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TRITIUM NMR IN INVESTIGATIONS OF BRANCHED-CHAIN AMINO ACID METABOLISM

THE SYNTHESIS OF CHIRAL-METHYL VALINE

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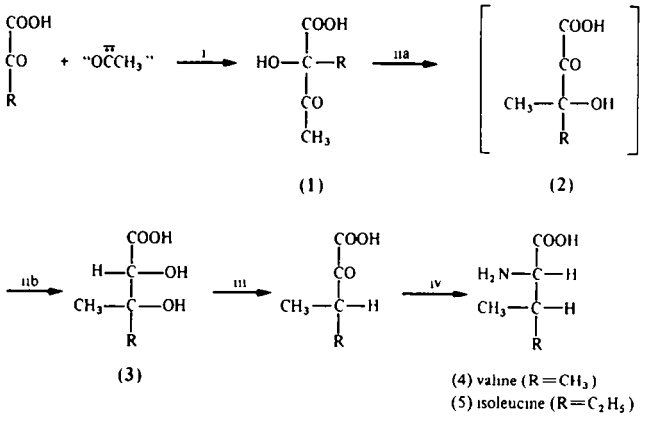
Abstract—A synthesis of chiral-methyl valine has been carried out in order to exploit the principle of the linked configurations of pro, prochiral and prochiral centres in a biosynthetic experiment.

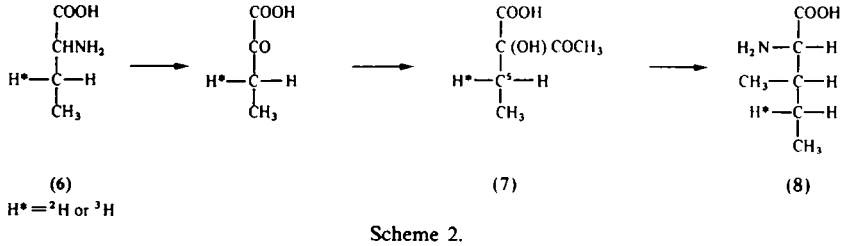
In their later stages, the pathways of biosynthesis of the branched-chain amino acids valine and isoleucine share a common set of enzymes (Scheme 1).[1] This pathway exhibits many interesting stereochemical features, amongst which those associated with the transformations catalysed by the enzyme reductoisomerase ((1)→(2)→(3)) are of particular interest. It is during the course of these transformations that the carbon skeletons of the final metabolites are generated, through tertiary ketol rearrangement of an α-hydroxy-β-keto acid precursor. In order to provide a complete stereochemical description of the transformations undergone by the substrates in the reductoismoerase reaction, the answers to four questions must be sought, giving a total of eight questions for the two pathways. The answers to seven of these questions have been obtained (Table 1).[2,3,5-7] The one remaining concerns the stereochemistry at the migrating centre during the Me migration step in valine biosynthesis ((1)→(2), R=Me, Scheme 1). This process has been shown to proceed with retention of configuration during migration of the Et group in the isoleucine pathway in both a bacterium (*Serratia marcescens*)[3] and higher plants (*Senecio* species).[4] In order to secure such a result it was necessary that the rearrangemeent should be carried out with a substrate in which the migrating centre was chiral and of known configuration, and that a method should be at hand for the elucidation of the configuration at the corresponding centre in the product. The migrating centre in the rearrangement step of the isoleucine pathway is prochiral and the H atoms at C-5 in the substrate α-acetohydroxybutyrate (7, Scheme 2), are diastereotopic. In order to solve the problem, α-acetohydroxybutyrate (**7**) was therefore generated *in situ* in the chosen organism from 3-deuterio- or tritio-2-aminobutyrate (**6**) of known (3*S*) configuration at C-3. The configuration at the corresponding centre in the product was determined by NMR (for D labelling) or by enzymatic analysis (for T labelling). This experiment illustrates the direct approach to the solution of such problems: the required configuration at the centre of interest is generated directly by stereospecific (chemical or enzymatic) methods.

However, during biological investigations, advantage can often be taken of the stereoselectivity of enzymatic systems. In this way a difficult resolution or stereospecific synthesis often may be avoided. This approach was used during an investigation into the destination of the methyl group in the rearrangement step of valine biosynthesis.[5] The question was whether the migrating Me group becomes the *pro-R* or the *pro-S* Me group in the product 2-keto-3-hydroxy acid (**10**, Scheme 3). For this investigation, a cell-free system from *Salmonella typhimurium* was used, the reductoisomerase of which was known to operate only on the (*S*)-isomer (**9**) of the substrate α-acetolacate (Scheme 3).[6] Accordingly, the triply ^{13}C-labelled *racemic* α-acetolactate (**11**) (Scheme 4) was incubated in this system to give a product, L-valine (**13**), derived solely from the (*S*)-component of the substrate and in which the label was shown by ^{13}C NMR spectroscopy to reside in the *pro-S* Me group. Since the transformation of the intermediate dihydroxy acid (**12**) into valine was known to proceed with overall retention of configuration at C-3,[7] it was concluded that the migrating Me group becomes the *pro-R* Me group in the 2-oxo-3-hydroxy acid (**10**).

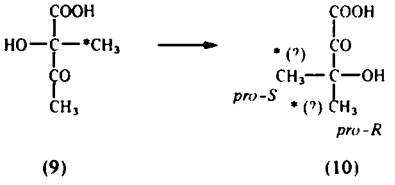
This approach can be extended to investigations of stereochemical changes involving a prochiral centre in the substrate (as opposed to the product) of an enzymatic reaction or sequence of reactions. Thus it is often possible to generate stereospecifically a certain absolute configuration at a prochiral centre in such a way that the absolute configuration at this centre is linked in a precisely defined way to the configuration at a neighbouring *chiral* centre. When an enzyme then selects substrate molecules with a particular configuration at the chiral centre, a particular configuration at the prochiral centre is simultaneously selected, since the configurations at the two centres are linked by the stereochemistry of their mode of formation. Thus, for example, reduction of the racemic N-acetylisodehydroisoleucine (**14**) (Scheme 5) with tritiated di-imide gave, after hydrolysis and chromatographic separation of the isoleucine produced from the simultaneously formed alloisoleucine, a product DL-isoleucine in which the L-component (**15**) was labelled in the 4-*pro-S* position and the D-component (**16**) in the 4-*pro-R* position (Scheme 5).[4] This conclusion follows from the *cis*-stereospecificity of the addition of H from di-imide to the (*E*)-double bond of the unsaturated starting material. When the racemic tritiated isoleucine was administered to *Senecio magnificus* plants, together with uniformly ^{14}C-labelled L-isoleucine as an internal



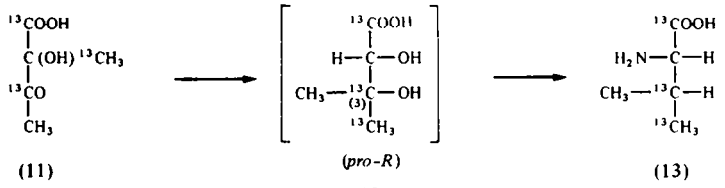
Scheme 1. Enzymes: (i) acetolactate synthase (acetolactate pyruvate-lyase (carboxylating), EC 4.1.3.18); (iia, b) ketol-acid reductoisomerase (2,3-dihydroxy-isovalerate: $NADP^+$ oxidoreductase (isomerising), EC 1.1.1.86; (iii) dihydroxyacid dehydratase (2,3-dihydroxyacid hydrolyase, EC 4.2.1.9); (iv) branched-chain-amino-acid aminotransferase (branched-chain-amino-acid: 2-oxoglutarate aminotransferase, EC 2.6.1.42).



Scheme 2.



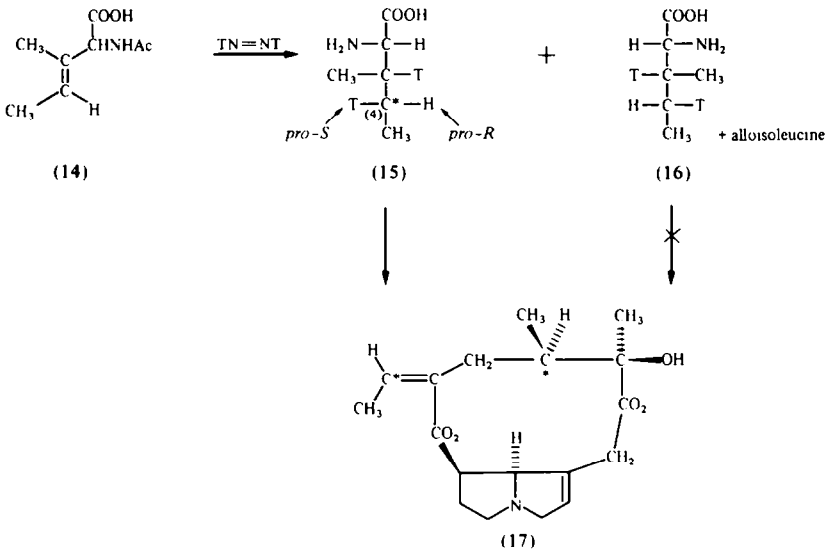
Scheme 3.



Scheme 4.

standard, the senecionine (**17**) produced was essentially devoid of tritium (Scheme 5). Senecic acid, the "necic acid" component of senecionine (**17**) is derived from two molecules of isoleucine with loss of the carboxyl groups.[8] An H atom is lost from C-4 of both the precursor molecules. The foregoing result therefore shows that it is the 4-*pro-S* H that is lost in both cases. The justification for this conclusion depends on a result from previous work in which it was shown that of the four stereoisomers of isoleucine–alloisoleucine, only L-isoleucine (as **15**) is incorporated into senecionine (**17**) (exclusively into the necic acid component).[9] In selecting a precursor with the 3*S*-configuration (i.e. that of L-isoleucine), the enzyme system in the plant was therefore selecting a precursor labelled in the 4-*pro-S* position and discriminating against the component in the mixture with the 3-*R* configuration (D-isoleucine **16**) and labelled in the stereochemically linked 4-*pro-R* position.

The approach just described depends on the ability of enzymes to discriminate between enantiomers. However, the interactions between both diastereotopic and enantiotopic groups of a substrate with an enzyme are diastereoisomeric; consequently enzymes usually differentiate between diastereotopic



Scheme 5.

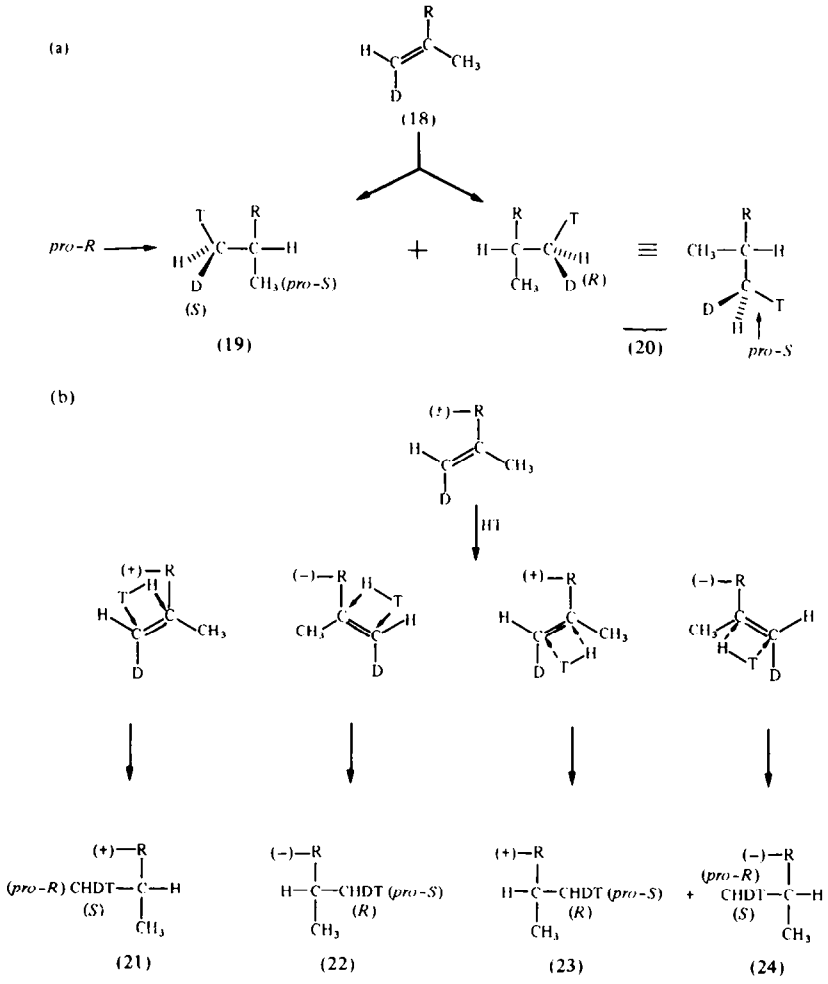
and enantiotopic substituents at prochiral centres as rigorously as they differentiate between enantiomers. The principle of stereochemical linking can therefore be extended to cases where a fixed configurational relationship can be established between a *prochiral* centre and a *pro,prochiral* centre. A pro,prochiral centre is one from which by isotopic labelling of one substituent a prochiral centre can be generated, and from which, by further isotopic labelling (with, for example, a different isotope) of a second substituent, a chiral center can be generated. Thus the methyl carbon atom of acetic acid (Scheme 6) is a pro, prochiral centre. Substitution, for example, of one hydrogen atom by deuterium, creates a prochiral centre, since on further substitution of each of the remaining hydrogen atoms in turn by tritium, the two enantiomers of a chiral system are generated.

The pro, prochiral Me groups of an isopropyl unit, considered as heterotopic substituents of the methine C centre, are enantiotopic in an achiral molecule or diastereotopic in a chiral one. The heterotopic Me groups of such a system may be generated in a chiral form and in such a way that their configurations are linked to the configuration at the prochiral methine C atom, as illustrated in Scheme 7. Thus, starting from the (*E*)-[^{2}H]isopropenyl system (**18**) (Scheme 7a) by, for example, *cis* addition of HT (obtainable by equilibration of H_2 and T_2), an isopropyl system bearing chiral Me groups will be generated. Note that the product consists of a mixture of species bearing a T label in a Me group comprising one (**19**) in which the *pro-R* Me group has the *S*-configuration and one (**20**) in which the *pro-S* Me group has the *R*-configuration. These configurations are determined solely by the configuration of the starting alkene and by the mode of addition of HT (taken to be *cis* for purposes of illustration). (Species in which neither Me group bears a T atom are ignored). To generate an isopropyl group with the opposite set of configurations, all that is required is that the starting material should be the geometrical isomer of the labelled alkene (**18**).

The configurations of the products are entirely independent of the nature of the group *R*. This group affects the stereochemical outcome of the synthesis only in that if it is chiral (Scheme 7b), the transition states attained during the hydrogenation step will be diastereoisomeric and the consequent asymmetric induction will lead to the production of unequal amounts of the enantiomeric pairs [**21** + **22**] and [**23** + **24**].

A mixture of species such as [**19** + **20**] or [**21** + **22** + **23** + **24**] may be used, without separation, in a biosynthetic experiment provided only that the usual discrimination between heterotopic substituents obtains. An example of such a system is the one in which valine is incorporated *via* the Arnstein tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (**25**), isopenicillin N (**26**) and penicillin N (**27**) into cephalosporin C (**28**) (Scheme 8).[10] Strict stereochemical control is exercised throughout this sequence with respect to changes taking place at the isopropyl group of valine. Thus the insertion of sulphur during generation of the thiazolidine system of the penicillins takes place with overall retention of configuration.[11] Further, the ring expansion to the dihydrothiazine system of cephalosporin C takes place in such a way that the original 4-*pro-S* Me carbon of valine becomes the exocyclic 3′-C atom of cephalosporin C and the *pro-R* Me group becomes the 2-methylene carbon atom of the dihydrothiazine system (Scheme 8).[11a,b,12] The conversion of the original Me groups of valine into the corresponding methylene groups of cephalosporin C have stereo-

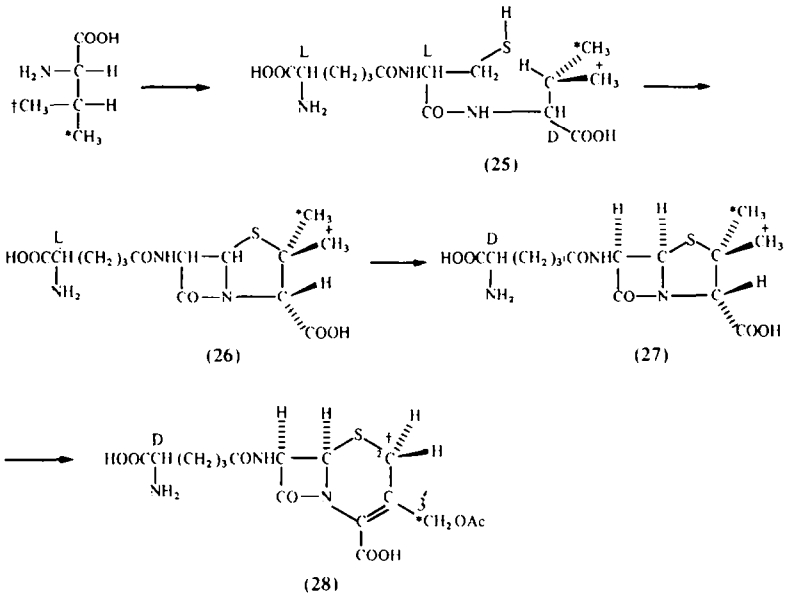
Scheme 6.



Scheme 7.

chemical implications of considerable importance with respect to the mechanisms of the processes of sulphur insertion and acetoxylation that accompany the transformation of penicillin N into cephalosporin C (Scheme 8). Further, if stereospecific, these changes, and in particular the acetoxylation process which operates on the Me group of valine (the *pro-S*) that has migrated during the reductoisomerase reaction, permit, in principle, the use of the cephalosporin C biosynthetic pathway as a tool for the analysis of the configurations of the Me groups of chiral-methyl valine.

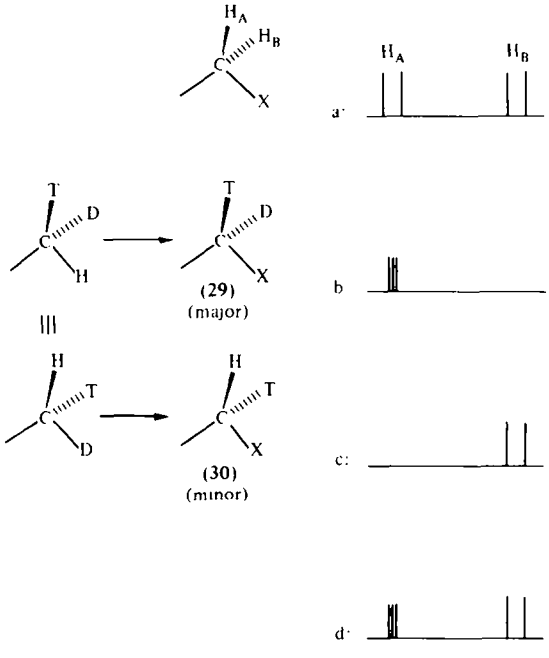
If this system were to be used for such stereochemical studies, it would be necessary to have available methods for the analysis of the configurations, with respect to isotopic substitution, of centres of interest in the product. There are two general approaches to this problem, both of which depend on the presence of tritium at the centres the configurations of which are to be analysed. The first approach, exemplified in the elegant work of Arigoni and Cornforth and their respective co-workers, depends on the operation of a deuterium isotope effect during conversion of the methyl into a methylene group.[13] In a stereospecific reaction (*cf* Scheme 9) which for the sake of argument may be assumed to proceed with retention of configuration, the product species containing tritium arise by replacement (by a substituent "X" in the illustration) of a proton and a deuteron respectively. If the latter process were subject to a normal (positive) isotope effect, the corresponding product would be the minor component of the product mixture. Assuming that the tritium content of the heterotopic H atoms H_A and H_B in the product could be determined by chemical, physical or enzymatic analysis, it would be possible to infer, by reversing the logic of the above argument, the stereochemistry of the substitution process. Thus if the transformation were to proceed with *inversion* of configuration, the relative tritium contents of the hydrogen atoms H_A and H_B would be reversed, so that if H_A were the site expected to be most heavily labelled for a retention mode (Scheme 9), H_B would be more heavily labelled for an inversion mode. However, unless the magnitude of the deuterium isotope effect is known independently, such an experiment does not give a quantitative idea of the stereospecificity of the transformation. Thus a given ratio of tritium content for the hydrogens H_A and H_B might result from any number of combinations of deuterium isotope effect with stereospecificity. (A high deuterium isotope effect combined with low



Scheme 8.

stereospecificity in the enzymatic reaction could give the same tritium ratio as a low deuterium isotope effect combined with high stereospecificity.) Further, in order to apply such an analysis to the transformations of an isopropyl group labelled as in Scheme 7, a way of investigating separately both labelled sites in the product (e.g. C-2 and C-3′ in cephalosporin C, Scheme 8) would be required.

Both of these difficulties can be overcome by the application of ^{3}H NMR to product analysis. Normally, as in the incorporation of valine into cephalosporin C (Scheme 8), the original methyl C atoms become constitutionally non-equivalent in the product. The attached H nuclei consequently will be magnetically non-equivalent at the two centres and further, if diastereotopic, the nuclei attached to the individual centres will also be non-equivalent and generate an AB system in the NMR spectrum or an AB component of a more complex spin system if coupled to protons at other positions. Thus in the spectrum of cephalosporin C, the C-2 and C-3′ methylene groups give rise to well separated AB quartets in the ^{1}H NMR spectrum.[14] Because of the close correspondence between proton and triton chemical shifts,[15] corresponding signals will appear in the ^{3}H NMR spectrum. It will be evident therefore that provided the signals due to the C-2 and C-3′ methylene protons can be assigned in the ^{1}H NMR spectrum of cephalosporin C the location of tritium at the corresponding positions can be inferred immediately from the ^{3}H NMR spectrum. The ratio of tritium in the two heterotopic substituents of the prochiral methylene groups can therefore be determined directly from the integrated signals of the ^{3}H NMR spectrum. The analysis outlined above and depicted in Scheme 9 is thereby performed. However, the use of ^{3}H NMR further provides, in principle, a method for the *quantitative* evaluation of the stereospecificity of a transformation in a way that does not involve assumptions about deuterium isotope effects. Measurement of signal areas are best performed on a proton decoupled spectrum. However, if proton coupling is retained, additional information becomes available. Thus in the example in Scheme 9, if it is assumed, for purposes of illustration, that the signal due to H_A appears at lower field than that due to H_B (spectrum a), the tritium NMR signal (undecoupled) due to species (29) (spectrum b) will appear at the downfield position as a 1:1:1 triplet due to T:D coupling, whereas the tritium signal due to species (**30**) (spectrum c) will appear in the upfield position as a doublet due to T:H coupling. The combination of the signals of these two species would appear as in (d) (with relative integrations determined by the magnitude of the deuterium isotope effect in the substitution process, as discussed above). With a starting Me group of the opposite



Scheme 9.

configuration, the reverse pattern would be obtained (downfield doublet:upfield triplet). Alternatively, if the introduction of the group "X" were accompanied by overall inversion of configuration, the pattern of signals would again be reversed. A transformation accompanied by an intermediate stereochemical course (partial retention or inversion) would give rise to a spectrum containing corresponding combinations of signals, from the integrations of which the extent of retention or inversion of configuration could, in principle, be estimated. The variables in the system depicted in Scheme 9 are (i) the configuration of the initial chiral Me group; (ii) the stereochemistry of the substitution process and (iii) the assignment of the signals in the NMR spectrum. It is evident that if any two of these variables can be determined, the third can be inferred by the use of this technique.

The greatest single obstacle to the use of ^{3}H NMR is the amount of radioactivity that needs to be handled. The final sample must be obtained in sufficient quantity to give an acceptable signal-to-noise ratio in the ^{3}H NMR spectrum. Also, related to the final sample size by the efficiency of the biological or chemical transformation under investigation, is the amount of initial radioactive substrate required. With modern pulsed Fourier transform equipment, useful signal intensities can be obtained on samples containing the tritium equivalent of a few hundred microcuries per labelled position. However, in order to achieve such levels in biological experiments with overall conversion efficiencies of sometimes less than 1%, it may be necessary to handle many tens or hundreds of millicuries of a radioactive precursor.

It is necessary therefore to devise methods for the preparation of labelled precursors which allow for the efficient introduction of tritium. The experiments described below were designed to solve this problem with respect to a planned study of the incorporation of "chiral-methyl valine" into cephalosporin C (**28**) according to the principles described above.

RESULTS

The principal synthetic objectives were (i) the generation of a stereospecifically deuterated isopropenyl system (*cf* Scheme 7), (ii) tritiation of this isopropenyl system in such a way that the configurations of the chiral Me groups produced would be related to the configuration of the adjacent centre in a strictly defined manner.

The first of these problems was solved using a method we had developed for the synthesis of acrylates stereospecifically labelled in the vinylic methylene group, by reduction of the corresponding bromo derivative using sodium amalgam in D_2O (Scheme 10a).[16] In this way, using (*E*)-3-bromomethacrylic acid (**31**), (*E*)-[3-^{2}H]methacrylic acid (**32**) was obtained. In principle, the corresponding (*Z*)-isomer (**32a**) could be prepared by the sequence shown in Scheme 10b. However, this multistep process was avoided by making use of Aberhart's observation that treatment of (*E*)-3-bromomethacrylic acid with *tert*-BuLi gave the Li derivative (**43**) (Scheme 10c) rather than the corresponding acrylate derivative (**44**).[17] We have found that this reaction is readily executed using n-BuLi. Quenching of the Li derivative (**43**) with D_2O followed by sodium amalgam reduction in H_2O then gave the required (*Z*)-[3-^{2}H]methacrylic acid (**32a**). Because compound (**42**) lacked vinylic protons, the NMR signals from which might have been reliably used to confirm its stereochemistry, it was treated with BuLi and the resulting dianion was quenched with H_2O. The product was the starting (*E*)-3-bromomethacrylic acid (**31**), confirming the stereospecificity of the exchange reaction (Scheme 10c).

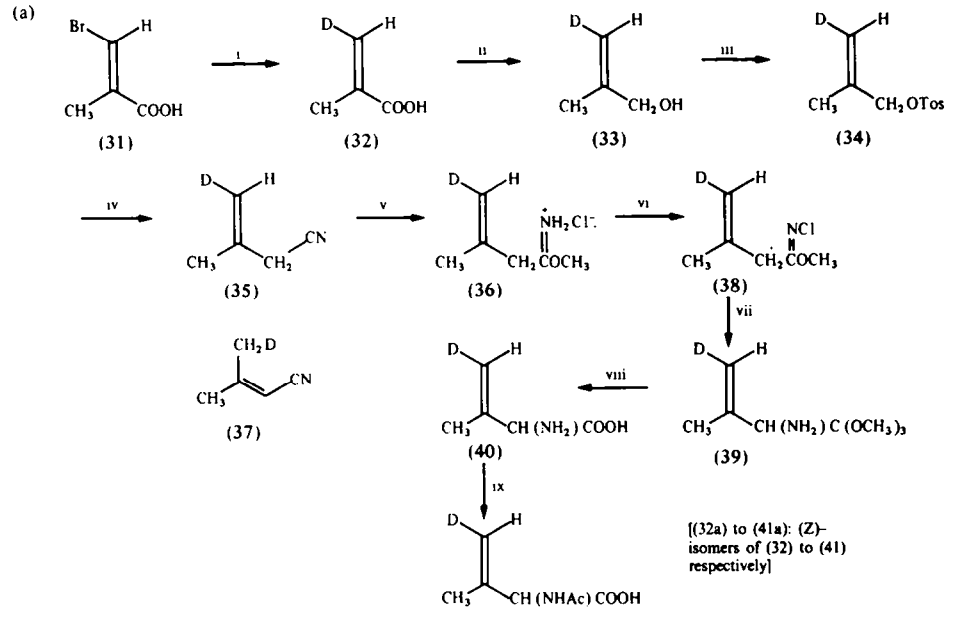
The following step in the synthesis required reduction of the methacrylic acid to the corresponding alcohol (Scheme 10a). This subsequently permitted the use of an even more economical route to the (*Z*)-isomer of alcohol (**33**) using a minor modification of the method of Duboudin *et al.* (Scheme 10d).[18]

An attractive method for elaboration of methacryl alcohol (as **33**) to isodehydrovaline (as **40**) was suggested by the observation of Graham that the Neber rearrangement of N-chloroimidates with a β-activating group was particularly favourable (Scheme 11a),[19] an observation supported by the remarkable and pertinent observation of Olesen Larsen *et al.* of the nearly quantitative conversion of sinigrin (potassium allyl glucosinolate **45**) into vinylglycine (**46**) on treatment with aqueous alkali (Scheme 11b).[20]

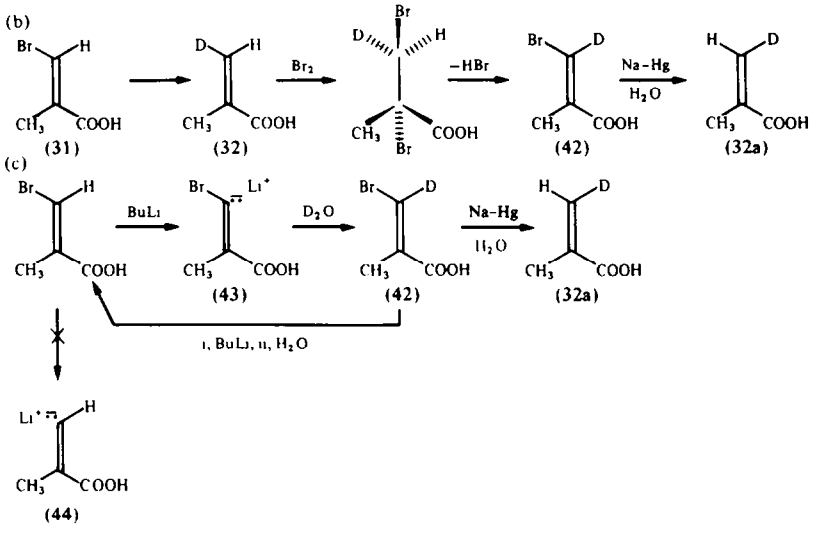
Methacryl alcohol (**33**) was converted *via* the tosylate (**34**) into the nitrile (**35**). Although this reaction could only be induced to proceed in poor yield ($\sim$ 20%) owing partly to the concomitant formation of the conjugated isomer (**37**), the subsequent conversions into the imidate hydrochloride (**36**) and N-chloroimidate (**38**), Neber rearrangement of the latter and hydrolysis of the resulting orthoester (**39**) to isodehydrovaline (**40**) all proceeded in moderate to good yield. The (*Z*)-isomer (**40a**) of the isodehydrovaline (**40**) was prepared by a similar sequence starting either from the (*Z*)-[3-^{2}H]methacrylic acid (**32a**) or from the corresponding alcohol (**33a**).

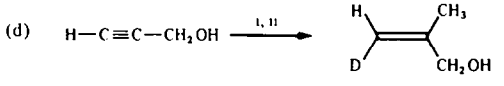
The saturated product, 2-methylpropanoic acid, produced by overreduction in the first stage of the synthesis, was carried through to the corresponding tosylate but, presumably because of the greater reactivity of the allylic analogue (**34**) in the subsequent displacement with limiting amounts of cyanide ion, it was eliminated. The nitrile (**37**) was similarly carried through to the corresponding N-chloroimidate, but, since the latter could not undergo the Neber rearrangement, it was not converted into an amino acid product.

The overall stereospecificities of these synthetic procedures were verified by the ^{1}H NMR spectra of the N-acetyl derivatives of the monodeuterioisodehydrovalines (Table 2). At 400 MHz, the signals due to the vinylic protons in the undeuterated compound (as **41**) were fully resolved. The downfield signal appeared as a broad singlet, and the upfield signal appeared as a multiplet. This assignment was confirmed by the spectrum of the (*E*)-deuterio isomer (**41**) which contained essentially the downfield signal only. This signal appeared with an upfield deuterium isotope shift of 0.014 ppm. The presence of a small amount of the (*Z*)-isomer (< 5%) was indicated by a weak multiplet at the same position as the signal due to the vinylic proton in the (*Z*)-isomer (**41a**), shifted upfield by 0.014 ppm from the corresponding signal in the undeuterated compound. The presence of 12% of the unlabelled derivative was also indicated by weak signals at the unshifted positions. A similar analysis of the spec-



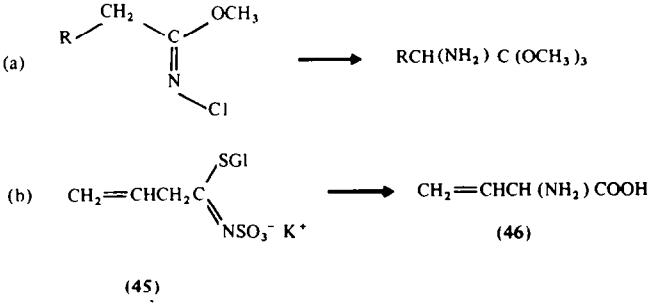
Reagents: i, Na–Hg, D_2O; ii, $LiAlH_4$; iii, TosCl, C_5H_5N; iv, KCN, 18-crown-6; v, HCl, CH_3OH; vi, NaOCl; vii, $NaOCH_3$; viii, H^+/H_2O; ix, Ac_2O.





Reagents: i, CH_3MgBr–CuI; ii, D_2O.

Scheme 10.



Scheme 11.

trum of the (*Z*)-isomer (**41a**) indicated the presence of 12% unlabelled material and none of the isomeric derivative (**41**). In this spectrum the signal due to the vinylic proton appeared as a quartet (J 1.5 Hz) and the signal due to the adjacent Me group appeared as a corresponding doublet (J 1.5 Hz). The sample of the (*Z*)-isomer (**41a**) subjected to this analysis was prepared from the corresponding (*Z*)-isomer of the alcohol (**33**) by the method of Scheme 10d.

It was proposed to tritiate the isopropenyl system as in Scheme 7. To give maximum assurance of a stereospecific reduction, Wilkinson's catalyst was used, with benzene–ethanol as solvent. To bring the isodehydrovalines into a form soluble in this solvent they were converted into the N-acetyl derivatives described above. This had the additional advantage that the product N-acetylvaline could be resolved directly by enzymatic hydrolysis of the L-component using acylase I. In some subsequent experiments it was found advantageous to resolve the N-acetylisodehydrovaline (as **47** + **48**) using acylase I, followed by re-acetylation of the L-component. This procedure made available for reduction both enantiomers of the derivative (**41** and **41a**). However, resolution is *not* a prerequisite for biological studies with this material (*vide infra*), because the configuration at C-2 is in no way related to or affects the configuration of the isopropyl system.

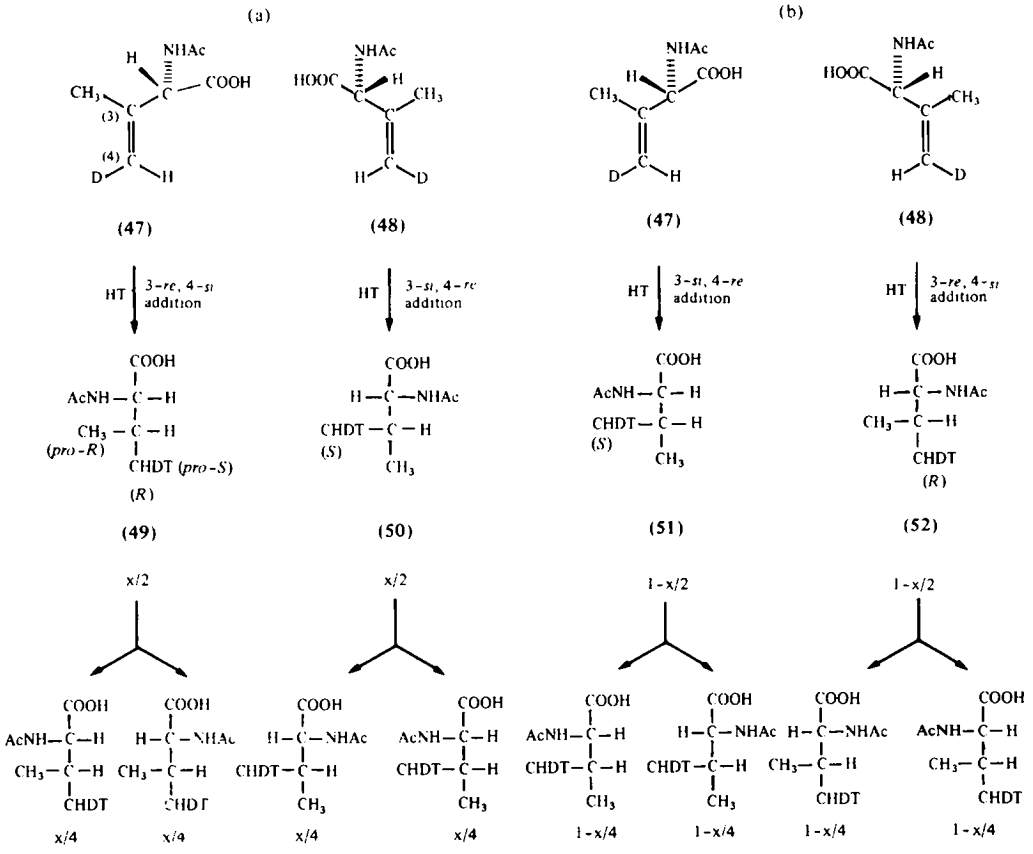
An initial reduction was carried out with a mixture of HT and H_2, produced by equilibrating H_2 gas and carrier-free T_2 gas (1:7) so as to give a product which contained essentially no doubly-labelled species.

The ^1H NMR spectrum (Fig. 1b) of the (*R*,*S*)-[4-^{3}H,^{2}H]valine produced (after hydrolysis of the N-acetyl derivative) indicated a high degree of asymmetric induction in the reduction. The integrated area of the upfield Me doublet, corresponding to the 4-*pro*-*S* Me group of species **49** (magnetically equivalent to the 4-*pro*-*R* Me group of species **50**) was only two thirds that of the downfield doublet, indicating that of the two modes of addition of hydrogen that to the 3-*re*,4-*si* face of the double bond in the 2*S* component (**47**) (isoenergetic with addition to the 3-*si*,4-*re* face of the 2*R*-component **48**, Scheme 12a) was preferred almost exclusively to the alternative mode of addition (Scheme 12b). Exclusive formation of the enantiomer pair [**49** + **50**] would have led to relative areas of 2:3 for the integrations of the upfield and downfield signals due to the Me groups.

This interpretation was supported by the ^3H NMR spectrum (Fig. 1a), which shows a correspondingly large signal due to the tritium in the 4-*pro*-*S* and 4-*pro*-*R* Me groups of species (**49** and **50**) respectively and a much smaller signal due to the tritium in the 4-*pro*-*R* and 4-*pro*-*S* Me groups of species **50** and **51** respectively. The relative areas of the signals (5:95) indicate the degree of asymmetric induction that occurred.

Subsequent experiments were carried out using carrier-free T_2 gas. Typically, from 10 Ci of T_2, 3–5 Ci of valine could be recovered after hydrolysis of the N-acetyl derivative.

A paradoxical situation now arose, created by the unexpectedly high degree of asymmetric induction in



Scheme 12.

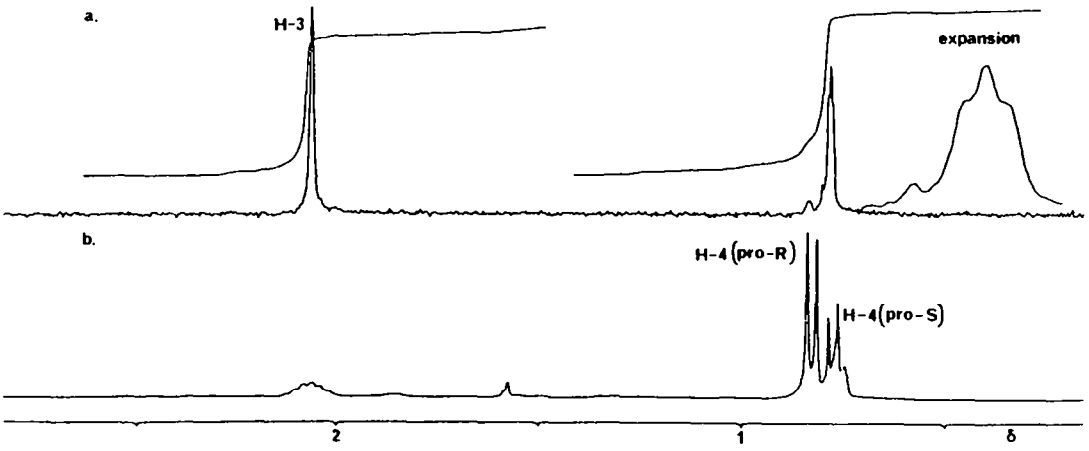


Fig. 1a. ^{3}H NMR spectrum (320 MHz, broad band proton decoupled) and b. ^{1}H NMR spectrum (300 MHz) of (RS)-valine obtained by hydrogenation of (*E*)-[4-^{2}H]-2-acetamido-3-methyl-3-butenoic acid (41) with HT/H_2 followed by hydrolysis.

the hydrogenation of N-acetylisodehydrovaline. This turns out to be disadvantageous in experiments in which use is to be made of the linked stereochemistry of pro, prochiral and prochiral centres and leads to the conclusion that the products should be *racemised* in order to improve the efficiency of the biological experiment! The advantage of employing the linked stereochemistry of *prochiral* and *chiral* centres in a biosynthetic investigation is paid for in the rejection of one stereoisomer by the biological system (*cf* the experiment illustrated in Scheme 5). In such experiments, half of the labelled precursor plays no part in the experiment and is wasted. With stereochemically linked *pro, prochiral* and *prochiral* centres, both isotopically labelled forms of the system are accepted. Provided that the pro, prochiral centres are converted into prochiral centres that can be separately analysed (by, for example, the ^{3}H NMR method outlined above), both isotopically stereoisomeric forms of the precursor can give information in a single experiment. However, with the valine produced by the route of Scheme 12, the additional chiral centre at C-2 perturbs the distribution of label in the experimentally useful L-isomers, which are used much more efficiently than the D-isomers for penicillin production. Thus, considering the L-isomers only, species **49** with a labelled *pro-S* Me group greatly predominates over species **51** with a labelled *pro-R* Me group. If the mixture [**49** + **50** + **51** + **52**] were to be converted biosynthetically into cephalosporin C, C-3′ would be labelled much more heavily than C-2 (*cf* Scheme 8). If the labelling of both *pro-R* and *pro-S* Me groups is to be equalised so as to make more certain that useful information can be obtained relating both to C-2 and to C-3′ in cephalosporin C (**28**), each of the derivatives **49** to **52** must be racemised. In this way, a mixture is obtained in which the L-(2*S*) component is equally labelled in both the *pro-R* and *pro-S* Me groups (Scheme 12).

Racemisation of the N-acetylated product of the reduction of the N-acetylisodehydrovalines was readily accomplished by brief boiling in Ac_2O–AcOH. The ^{3}H NMR spectrum of the valine obtained by hydrolysis of the N-acetylvalines following such a treatment is shown in Fig. 2. The Me signals are now of equal intensity. They consist of an apparent doublet (the ^{2}H splitting is not resolved in this spectrum), superimposed on an apparent singlet which coincided with the downfield component of the doublet. The singlet arose from monotritiated species generated by exchange with the ethanol co-solvent during hydrogenation. Each doublet exhibits an upfield shift of 0.013 ppm. A similar pattern is seen in the signal due to the T at C-3. The relative

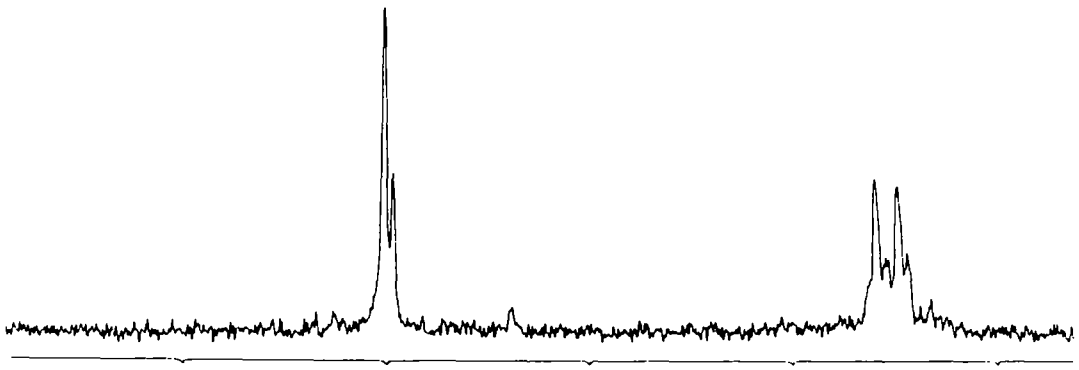


Fig. 2. ^{3}H NMR spectrum (320 MHz, broad band proton decoupled) of (RS)-valine obtained by hydrogenation of (*Z*)-[4-^{2}H]-2-acetamido-3-methyl-3-butenoic acid (41a) with T_2, followed by racemisation and hydrolysis.

intensities of the doublets and corresponding singlets indicate that the product in the experiment illustrated consisted of approx. 15% monotritiated and 85% ditritiated species.

If only one centre were of interest in the product of a biological experiment with chiral-methyl valine, it would be advantageous to resolve the isodehydrovaline before hydrogenation. The L-(2*S*)-N-acetylisodehydrovaline would then give a product with the all-L configuration labelled predominantly in the *pro-S* Me group. Similar hydrogenation of the D-isomer followed by racemisation would give a product in which the L-component would be labelled predominantly in the *pro-R* Me group. The latter conclusion can be verified by tracing the fate of the D-component in Scheme 12.

The methods described in this paper have led to the ready availability of stereospecifically labelled valines of high specific radioactivity, which can be used to investigate the stereochemistry of changes taking place at the Me groups in biological reactions of valine. By the application of the principle of configurationally linked pro, prochiral and prochiral centres it is possible to produce the precursors required for such investigations without recourse, at any stage, to a resolution step, chemical or enzymatic, and to obtain, in principle, from a single experiment, information relating to the fate of two prochiral centres during a biological transformation. Experiments are currently underway to test these ideas in an investigation of the stereochemistry of the conversion of "chiral methyl valine" into cephalosporin C.

As an addendum, it should be noted that a pro, prochiral centre may be configurationally linked to a *chiral* centre. Selection for a given configuration of the chiral centre would lead to concomitant selection of a given configuration at the pro, prochiral centre. However, in such an experiment, the isomer with the enantiomeric configuration would not be utilised. An example of such a system is the chiral methyl lactic acid synthesised by Fryzuk and Bosnich.[21]

Table 1. Stereochemistry of the reductoisomerase reaction (*cf* Scheme 1)

	Valine pathway	Isoleucine pathway
1. Configuration of the substrate (1)	S	S
2. Configuration of the intermediate (2)	3-R[a]	3-R
3. Configuration of the product (3)	2R,3R[a]	2R,3R
4. Stereochemistry with respect to the migrating centre in step iia	?	Retention

[a] Giving higher priority in applying the sequence rules to the migrating methyl group (equivalent to the ethyl group of the isoleucine pathway).

Table 2. 400 MHz NMR Spectra of 2-acetamido-3-methyl-3-butenoic acid and of the corresponding (*E*)-[4-^{2}H]- (41) and (*Z*)-[4-^{2}H]- (41a) acids

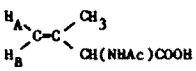
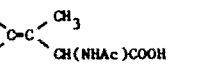
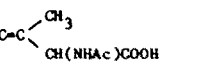
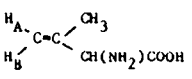
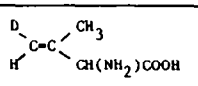
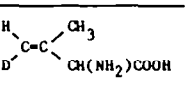
			
NH	8.24 (d, J 7.9 Hz)	8.25 (d, J 8.0 Hz)	8.25 (d, J 7.9 Hz)
H_A	4.95 (m)	-	4.94 (q, J 1.5 Hz)
H_B	4.98 (broad s)	4.96 (broad s)	-
CH	4.75 (d, J 8.0 Hz)	4.75 (d, J 8.0 Hz)	4.75 (d, J 8.0 Hz)
CH_3CO	1.87	1.87	1.87
$CH_3C=$	1.74 (broad s)	1.74 (broad s)	1.74 (d, J 1.5 Hz)

Table 3. NMR spectra of 2-amino-3-methyl-3-butenoic acid and of the corresponding (*E*)-[4-^{2}H]- (**40**) and (*Z*)-[4-^{2}H]- (**40a**) acids

			
H_A	5.49	-	5.50 (q, J 1.5 Hz)
H_B	5.43	5.44	-
CH	4.84	4.84	4.85
CH_3	2.00	2.00	2.00 (d, J 1.5 Hz)

Solvent: trifluoroacetic acid. All signals apparent singlets, unless otherwise stated.

EXPERIMENTAL

All m.ps are corrected. NMR spectra at 100 MHz were determined with a JEOL MH 100 spectrometer, at 300 MHz with a Brucker WH-300 spectrometer and at 400 with a Brucker WH-400 spectrometer. Unless otherwise stated, all NMR data refer to spectra obtained at 100 MHz and for soln in $CDCl_3$. Optical rotations were determined with an NPL Automatic Polarimeter Model 243 (Thorn Automation, Nottingham, England). Bulb-tube distillations were carried out with a Bulb-tube oven Model GKR 50 (Buchi AG, Flawil, Switzerland). Gas chromatography was carried out using Model 104 or GCD gas chromatographs (Pye Ltd., Cambridge, England). The following columns and conditions were used: system 1) 3% OV1 on Gas Chrom Q, 60–80 mesh at 90°; system 2) as (1) but at 80°; system 3) 6% Carbowax-terephthalic acid on Gas Chrom Q, 100–120 mesh at 80°; system 4) as (3) but at 150°. D_2O was purchased from B.D.H. Ltd, Poole, Dorset, England and tritium gas from the Radiochemical Centre, Amersham, Bucks, England. Paper chromatography of amino acids was carried out using Whatman No. 1 paper in the solvent system n-BuOH : AcOH : H_2O (37 : 9 : 25). TLC of carboxylic acids was carried out using Kieselgel PF-254 plates in the solvent system EtOAc : CCl_4 (1 : 3).

(E)-[3-^{2}H]-3-*Bromo*-2-*methyl*-2-*propenoic acid* (**42**). Compound **31** (41.4 g, 0.25 mol) in THF (500 ml, dried over LAH) was stirred and treated dropwise at −78° with a soln of BuLi in ether (1.5 M, 329 ml, 0.5 mol), at such a rate that the temp remained below −60°. The mixture was stirred for a further 30 min at −78°, D_2O (10 ml, 99.7 at.%) was added, the mixture was allowed to warm to room temp and evaporated to dryness. The residue was dissolved in the minimum volume of water, the soln was acidified (Congo red), extracted with CH_2Cl_2 (2 × 400 ml), dried ($MgSO_4$) and evaporated. The product was crystallised (light petroleum b.p. 60–80°) to give **42** (39.3 g, 95%) m.p. 64.5–65.5° (lit. m.p. for material of normal isotopic composition: 64–65°.[22] δ 2.02 (3H, s, CH_3); (for material of normal isotopic composition): 2.02 (3H, s), 7.70 (1H, s HC=). The acid (200 mg) was recycled through the above procedure and the final quenching was carried out with H_2O to give (*E*)-3-Bromo-2-methyl-2-propenoic acid, identical (m.p., NMR) with authentic material.

(Z)-[3-^{2}H]-2-*Methyl*-2-*propenoic acid* (**32a**). Compound **42** (10 g, 0.06 mol) in freshly distilled THF (70 ml) was treated with a soln of NaOH (2.38 g) in water (20 ml). The mixture was coooled to 0°, sodium amalgam (170 g, 2.5% w/w, 0.18 mol) was added. The mixture was stirred for 15 min, and allowed to warm to room temp. The course of the reaction was followed by TLC and by GLC (system 4) and stopped before the proportion of saturated product rose above 20%. (The time for complete reaction depended on the rate of stirring.) On completion of the reaction, the soln was separated from the Hg by decantation. The soln was evaporated to dryness at 40–50°, the residue was dissolved in the minimum quantity water, treated with hydroquinone (20 mg) and diethyl ether (40 ml) and the soln was stirred and cooled to 0°. The aqueous layer was acidified (Congo red) with conc HCl and extracted with diethyl ether (2 × 200 ml). The combined ethereal solns were dried (Na_2SO_4) and evaporated. The residue was distilled (38°, 1 mmHg) into a flask containing hydroquinone (20 mg). Typically a crude yield of 60% was obtained as a mixture of **32a** (80%) and the saturated acid $CH_2DCH(CH_3)COOH$ (20%) (GLC, system 4). δ 1.9 (3H, d, J 1.8 Hz, CH_3). 5.62 (1H, q, J 1.8 Hz, HC–). (major product).

(Z)-[3-^{2}H]-2-*Methyl*-2-*propenol* (**33a**). A soln of (*Z*)-[3-^{2}H]-2-methyl-2-propenoic acid (14.2 g) in diethyl ether (40 ml) was added dropwise to a stirred suspension of LAH (7.2 g) in diethyl ether (200 ml) containing hydroquinone (30 mg) at −10°. The mixture was stirred for a further 20 min and treated slowly with ice-water (40 ml) with stirring. The ethereal soln was dried (Na_2SO_4) and evaporated. The product (75%) was shown (GLC (system 3), NMR) to consist of a mixture of **33a** (69%) and the corresponding saturated alcohol $CH_2DCH(CH_3)CH_2OH$ (31%). δ 1.74 (3H, m, CH_3), 4.01 (2H, s, CH_2), 4.83 (1H, m, HC=); for material of normal isotopic composition: 1.71 (3H, m, CH_3), 3.98 (2H, s, CH_2), 4.82, 4.95 (each 1H, m, HC=).

(Z)-[3-^{2}H]-2-*Methyl*-2-*propen*-1-*yl* p-*toluenesulphonate* (**34a**). A soln of **33a** (6.07 g) in dry CH_2Cl_2 (100 ml) was added dropwise to a soln of *p*-toluenesulphonyl chloride (11.87 g) and Et_3N (18 ml) in dry CH_2Cl_2 (60 ml) at 0°. The mixture was stirred until reaction was complete (as judged by the disappearance of the ^{1}H NMR signal of the CH_2 group of **33a**). The mixture was washed with water, HCl (2 M), K_2CO_3 aq (2 M) and water, and dried ($MgSO_4$). The solvent was removed under reduced (1 mmHg) pressure at room temp to give **34a** (9.43 g, 50%) containing 31% of the saturated compound $CH_2DCH(CH_3)CH_2OTs$. δ 1.62 (3H, s, CH_3), 2.36 (3H, s, CH_3Ar), 4.32 (2H, s, CH_2), 4.81 (1H, broad s, CHD=), 7.25, 7.73 (each 2H, d, J 9 Hz, Ar–H); (*E*)-isomer (**34**): 1.65 (3H, s, CH_3), 2.38 (3H, s, $ArCH_3$), 4.31 (2H, s, CH_2), 4.84 (1H, s, HC=), 7.14, 7.58 (each 2H, d, J 8 Hz, Ar–H); for material of normal isotopic composition: 1.64 (3H, s, CH_3), 2.36 (3H, s, $ArCH_3$), 4.29 (2H, s, CH_2), 4.79, 4.82 (each 1H, s, HC=), 7.10, 7.53 (each 2H, d, J 8 Hz, Ar–H).

(Z)-[4-^{2}H]-3-*Methyl*-3-*butenenitrile* (**35a**). A soln of 18-crown-6 (8.31 g) and KCN (2.05 g) in MeOH (250 ml) was evaporated to dryness and the residue was dried at room temp for 2 hr at 1 mmHg. To the resulting solid a soln of **34a** (9.43 g) in diethyl ether (250 ml) was added. The soln was stirred and the reaction was followed by GLC (system

2) until the ratio of **35a** to the conjugated isomer $CH_2D(CH_3)C{=}CHCN$ was at a maximum (*ca* 16 h). The mixture was filtered, washed with a sat soln of NaCl aq (70 ml), dried ($MgSO_4$) and evaporated. The residual oil was distilled in a bulb-tube apparatus at 100°, 1 mmHg, to give 0.91 g of a mixture of **35a** (79%) and the conjugated isomer $CH_2D(CH_3)C{=}CHCN$ (21%). δ 1.78 (3H, s, CH_3), 3.00 (2H, s, CH_2), 4.93 (1H, m, CHD) (major product); (*E*)-isomer (**35**): 1.85 (3H, s, CH_3), 3.05 (2H, s, CH_2), 5.1 (1H, s, HC=); for material of normal isotopic composition: 1.85 (3H, s, CH_3), 3.06 (2H, s, CH_2), 5.0, 5.09 (each 1H, s, HC=).

Methyl 1-((Z)-[4-^{2}H]-3-*methyl*-3-*butenyl*)-*imidate hydrochloride* (**36a**). **35a** (0.91 g) in dry MeOH (0.45 ml) was cooled in an ice-salt bath. Dry HCl was passed into the soln for 1 hr. The reaction vessel was sealed and kept at −10° overnight. Dry ether (10 ml) was added to the stirred soln. The ppt was washed with dry ether and dried *in vacuo* over silica gel to give **36a** as a hygroscopic crystalline solid (0.85 g, 51%), δ (CD_3SOCD_3) 1.69 (3H, s, CH_3C–), 3.34 (2H, s, CH_2), 4.01 (3H, s, OCH_3), 4.89 (1H, s, HC=), 11.9 (2H, broad s, $^+NH_2$); (*E*)-isomer (**36**): 1.78 (3H, s, CH_3), 3.47 (2H, s, CH_2), 4.14 (3H, s, OCH_3), 4.98 (1H, s, HC=); for material of normal isotopic composition (400 MHz): 1.75 (3H, s, CH_3), 3.37 (2H, s, CH_2), 4.09 (3H, s, OCH_3), 4.93, 5.00 (each 1H, s, HC=).

Methyl (Z)-[4-^{2}H]-*N*-*chloro*-3-*methyl*-3-*butenyl imidate* (**38a**). Compound **37a** (525 mg) was treated dropwise at 0° with NaOCl aq (10–14%, 24 ml). The mixture was stirred at 0° for 1 hr and extracted with light petroleum (b.p. 40–60°, 2 × 50 ml), dried ($MgSO_4$) and evaporated to give **38a** as a colourless liquid (386 mg, 73%). δ 1.78 (3H, s, CH_3C), 3.29 (2H, s, CH_2), 3.78 (3H, s, OCH_3), 4.90 (1H, broad s, HC=). (*E*)-isomer (**38**): 1.80 (3H, s, CH_3C), 3.30 (2H, S CH_2), 3.75 (3H, s OCH_3), 4.90 (1H, broad s, HC=).

(Z)-[4-^{2}H]-2-*Amino*-3-*methyl*-3-*butenoic acid* (**40a**). A soln of NaOMe (1.35 M, 2 ml) in MeOH was added to a stirred soln of **38a** (358 mg) in light petroleum (b.p. 40–60°, 3 ml). The mixture was stirred for 2 days, dil HCl (2 M, 3 ml) was added and the soln was boiled under reflux for 2 hr. The soln was cooled and evaporated to dryness under reduced pressure at 40–50°. The residue was dissolved in water (5 ml) and passed down an ion exchange column (Dowex 50W × 8, H^- form, 5 g). The column was washed with deionised water until the eluate was neutral and the product was eluted with dilute ammonia (2 M) until the eluate no longer gave a reaction with ninhydrin. The eluate was evaporated under reduced pressure to give **40a**, (230 mg, 83%). (For the NMR data, see Table 3). M.p. for material of normal isotopic composition 207–208.5° (dec) (lit.[23] m.p. 212–215° (dec, uncorrected)).

(Z)-[4-^{2}H]-2-*Amino*-3-*methyl*-3-*butenoic acid* (**40a**). A To a vigorously stirred soln of **40a** (230 mg) and K_2CO_3 (1.58 g) in water (25 ml) at 0° was added dropwise a soln of Ac_2O (1.12 ml) in ether (11 ml). The mixture was stirred for a further 45 min, allowed to warm to room temp and concentrated to *ca* 10 ml under reduced pressure. The soln was passed through a column of Dowex 50W × 8 ion exchange resin (H^+ form, 10 g) and the column was eluted with water. The eluate was evaporated under reduced pressure and the product was dried overnight (75°, 1 mmHg) to give **41a**, (180 mg) (57%). For the NMR data, see Table 2. In trial experiments, the unlabelled compound, prepared as above, was recrystallised from MeOH–EtOAc, m.p. 184–187°. (Found C, 53.2; H, 7.1; N, 8.9. $C_7H_{11}O_3N$ requires: C, 53.5; H, 7.05; N, 8.9%.) (For the NMR data, see Table 2.)

Enzymatic hydrolysis of (Z)–(R,S)-(4-^{2}H]-2-*acetamido*-3-*methyl*-3-*butenoic acid* (**41a**). Compound **41a** (100 mg) in distilled water (4 ml) at pH 7.2 (pH adjusted with LiOH (0.3 M)) was treated with acylase I (1 mg) and the mixture was incubated at 37° for 23 hr. The soln was applied to a column of Dowex 50W × 8 ion exchange resin (H^+ form, 5 g), which was eluted with water. The column was eluted with aqueous ammonia (2 M) and the eluate was evaporated to give (*Z*)-(*S*)-[4-^{2}H]-2-amino-3-methyl-3-butenoic acid (as **40a**) (29 mg), m.p. 207–208°. (dec). (Lit.[23] m.p. for material of normal isotopic composition 212-215° (dec, uncorrected)). The initial eluate was evaporated under reduced pressure to give a residue (64 mg) which was again hydrolysed as before. From the second treatment, a further quantity of the amino acid (6 mg) was obtained to give a total of 35 mg. From the initial eluate the (*Z*)-(*R*)-[4-^{2}H]-2-acetamido-3-methyl-3-butenoic acid (46.3 mg) was obtained. The (*S*)-component was reacetylated as before to give the (*Z*)-(*S*)-[4-^{2}H]-2-acetamido-3-methyl-3-butenoic acid (34 mg, 85%). $[\alpha]_D = 97.8 \pm 2°$ (c, 2, EtOH).

Hydrogenation of (Z)-[4-^{2}]-2-*acetamido*-3-*methyl*-3-*butenoic acid* (**41a**). In a typical procedure, a soln of the N-acetylisodehydrovaline (20 mg) in benzene (6 ml) and dry EtOH (1.5 ml) was hydrogenated with T_2 gas (10 Ci, > 90 atm%, 4 ml) in the presence of Wilkinson's catalyst (4 mg). Following the reduction with T_2, the soln was hydrogenated with H_2, to ensure complete chemical reduction. The residue was dissolved in EtOH (1 ml), HCl (4 M, 10 ml) was added, the mixture was boiled under reflux for 2 hr, and evaporated to dryness under reduced pressure. The residue was dissolved in the minimum quantity of aqueous EtOH and applied to a column of Dowex 50W × 8 ion exchange resin (H^- form, 10 g). The column was eluted with water followed by aqueous ammonia (2 M) until the eluate no longer gave a positive reaction with ninhydrin. The product was shown to be chemically and radiochemically pure (> 98%) by paper chromatography followed by strip counting of the paper chromatogram in the liquid scintillation counter. Dilution analysis against authentic DL-valine indicated a radiochemical purity of 104 ± 5%. (N.b. during these studies it was discovered that carrier free T_2 gas when stored for several months in glass vials underwent radiolysis with adsorbtion of the radiolysis products on to the glass, generating a partial vacuum inside the vials and leading to substantial losses of the tritium gas. The glass of the vials became highly radioactive. To avoid this problem, the gas was subsequently used as soon as practicable after delivery.)

The first reduction with HT was carried out by the Tritium Labelling Service of the Radiochemical Centre, Forest Farm Industrial Estate, Whitchurch, Cardiff, Wales. For this reduction, carrier free T_2 (10 Ci) and H_2 gas (1 : 7, v/v) was maintained over the catalyst (280 mg) dissolved in benzene (70 ml) and EtOH (32 ml) for 1 hr before the substrate ((41), 178 mg) was added. The mixture was hydrogenated for a further 3.5 hr before work-up.

Racemisation of N-*acetylvaline*. In a typical experiment, the product from an hydrogenation in the presence of Wilkinson's catalyst (above) was boiled under reflux with Ac_2O (1 ml) and water (0.1 ml) for 15 min. The soln was treated with HCl (4 M, 10 ml) and the N-acetyl derivative was hydrolysed to valine as before.

(Z)-[3-^{2}H]-2-*Methyl*-1-*prop*-2-*enol* (**33a**) *from propargyl alcohol*. The following modification of the published procedure[18] was used. To a soln of propargyl alcohol (5 g) in dry THF (150 ml), cuprous iodide (1.9 g) was added. The mixture was stirred, cooled to −78° and treated with MeMgBr prepared from MeBr (15 ml) and Mg (6.5 g) in THF (250 ml). The rate of addition was controlled so that the temp of the mixture remained below −60°. The mixture was allowed to warm to room temp overnight, it was treated with D_2O (10 ml), added to diethyl ether (1 : 1) and the aqueous phase was brought to pH 7 (dil HCl). The aqueous layer was removed, the ethereal fraction was washed with dil HCl, dried (Na_2CO_3 + $MgSO_4$), and the ether and THF were removed by careful distillation, finally under reduced pressure at 10°. The yield obtained varied between 70 and 90%, purity >95% by GLC (system 3). δ 1.74 (3H, d, J 1.5 Hz, CH_3), 2.7 (1H, broad s, OH), 4.0 (2H, s, CH_2), 4.83 (1H, q, J 1.5 Hz, CHD). The NMR spectrum indicated the presence of <6% of the non-deuterated compound.

(E)-[4-^{2}H]-2-*Amino*-3-*methyl*-3-*propenoic acid* (**40**). This

was synthesised by the route above for the (*Z*)- **40a** with the following modifications: (E)-[3-^{2}H]-2-*Methyl*-2-*propenoic acid* (**32**). In a typical procedure, to a soln of **31** (10 g, 60.6 mmol) in THF (70 ml), NaH (1.5 g, 62.5 mmol) was added. The mixture was stirred until no more white ppt appeared. D_2O (20 ml) was added dropwise. The clear soln was cooled to 0°, treated with NaHg (2.5%, 170 g, 3.1 equiv). The mixture was allowed to warm up to room temp and was stirred until no more starting material was detectable by TLC (20–40 hr, depending on the rate of stirring). If a ppt formed, it was brought into soln by adding a few drops of D_2O. The soln was separated from the Hg by decantation, concentrated under reduced pressure to approx. 20 ml, acidified (Congo red) with dil H_2SO_4 and extracted with ether (3 × 100 ml). The ethereal extracts were dried (Na_2SO_4), the ether was evaporated and the product was distilled (38°, 1 mmHg) into a flask containing hydroquinone (20 mg) to give **32**, 3.89 g (74%) contaminated with 8% of the saturated acid $CH_2DCH(CH_3)COOH$. δ 1.93 (3H, s, CH_3), 6.08 (1H, s, CHD), 10.6 (1H, broad s, COOH). (E)-[4-^{2}H]-2-*Amino*-3-*methyl*-3-*propenoic acid* (**40**). A stirred soln of the (*E*)-isomer **38** of the N-chloroimidate (358 mg) in light petroleum was treated dropwise with a soln of NaOMe in MeOH (1.95 ml, 30.6 mg Na ml^{-1}). The mixture was stirred at room temp until starting material could no longer be detected by GLC (system 1) (approx. 18 hr). The mixture was treated with HCl (2 M, 3.1 ml) and the solvents were evaporated under reduced pressure. The residue was extracted with hot MeOH (2 × 10 ml) and the extracts were evaporated to dryness to give the hydrochloride of the methyl ester of **40** δ (CD_3SOCD_3) 1.75 (3H, s, CH_3), 3.69 (3H, s, OCH_3), 4.51 (1H, s, CH), 5.17 (1H, s, HC=), 8.94 (3H, broad s, NH_3^+); for material of normal isotopic composition: 1.83 (3H, s, CH_3), 3.80 (3H, s, OCH_3), 4.62 (1H, broad s, CH), 5.28, 5.34 (each 1H, s, HC=), 9.11 (3H, broad s, NH_3^+). The hydrochloride (440 mg) was boiled under reflux in HCl (1 M, 8.8 ml) for 4.5 hr. The cooled soln was applied to a column of Dowex 50W × 8 ion exchange resin (H^+ form, 24 ml wet volume). The column was eluted with water (200 ml) followed by aqueous ammonia (2 M, 140 ml). The ammoniacal eluate was treated with activated charcoal, filtered and evaporated under reduced pressure to give **40**, 210 mg. For the NMR spectrum, see Table 3. NMR data for the remaining intermediates in the synthesis of the (*E*)- **40** are included with the data for the corresponding (*Z*)-isomers, above.

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