaction was poured into ice water, the organic layer washed ($NaHCO_3$, 2 × 10 ml; H_2O, 1 × 10 ml), dried by filtration through $MgSO_4$ and then refluxed for 16 h. Solvent evaporation *in vacuo* afforded a mixture consisting of the derived acid, its α-methylbenzyl ester and styrene. This mixture was hydrolysed by refluxing with 6M HCl (15 ml) for 5 h and then extracting the organic material into $CHCl_3$ (4 × 15 ml). The acid was recovered by extraction into NaOH (4 × 10 ml), acidification at 0 °C with conc. HCl and re-extraction into $CHCl_3$ (5 × 20 ml). The extracts were washed with brine, dried ($MgSO_4$) and the solvent evaporated under reduced pressure to give (2R)-[1-^{13}C]-2-*methyl-4-phenylbutanoic acid* (*R*)-(iii) (740 mg, 83%) as a colourless oil, $[\alpha]_D$ − 20.9° (*c* 0.141 in $CHCl_3$). δ 1.20 (3 H, dd, J_{HH} 7 Hz, $^3J_{CH}$ 6 Hz, CH_3), 1.50—2.35 (2 H, m, $-CH_2-$), 2.3—2.8 (3 H, m, $ArCH_2$ + $CHCO_2H$), and 7.20 (5 H, Ph); *m/z* 179 (11), 105 (83), 91 (80), 75 (100), and 65 (20) (Found M^+ 179.1027, $^{12}C_{10}{}^{13}CH_{14}O_2$ requires 179.1027). Similarly, decomposition of the (*SR*)-amide (1.2 g) afforded (2S)-[1-^{13}C]-2-*methyl-4-phenylbutanoic acid*, (*S*)-(iii), (596 mg, 78%) as a colourless oil, $[\alpha]_D$ + 23.7° (*c* 0.63 in $CHCl_3$), δ 1.10 (3 H, dd, J_{HH} 7.5 Hz, $^3J_{CH}$ 6 Hz, CH_3), 1.5—2.2 (2 H, m, $-CH_2-$), 2.3—2.8 (3 H, m, $ArCH_2$ + $CHCO_2H$), and 7.2 (5 H. Ph); *m/z* 179 (17), 105 (70), 91 (60), and 75 (100) (Found: M^+ 179.1031). The configurations of (2*R*)- and (2*S*)-2-methyl-4-phenylbutanoic acids are based on correlation by ozonolysis with (2*R*)-2-methylglutaric acid [45,46] by Fuganti and his colleagues. We are indebted to Prof. Claudio Fuganti and Dr. Giuseppe Pedrocchi-Fantoni for unpublished data. Racemic amide (4.5 g) similarly afforded racemic acid (2.31 g, 80%).

(2R)-[1-^{13}C]-2-*Methyl-4-phenylbutanol*, (R)-(v).—(2*R*)-[1-^{13}C]-2-methyl-4-phenylbutanoic acid (735 mg, 4.1 mmol) in ether (20 ml) was added to $LiAlH_4$ (500 mg, 13.15 mmol) suspended in ether (30 ml). The suspension was refluxed for 4 h, cooled to 0 °C and the excess of $LiAlH_4$ decomposed by adding saturated NH_4Cl. Evaporation of ether from the filtered and dried solution afforded the *alcohol*(R)-(v) (644 mg, 95%) as a colourless oil, δ 0.95 (3 H, dd, J_{HH} 7.5 Hz, $^3J_{CH}$ 6 Hz, $-CH_3$), 1.45 (1 H, br s, OH), 1.3—1.9 (3 H, m, CH_2, CH), 2.60 (2 H, m, $ArCH_2$), 3.50 (2 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 140 Hz, $-*CH_2-$), and 7.20 (5 H, Ph); *m/z* 165 (12), 147 (9), 132 (15), 117 (21), 104 (64), 91 (100), and 65 (19). (Found: M^+, 165.1237. $C_{10}{}^{13}CH_{16}O$ requires 165.1235). Similarly, (2*S*)-[1-^{13}C]-2-methyl-4-phenylbutanoic acid (596 mg) afforded the *alcohol* (S)-(v) (497 mg), δ 0.97 (3 H, dd, J_{HH} 7 Hz, $^3J_{CH}$ 6 Hz, CH_3), 1.30 (1 H, br s, OH), 1.35—2.0 (3 H, m, $-CH_2-$, $-CH-$), 2.70 (2 H, m, $ArCH_2$), 3.45 (2 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 140 Hz, $-*CH_2-$), and 7.20 (5 H, Ph); *m/z* 165 (14), 147 (10), 132 (18), 117 (20), 104 (65), 91 (100), and 65 (18) (Found: M^+, 165.1237).

Toluene-p-*sulphonate of* (2R)-[1-^{13}C]-2-*Methyl-4-phenylbutanol* (R)-(vi).—This was prepared by storing the alcohol (*R*)-(v) (640 mg) and toluene-*p*-sulphonyl chloride (1.86 g) in anhydrous pyridine (5 ml) for 16 h at 0 °C. Usual work-up afforded the *toluene*-p-*sulphonate* (*R*)-(vi) (1.19 g, 97%), δ 0.92 (3 H, dd, J_{HH} 7 Hz, $^3J_{CH}$ 6 Hz, CH_3), 1.4—2.0 (3 H, m, $-CH_2$ + $-CH-$), 2.40 (3 H, s, $ArCH_3$), 2.45 (2 H, t, J 7 Hz, $ArCH_2$), 3.90 (2 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 150 Hz, $*CH_2O$), and 7.1—7.5 and 7.75 (9 H, Ph + C_6H_4); *m/z* 147 (15), 132 (17), 104 (79), 91 (100), and

65 (24) (Found: $M^+ - HSO_3C_7H_7$, 147.1131. $^{12}C_{10}{}^{13}CH_{14}$ requires 147.1126).

Toluene-p-*sulphonate of* (2S)-[1-^{13}C]-2-*Methyl*-4-*phenylbutanol* (S)-(vi).—Reaction of the alcohol (*S*)-(v) (497 mg) by the above method afforded *toluene*-p-*sulphonate* (S)-(vi) (886 mg, 94%), δ 0.92 (3 H, dd, J_{HH} 7 Hz, $^3J_{CH}$ 6 Hz, CH_3), 1.35—2.0 (3 H, m, CH_2 + CH), 2.4 (3 H, s, ArCH_3), 2.45 (2 H, t, *J* 7 Hz, ArCH_2), 3.90 (2 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 150 Hz, *CH_2O), and 7.1—7.4 and 7.75 (9 H, Ph + C_6H_4); *m/z* 319 (3), 147 (24), 131 (14), 104 (95), 91 (100), and 65 (23). (Found: M^+, 319.1324. $C_{17}{}^{13}CH_{22}O_3S$ requires 319.1323).

(2S)-[1-^{13}C]-2-*Methyl*-4-*phenylbutane* (S)-(vii).—(2*R*)-[1-^{13}C]-2-Methyl-4-phenylbutanol toluene-*p*-sulphonate (1.18 g, 3.76 mmol) in ether (10 ml) was added to a stirred suspension of $LiAlH_4$ (428 mg, 3 equiv.) in ether (20 ml) under argon, and the reaction refluxed for 4 h. Usual work-up afforded the *hydrocarbon* (S)-(vii) (385 mg, 70%) as an oil, δ 0.90 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 120 Hz, *CH_3), 0.94 (3 H, dd, J_{HH} 6 Hz, $^3J_{CH}$ 2.4 Hz, CH_3), 1.43—1.55 (3 H, br, CH_2 + CH), 2.61 (2 H, t, *J* 7.3 Hz, ArCH_2), and 7.25 (5 H, Ph); *m/z* 149 (3), 105 (28), 91 (99), 77 (8), and 57 (18) (Found: M^+, 149.1291. $C_{10}{}^{13}CH_{16}$ requires 149.1284).

In a similar manner (2*S*)-[1-^{13}C]-2-methyl-4-phenylbutanol toluene-*p*-sulphonate (885 mg) afforded (2R)-[1-^{13}C]-2-*methyl*-4-*phenylbutane* (R)-(vii) (313 mg, 74%), δ 0.90 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 126 Hz, *CH_3), 0.93 (3 H, dd, J_{HH} 6 Hz, $^3J_{CH}$ 2 Hz, CH_3), 1.4—1.7 (3 H, m, CH_2 + CH), 2.6 (2 H, t, *J* 7 Hz, ArCH_2), and 7.2 (5 H, Ph); *m/z* 105 (13), 91 (100), 77 (10), and 57 (10) (Found: $M^+ - C_2{}^{13}CH_7$, 105.0671. C_8H_9 requires 105.0704).

(2S)-[1-^{13}C]-4-*Bromo*-2-*methyl*-4-*phenylbutane* (S)-(viii).—The hydrocarbon *S*-(vii) prepared as above (380 mg, 2.55 mmol), NBS (451 mg, 2.55 mmol) and dibenzoyl peroxide (61 mg, 0.255 mmol) in dry CCl_4 (10 ml) were refluxed for *ca.* 15 min. Cooling to 0 °C, filtration and evaporation of solvent left the *bromide* (*S*)-(viii) (580 mg, *ca.* 100%) as a viscous oil, δ 0.89 (3 H, dd, *J* 6 Hz, $^1J_{CH}$ 126 Hz, *CH_3), 0.91 (3 H, br s, CH_3), 1.5—2.2 (3 H, m, CH_2 + CH), 5.05 (1 H, t, *J* 7.5 Hz, Ar*CH*Br), and 7.3 (5 H, Ph); *m/z* 227/229 (2/1), 148 (30), 122 (32), 105 (80), 91 (100), and 77 (46) (Found: $M^+ - Br$, 148.1207. $C_{10}{}^{13}CH_{15}$ requires 148.1207). (2R)-[1-^{13}C]-4-*Bromo*-2-*methyl*-4-*phenylbutane* (R)-(viii) (470 mg) was obtained similarly from (2*R*)-[1-^{13}C]-2-methyl-4-phenylbutane (310 mg), δ 0.89 (3 H, dd, *J* 6 Hz, $^1J_{CH}$ 132 Hz, *CH_3), 0.92 (3 H, br s, CH_3), 5.05 (1 H, t, *J* 7 Hz, Ar*CH*Br), and 7.4 (5 H, Ph); *m/z* 148 (35), 122 (27), 105 (49), 91 (100), and 77 (24) (Found: $M^+ - Br$, 148.1196).

(2S)-[1-^{13}C]-4-*Azido*-2-*methyl*-4-*phenylbutane* (S)-(ix).—A solution of the above bromide *S*-(viii) (578 mg, 3 mmol) in acetone (6 ml) was added to NaN_3 (795 mg, 4 equiv.) in water (9 ml) and the resulting suspension refluxed for 20 h, when all bromide had reacted (t.l.c.). The product was extracted into ether (5 × 6 ml), washed with water (6 ml), dried ($MgSO_4$) and evaporated cautiously under reduced pressure to afford the *azide* (S)-(ix) (400 mg, 83%) as a viscous oil, δ 0.9 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 124 Hz, *CH_3), 0.9 (3 H, dd, *J* 6 Hz, 2 Hz, CH_3), 1.19—1.63 (3 H, m, CH_2 + CH), 4.46 (1 H, t, *J* 7.5 Hz, Ar*CH*N_3), and 7.20 (5 H, Ph).

(2R)-[1-^{13}C]-4-*Azido*-2-*methyl*-4-*phenylbutane* (R)-(ix) (353 mg, 89%) was similarly obtained from (2*S*)-[1-^{13}C]-4-bromo-2-methyl-4-phenylbutane (475 mg), δ 0.89 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 124 Hz, *CH_3), 0.9 (3 H, dd, *J* 6 Hz, 2 Hz, CH_3), 1.27—1.73 (3 H, m, CH_2 + CH), 4.53 (1 H, t, *J* 7.5 Hz, ArCHN_3), 7.25 (5 H, Ph).

(2S)-[1-^{13}C]-4-*Amino*-2-*methyl*-4-*phenylbutane* (S)-(x).—The azide *S*-(ix) (400 mg, 2.1 mmol) in ether (5 ml) was added dropwise to a stirred suspension of $LiAlH_4$ (239 mg, 6.3 mmol) in ether (15 ml) under argon and then refluxed during 20 h. The reaction was cooled to 0 °C, a few drops of ice-water were added followed by ice-cold 4M NaOH. The gelatinous aluminium salts were filtered off and washed with ether (4 × 10 ml). The combined ether solutions were extracted with 2M HCl (3 × 15 ml), the acid solution washed with ether, basified with 4M NaOH and the amine extracted into ether (5 × 15 ml), washed with brine, dried ($MgSO_4$) and the solvent evaporated under reduced pressure to afford the amine (*S*)-(x) (217 mg, 63%) as a colourless oil, δ 0.9 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 126 Hz, *CH_3), 0.9 (3 H, br s, CH_3), 1.55 (3 H, m, CH_2 + CH), 3.96 (1 H, t, *J* 7 Hz, Ar*CH*NH_2), and 7.3 (5 H, Ph), *m/z* 106 (100), 91 (2), and 79 (13). [Found: ($M^+ - C_3{}^{13}CH_9$), 106.0655. C_7H_8N requires 106.0657]. Similarly, (2*R*)-[1-^{13}C]-4-azido-2-methyl-4-phenylbutane (353 mg) furnished (2*R*)-[1-^{13}C]-4-amino-2-methyl-4-phenylbutane (*R*)-(x) (192 mg, 63%), δ 0.9 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 126 Hz, *CH_3), 0.9 (3 H, br s, CH_3), 1.55 (3 H, m, CH_2 + CH), 3.96 (1 H, t, *J* 7 Hz, Ar*CH*NH_2), and 7.3 (5 H, Ph); *m/z* 106 (100), 91 (4), and 79 (10) (Found: $M^+ - C_3{}^{13}CH_9$, 106.0664).

(2S)-[1-^{13}C]-4-*Acetamido*-2-*methyl*-4-*phenylbutane* (S)-(xi).—To the amine *S*-(x) (217 mg, 1.33 mmol) in CH_2Cl_2 (5 ml) at −10 °C was added triethylamine (1 ml in 2 ml CH_2Cl_2) dropwise and then acetyl chloride (1 ml in 3 ml CH_2Cl_2) also dropwise with stirring under argon. After being left for a further 2 h at 0 °C, the reaction mixture was poured into ice water, the aqueous layer washed with CH_2Cl_2 and the combined organic solutions were washed with 2M HCl, H_2O, 4M NaOH, H_2O, dried ($MgSO_4$) and the solvent evaporated under reduced pressure, affording the *acetamide* (S)-(xi) (227 mg, 84%) as an oil, δ 0.9 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 126 H, *CH_3), 0.9 (3 H, br s, CH_3), 1.58 (3 H, m, CH_2 + CH), 1.88 (3 H, s, CH_3CO), 5.07 (1 H, m, C*H*NHAc), and 7.26 (5 H, Ph); *m/z* 206 (11), 163 (5), 148 (56), 120 (23), and 106 (100). (Found: M^+, 206.1497, $C_{12}{}^{13}CH_{19}NO$ requires 206.1500). (2*R*)-[1-^{13}*C*]-4-*Amino*-2-*methyl*-4-*phenylbutane* (190 mg), similarly furnished (2R)-[1-^{13}*C*]-4-*acetamido*-2-*methyl*-4-*phenylbutane* (R)-(xi) (223 mg; 94%), δ 0.9 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 126 Hz, *CH_3), 0.9 (3 H, br s, CH_3), 1.62 (3 H, m, CH_2 + CH), 1.91 (3 H, s, CH_3CO), 5.08 (1 H, m, Ar*CH*NHAc), and 7.3 (5 H, Ph); *m/z* 206 (9), 163 (2), 148 (50), 120 (20), and 106 (100) (Found: M^+, 206.1505).

(4S)- *and* (4R)-[5-^{13}C]*Leucines*.[47]—To a magnetically stirred solution of the amide (*S*)-(xi) (227 mg, 1.10 mmol) in CCl_4 (4.5 ml) and CH_3CN (4.5 ml) was added ruthenium trichloride trihydrate (10 mg, 0.03 mmol) followed by sodium metaperiodate (4.5 g, 21 mmol, 20 equiv.) dissolved in water (20 ml). The mixture became warm after *ca.* 1 h and a curdy precipitate began to appear. Stirring was continued for 24 h and then the solvents were evaporated under reduced pressure. The residue was taken up in hot methanol (20 ml) and silica gel (3 g) was added. The methanol was evaporated, the solid residue placed on top of a short silica gel column and the N-*acetyl-leucine* (S)-(xii) (173 mg) eluted with $CHCl_3$–MeOH (3:1, 200 ml). δ 0.95 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 126 Hz, *CH_3), 0.95 (3 H, br s, CH_3), 1.6 (3 H, m, CH_2 + CH), 2.05 (3 H, s, $COCH_3$), 4.7 (3 H, t, C*H*NAc). The (2*R*)-acetamide (223 mg) similarly afforded the (4*R*)-N-*acetyl-leucine* (R)-(xii) (174 mg), δ 1.0 (3 H, dd, J_{HH} 6 Hz, $^1J_{CH}$ 126 Hz, *CH_3), 1.0 (3 H, br s, CH_3), 1.68 (3 H, m, CH_2 + CH), 2.05 (3 H, s, CH_3CO), 4.7 (3 H, t, C*H*NAc). The *N*-acetyl-leucines were not purified but hydrolysed direct. Thus, (4*S*)-[5-^{13}C]-*N*-acetyl leucine (173 mg) was refluxed with 6M HCl (10 ml) for 16 h. The aqueous solution was taken to dryness *in vacuo*, the residue dissolved in water, again taken to dryness and the process repeated a further three times. The solid residue, in H_2O (1 ml), was added to a column containing Dowex 50

W × 8 (15 ml). The column was washed with water until the washings were free of Cl^- ions and then the leucine was eluted with 2M NH_4OH till the eluate gave a negative ninhydrin reaction. The ammonium hydroxide solutions were taken to dryness and the residue crystallised from H_2O–EtOH, affording (2*RS*,4*S*)-[5-^{13}C]-leucine (*S*)-(xiii) (95 mg), m.p. 290—295 °C (sealed tube); *m/z* 87 (100), 74 (35), and 57 (4) (Found: $M^+ - CO_2H$,[48] 87.0967. $C_4{}^{13}CH_{12}N$ requires 87.1002). Similarly, (4*R*)-[5-^{13}C]-*N*-acetyl-leucine (174 mg) afforded (2*RS*,4*R*)-[5-^{13}C]leucine (*R*)-(xiii) (89 mg), m.p. 293—295 °C (sealed tube); *m/z* 87 (100), 74 (35), and 57 (9) (Found: $M^+ - CO_2H$, 87.0954).

I. *Incorporation of* (2RS)-[2-^{13}C]*Leucine into Paniculide.*—(A) *Andrographis* suspension cultures (15 × 100 ml in 250 ml conical flasks) in a modified White's medium[41] were shaken on a horizontal rotary shaker for 21 days at 25 ± 1 °C. The culture medium was separated by filtration, and the callus re-suspended in fresh medium (1.5 l) containing (2*RS*)-[2-^{13}C]leucine (75 mg) and (2*S*)-[*U*-^{14}*C*]leucine (1 μCi). After a further 7 days, paniculide was isolated by concentrating the medium under reduced pressure at less than 40 °C to 500 ml and extracting with ethyl acetate (3 × 500 ml). Crystallisation of the ethyl acetate-soluble material afforded paniculide, m.p. 146—148 °C (34 mg, I_{spec} 13.0%). Incorporation of ^{13}C could not be detected by MS or by peak enhancement in the ^{13}C n.m.r. spectrum. The paniculide was converted to the diacetate [$(CH_3CO)_2O$–pyridine] which when purified by p.l.c. ($CHCl_3$–MeOH, 96:4; R_f 0.58) and crystallisation (ethyl acetate–hexane) had m.p. 98—99 °C. This, likewise, showed no evidence for ^{13}C incorporation by either MS or ^{13}C n.m.r. spectroscopy.

In a repeat experiment, (2*RS*)-[2-^{13}C]leucine (75 mg) mixed with (2*S*)-leucine (75 mg) and (2*S*)-[U-^{14}C]leucine were administered as above. Radioactivity was incorporated only into paniculide A, m.p., from ethyl acetate–hexane, 118—120 °C (18 mg, I_{spec} 28.3%) but again this showed no evidence of ^{13}C incorporation.

(B) *Andrographis* cultures were grown as in (A) in modified White's medium for *seven* days, leucine was added (as below) and the cultures were shaken for a further *sixteen* days and then harvested. Thus, (2*RS*)-[2-^{13}C]leucine (85 mg) and [U-^{14}C]-leucine (1.1 μCi), distributed between 8 flasks (800 ml) afforded paniculide, m.p. 145—148 °C (32 mg, I_{spec} 6.24%). Calculated enhancement per labelled site 104%. The ^{13}C n.m.r. spectrum is shown in Fig. 1C.

II. *Incorporation of* (2RS)-[3-^{13}C]*Leucine into Paniculide.*—Conditions of incubation were as in Experiment IB. (2*RS*)-[3-^{13}C]Leucine (800 mg) + (2*RS*)-[3-^{14}C]leucine (80 μCi) in 80 flasks (8 l) afforded paniculide, m.p. 145—147 °C (157 mg, I_{spec} 4.97%). The ^{13}C n.m.r. spectrum is shown in Fig. 1B.

III. *Incorporation of* (2RS,4R)-[5-^{13}C]*Leucine and* (2RS,4S)-[5-^{13}C]*Leucine into Paniculide.*—Conditions of incubation were as in Experiment IB. (2*RS*,4*R*)-[5-^{13}C]Leucine (85 mg) + [U-^{14}C]leucine (1 μCi) in 8 flasks (800 ml) afforded paniculide, m.p. 146—148 °C (34 mg, I_{spec} 7.58%). The ^{13}C n.m.r. spectrum is shown in Fig. 2C.

(2*RS*,4*S*)-[5-^{13}C]Leucine (76 mg) + [U-^{14}C]leucine (1 μCi) in 8 flasks (800 ml) afforded paniculide, m.p. 145—148 °C (29 mg, I_{spec} 6.83%). The ^{13}C n.m.r. spectrum is shown in Fig. 2B.

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