Pattern of Incorporation of Leucine Samples Asymmetrically Labelled with ^{13}C in the Isopropyl Unit into the C_5-Isoprenoid Units of Echinuline and Flavoglaucine

By ROSANNA CARDILLO, CLAUDIO FUGANTI,* DARIO GHIRINGHELLI, and PIERO GRASSELLI
(*Istituto di Chimica del Politecnico, Centro del C.N.R. per la Chimica delle Sostanze Organiche Naturali*, 20133 *Milano, Italy*)
and GIUSEPPE GATTI
(*Istituto di Chimica delle Macromolecole del C.N.R., I.C.M.*, 20133 *Milano, Italy*)

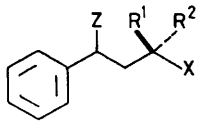
Summary The labelling pattern of the C_5-isoprenoid moieties of echinuline (**18**) and flavoglaucine (**19**) obtained in feeding experiments of *Aspergillus amstelodami* of 90% enriched (4*R*)[5-^{13}C]leucine (**11**) and of the (4*S*)-isomer (**12**), determined by ^{13}C-n.m.r. spectroscopy, appears in agreement with a preferential incorporation of the carbon atom of the *pro-S* methyl group of the stereospecifically labelled amino acid (**12**) into the C-3 methyl group of the intermediate mevalonic acid and of the carbon atom of the *pro-R* methyl group of (**11**) in position 4, respectively.

THE biological degradation of the amino-acid leucine involves[1] as relevant steps the $\alpha\beta$-desaturation of the intermediate isovalerylcoenzyme A (**13**) to β-methylcrotonylcoenzyme A (**14**), and the biotin-dependent carboxylation of the latter to *E*-β-methylglutaconylcoenzyme A (**15**).[2] This compound gives rise, in turn, upon addition of water to β-hydroxy-β-methylglutarylcoenzyme A (**16**), the precursor of mevalonic acid (**17**), thus accounting for the observed introduction of part of the leucine framework into the isoprenoid compounds.[3]

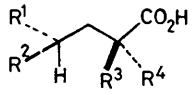
In view of the present interest in the stereospecificity of enzymic reactions[4] we undertook a stereochemical analysis of the abovementioned set of enzymic operations, and we are at present studying the mode of incorporation of asymmetrically labelled leucine samples into the C_5-isoprenoid units of the mould metabolite echinuline (**18**)[5] and flavoglaucine (**19**)[6]. We chose this approach because we expected in the case of a significant intact incorporation† of the leucine framework to take advantage for our stereoanalytical purposes of the detailed knowledge of the pattern and of the steric course of the incorporation of the intermediates following β-hydroxy-β-methylglutarylcoenzyme A (**16**) into this type of molecular fragment.[7]

We report now the synthesis of leucine samples asymmetrically labelled with ^{13}C in the isopropyl unit and the determination of their incorporation pattern into the isoprenylated metabolites echinuline (**18**) and flavoglaucine (**19**) by means of ^{13}C-n.m.r. spectroscopy from which the fate of the two distinguishable methyl groups of the amino-acid during the above transformations is tentatively inferred. Indeed, if the sequence reported in the Scheme is operating, we would expect incorporation of the ^{13}C-labelled carbon atoms of the *pro-S* and, respectively, *pro-R* methyl groups of the fed precursors (**12**) and (**11**) into the *Z*- and *E*-methyl groups of the isopentenyl chains, or *vice-versa*, depending upon the stereochemistry of the dehydrogenation of isovalerylcoenzyme A (**13**) to β-methylcrotonylcoenzyme A (**14**). Chiral [5-^{13}C]leucine was synthesized as follows. (2*RS*)[1-^{13}C]2-Methyl-4-phenylbutyric acid (**2**), obtained from carbonation with 90% enriched $^{13}CO_2$ of the Grignard reagent (**1**), was resolved through crystallisations of the salts with (−)- and (+)-α-phenylethylamine into the (2*R*) and the (2*S*) isomers (**3**) and (**4**), respectively. The absolute configuration and the optical purity of the acids (**3**) and (**4**) are based on the conversion of unlabelled (**3**)[8] upon ozonolysis into 2-methylglutaric acid and comparison of its optical

† Exploratory feeding experiments with [4,5-^{3}H; *U*-^{14}C]L-leucine showed incorporation into (**18**) with *ca.* 45% tritium retention.



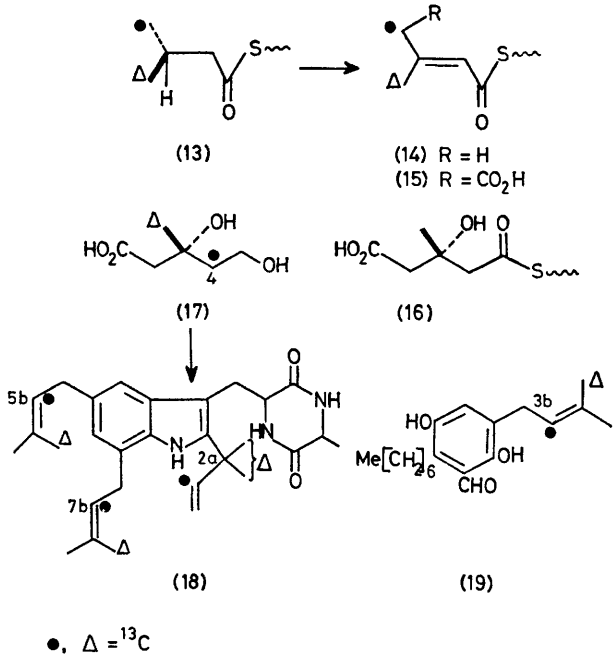
(1) R^1, R^2 = H, Me; X = MgBr; Z = H
(2) R^1, R^2 = H, Me; X = $^{13}CO_2H$; Z = H
(3) R^1 = Me; R^2 = Z = H; X = $^{13}CO_2H$
(4) R^1 = Z = H; R^2 = Me; X = $^{13}CO_2H$
(5) R^1 = Me; R^2 = Z = H; X = $^{13}CH_2OH$
(6) R^1 = Me; R^2 = Z = H; X = $^{13}CH_3$
(7) R^1 = Me; R^2 = H; X = $^{13}CH_3$; Z = Br
(8) R^1 = Me; R^2 = H; X = $^{13}CH_3$; Z = NH_2



(9) R^1 = $^{13}CH_3$; R^2 = CH_3; R^3, R^4 = H, NHCOMe
(10) R^1 = CH_3; R^2 = $^{13}CH_3$; R^3, R^4 = H, NHCOMe
(11) R^1 = $^{13}CH_3$; R^2 = CH_3; R^3, R^4 = H, NH_2
(11a) R^1 = $^{13}CH_3$; R^2 = CH_3; R^3 = NH_2; R^4 = H
(12) R^1 = CH_3; R^2 = $^{13}CH_3$; R^3, R^4 = H, NH_2
(12a) R^1 = CH_3; R^2 = $^{13}CH_3$; R^3 = NH_2; R^4 = H

properties with those of an authentic sample of (2*R*)-2-methylglutaric acid.[9] Reduction ($LiAlH_4$) of (**3**) (*ca.* 90% optical purity) gave the alcohol (**5**), transformed into (2*R*)-[1-^{13}C]2-methyl-4-phenylbutane (**6**) upon $LiAlH_4$ treatment of the toluene-*p*-sulphonyl derivative. The labelled hydrocarbon (**6**) was brominated (*N*-bromosuccinimide–CCl_4) to compound (**7**), which was converted upon azide displacement, followed by hydrogenation, into the amine (**8**). This material, after monoacetylation, was ozonised in 80% formic acid and oxidised to (2*RS*, 4*R*) [5-^{13}C]*N*-acetyl-leucine (**9**) in *ca.* 15% overall yield. Repetition of the above sequence afforded from the (2*S*)-acid (**4**) (*ca.* 80% optical purity) the (4*S*)-isomer (**10**). Acid hydrolysis of (**9**) and (**10**) gave the racemic materials (**11**) and (**12**). Enzymic resolution (hog kidney acylase) yielded from (**9**) and (**10**) (4*R*)[5-^{13}C]L-leucine (**11a**) and the (4*S*)-isomer (**12a**), respectively. The ^{13}C-n.m.r. spectra of the latter ^{13}C asymmetrically labelled leucine samples (NaOD, dioxan) allowed assignment of the signals at −44·032 and −45·110 p.p.m. (from internal dioxan) to the *pro-S* and *pro-R* methyl groups, respectively, and furthermore, confirmed the substantial enantiomeric purity at C-4 of the synthetic products.

Two sets of feeding experiments were performed. In the first, 60 mg of each chiral precursor (**11**) and (**12**) were fed separately, at the 3rd day, to 250 ml cultures of *Aspergillus amstelodami* grown on a synthetic medium, producing *ca.* 90 mg of echinuline (**18**) and the culture was harvested after one week. In the second experiment 70 mg samples were fed, at the same time, followed by 20 mg after a further 2 days, the fungus being harvested as before. The ^{13}C-n.m.r. spectra of echinuline (**18**) obtained from the (4*R*)-isomer (**11**), compared with a natural abundance sample,[10]‡ showed percentage enhancements as follows (1st, 2nd expt. given in parentheses) (the intensity of the signal due to α-carbon of the alanine unit was used as internal reference) of the signals assigned to C-2b (180, 140%), C-5, 7b (190, 160%), the methyl groups on C-2a (80, 40%), the *E*-methyl groups (130, 50%), and the *Z*-methyl groups (70, 50%), whereas in the samples biosynthesized from the (4*S*)-isomer (**12**) the signal enhancements were as follows: C-2b (30, 40%), C-5, 7b (70, 40%), the methyl groups on C-2a (120, 160%), the *E*-methyl groups (50, 60%), and the *Z*-methyl groups (160, 210%). Flavoglaucine (**19**) obtained in the same experiments showed a labelling pattern of the C_5-unit qualitatively in line with that observed in echinuline (**18**). The signals [$(CD_3)_2SO$]‡ due to C-3b and to the carbon atoms of the *E*- and *Z*-methyl groups appear at 121·81, 26·709, and 22·135 p.p.m. (from Me_4Si), respectively. The peak enhancements in the sample from the (4*R*)-isomer (**11**) for the above signals are: (160, 140%), (90, 90%), and (90, 80%), respectively, whereas in the 2nd experiment with the (4*S*)-isomer (**12**) the enhancements were as follows: (65%), (130%), and (320%), respectively. The labelling patterns of the C_5-units of the two metabolites (**18**) and (**19**) biosynthesized in the two sets of experiments from (**11**) and (**12**) thus formally indicate, within the limits of the method,[11] the trend to introduce the ^{13}C-label of the (4*S*)-precursor (**12**) into the *Z*-methyl group of the C_5-units, arising from the



(13) (14) R = H (15) R = CO_2H (17) (16) (18) (19)

●, Δ = ^{13}C

SCHEME

C-3 methyl group of mevalonic acid (**17**), with corresponding incorporation, with minor selectivity, of the ^{13}C-label of the (4*R*)-isomer (**11**) into position 2 of the chain, arising from position 4 of (**17**), this major pathway being accompanied by scrambling of the label, whose extent varies from (**18**) to (**19**) and with the feeding conditions.

An economic interpretation of the above results points to a stereospecific α,β-desaturation of the intermediate isovalerylcoenzyme A (**13**) to β-methyl crotonylcoenzyme A (**14**), with the stereochemistry shown in the Scheme.

‡ Full assignment to be published.

Subsequently, cleavage of β-hydroxy-β-methylglutarylcoenzyme A (**16**) to acetyl CoA and acetoacetate, incorporating regioselectively the labels of (**11**) and (**12**), followed by intact incorporation of this C_4-unit as acetoacetylCoA into mevalonate would account for the observed introduction of the ^{13}C label of (**11**) in position **4** of the latter intermediate. However, further work is needed to settle the problem completely. The observed incorporation pattern militates against participation in this mould of a direct pathway from leucine to dimethylallylpyrophosphate of the type claimed[12] to operate in *Cinnamomum camphora*.

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