Pyrrolizidine Alkaloid Biosynthesis: Stereochemistry of the Formation of Isoleucine in *Senecio* Species and of its Conversion into Necic Acids †

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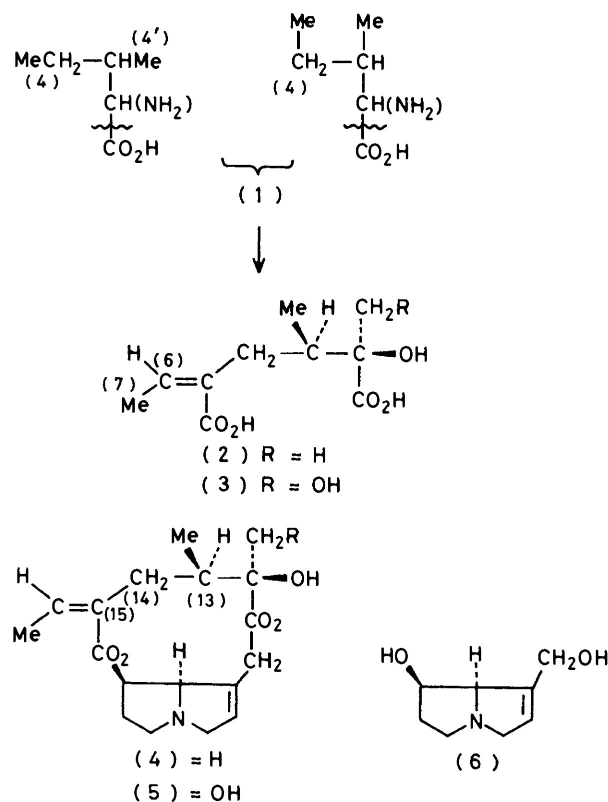
Incorporation experiments with isoleucine (1) and 2-aminobutanoic acid [as (20)], stereospecifically labelled with tritium at C-4 and C-3 respectively, have shown that the ethyl migration step during biosynthesis of isoleucine (1) in *Senecio* species takes place with retention of configuration. It was also shown that coupling of two molecules of isoleucine (1) to give the ten-carbon necic acids of the senecic (2) type takes place with loss of the C-4 *pro-S* and retention of the C-4 *pro-R* hydrogen from both components. A new synthesis of βγ-unsaturated amino-acids has been developed which provides for control of the configuration of the olefinic system.

A major group of ten-carbon necic acids of the pyrrolizidine alkaloids belong to the class exemplified by senecic (2) and isatinecic (3) acids. These acids are the esterifying acids of the alkaloids senecionine (4) and retrorsine (5), respectively, and are formed in nature from two molecules of L-isoleucine (1) with loss of the carboxyl-groups (Scheme 1).[1] A crucial step in the construction of the necic acid skeleton is the formation of the C-(13)–C-(14) bond between the formally unactivated C-4, C-4′ positions of the precursor molecules of L-isoleucine. In order to investigate the mechanism of this novel process, experiments have been carried out to define the level of oxidation to which the C-4 methylene carbon atom in L-isoleucine (1) is raised during its transformation into C-13 and C-14 of the alkaloids (4) and (5). The stereochemistry of the processes taking place at C-4 in L-isoleucine (1) have also been investigated.

Following an established procedure,[1a] [4-$^3H_2$]isoleucine (7) was synthesised as shown in Scheme 2. The product consisted of a mixture of (2*RS*)-isoleucine and (2*RS*)-alloisoleucine in the ratio 46 : 54 by amino-acid analysis. It has been shown that of the four isomers of isoleucine, only (*S*)-isoleucine (L-isoleucine) is an effective precursor of the necic acids.[2] Accordingly, the tritiated mixture was administered together with (*S*)-[U-^{14}C]isoleucine to *Senecio isatideus* plants growing in hydroponic solution. Following isolation and recrystallisation to constant activity, the product alkaloid retrorsine (5) was hydrolysed to give isatinecic acid (3) and the necine base retronecine (6) (Scheme 1). The results of this experiment (Table) show first that isoleucine (1) was a specific precursor of isatinecic acid (3) in agreement with earlier experiments,[1] and that half of the tritium of the administered (2*S*)-[4-$^3H_2$]isoleucine had been retained. This result shows that both the hydrogen atom at C-13 and the vinyl hydrogen atom of the C-15 ethylidene group in retrorsine (5) are derived from the C-4 methylene hydrogen atoms of (2*S*)-isoleucine [as (1)]. These impose a limit on the change in oxidation state at C-4 in isoleucine (1) during conversion into both C-13 and the vinyl carbon atom of the C-15 side-chain of retrorsine (5) to a two-electron conversion to the alkene or carbinol level.

In order to explore further this transformation, (2*RS*,4*RS*)- and (2*RS*,4*S*)-[3,4-$^3H_2$]isoleucine [(11) + (12) and (11), respectively] were synthesised as shown in Scheme 3. For the hydrogenation of the *N*-acetylisodehydroisoleucine (10), di-imide reduction was preferred over heterogeneous catalytic methods, in order to be certain of regiospecificity and clean



Scheme 1

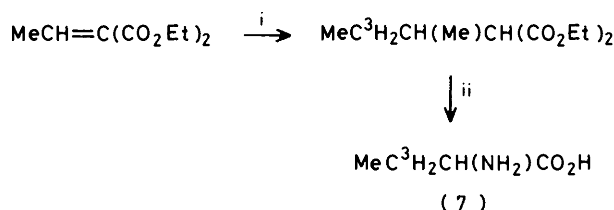
cis-stereospecificity in the reaction.[3] To confirm the regio- and stereo-specificity of this reduction, the reaction was repeated with di-deuteriodi-imide. The product, after hydrolysis, was purified by ion-exchange chromatography. In the 220 MHz n.m.r. spectrum of the (2*RS*)-isoleucine thus obtained, the downfield component of the two multiplets due to the C-4 protons integrated for 0.47 ± 0.03 protons and the upfield component integrated for 1.08 ± 0.05 protons, using the signals due to the methyl protons as an internal standard. Correspondingly, the integration of the signal due to the proton at C-3 corresponded to 0.42 ± 0.05 protons. This evidence confirms the stereospecificity of the reduction and makes possible the assignment of the n.m.r. signals due to the C-4 protons: the downfield multiplet corresponds to H-4(*S*) and the upfield multiplet to H-4(*R*) [*cf.* (11), Scheme 3].

† Preliminary report: R. Cahill, D. H. G. Crout, M. B. Mitchell, and U. S. Muller, *J. Chem. Soc., Chem. Commun.*, 1980, 419.

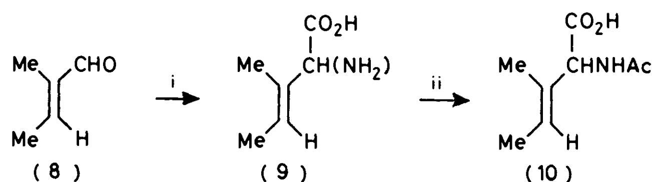
Table. Incorporation of 2-aminobutanoic acid [as (20)] and isoleucine (1) into alkaloids (4) and (5) in *Senecio* species

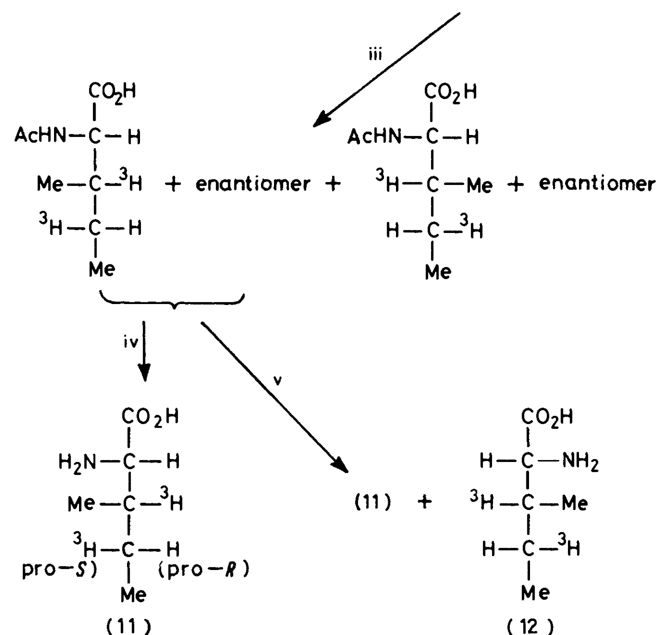
Precursor	$^3H/^{14}C$ ratio				% Retention of tritium			% ^{14}C Activity		
2-Aminobutanoic acid	Precursor	Alkaloid (4) or (5)	Necic acid [a]	C-6 7 unit of necic acid [b]	Alkaloid	Necic acid	C-6, 7 unit of necic acid	Necic acid	C-6, 7 unit of necic acid	% Incorporation (^{14}C)
(2*RS*,3*S*)-[3-$^3H_1$,3-^{14}C] [c]	2.85	0.18	—	—	6.5	—	—	—	—	0.015
(2*RS*)-[3-$^3H_2$,3-^{14}C] [d]	8.23	3.85	3.78	4.59	47	—	56	98	43	3.4
(2*S*)-[3-$^3H_2$,3-^{14}C] [d]	7.9	3.63	3.72	4.01	46	47.1	50.8	95	47	3.6
(2*R*)-[3-$^3H_2$,3-^{14}C] [d]	7.97	3.77	3.88	4.01	47.4	48.7	50.3	94	51	2.1
Isoleucine										
(2*S*)-[U-^{14}C] plus:										
(2*RS*,4*RS*)-[3,4-$^3H_2$] [c]	1.44 [e]	0.074	—	—	5.1	(5.1)	—	—	—	0.05
(2*S*,4*S*)-[3,4-$^3H_2$] [c]	1.84 [f]	0.12	—	—	6.5	(6.5)	—	—	—	0.004
(2*S*)-[4-$^3H_2$] [d]	2.36 [g]	1.42	1.60	—	50	56	—	94	—	0.21
(2*S*,4*R*)-[4-$^3H_1$] [d]	2.72 [f]	2.59	—	—	95.2	(95.2)	—	—	—	2.2

[a] After hydrolysis of the alkaloid. [b] Isolated as the dimedone derivative of acetaldehyde after ozonolysis of the necic acid. [c] Incorporation experiments in *S. magnificus*. Alkaloid: senecionine (4); necic acid: senecic acid (2). [d] Incorporation experiments in *S. isatideus*. Alkaloid: retrorsine (5); necic acid: isatinecic acid (3). [e] Corrected for the obligatory loss of ^3H at C-3 in isoleucine (1) and of the carboxy carbon in the 1-(2*S*)-[U-^{14}C]isoleucine and to take into account the specific incorporation of only (2*S*)-isoleucine.[2] [f] Corrected for the loss of the carboxy-carbon in the (2*S*)-[U-^{14}C]isoleucine. [g] Actually fed as a mixture of (2*RS*)isoleucine and (2*RS*)-alloisoleucine. Ratio corrected to take into account the specific incorporation of only (2*S*)-isoleucine into the necic acid.[2]



Scheme 2. *Reagents:* i, MeC^3H_2MgI; ii, OH^--H_2O, HN_3



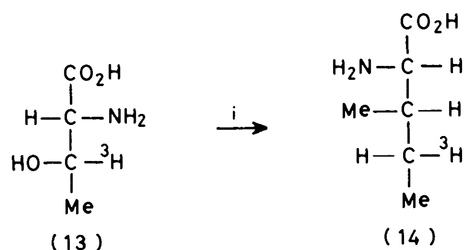


Scheme 3. *Reagents:* i, NH_4CN, H^+-H_2O; ii, Ac_2O; iii, $^3HN{=}N^3H$; iv, Acylase I; v, H^+-H_2O

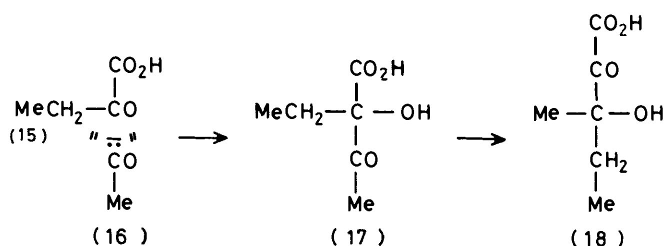
The high protium content at C-3 and C-4 reflects the operation of a substantial deuterium isotope effect during the reduction. If the protium content of the deuteriated di-imide were the same as that of the D_2O used to generate it (99.8%), an apparent deuterium isotope effect of 225 would be indicated. Normal precautions were taken during preparation of the dideuteriodi-imide. However, if, for example, the deuterium content of the generated di-imide fell to 99%, through the intrusion of adventitious water, the calculation would have shown an apparent deuterium isotope effect of 45. Most reports of the reduction of relatively unhindered di-substituted alkenes indicate that no substantial isotope effect is observed.[4] However, in at least one instance of the reduction of an acrylate derivative,[5] a substantial deuterium isotope effect was also observed.[6] There was an apparent tritium isotope effect of 275 for the reduction with tritiated di-imide. However, the validity of this figure again depends on the assumption that the tritiated water used to generate the di-imide was of the activity specified and that the activity was not diluted by adventitious water. Conclusions cannot readily be drawn from a comparison between the apparent deuterium and tritium isotope effects because the deuterium isotope effect reflected the relative rates of reaction of the di- and mono-deuteriated species, whereas the tritium isotope effect reflected the relative rates of reaction of monotritiated di-imide and di-imide of normal isotopic composition.

Thus although their exact magnitude cannot be defined precisely, it is evident that di-imide reduction of the unsaturated amino-acid derivative (10) was associated with substantial deuterium and tritium isotope effects.

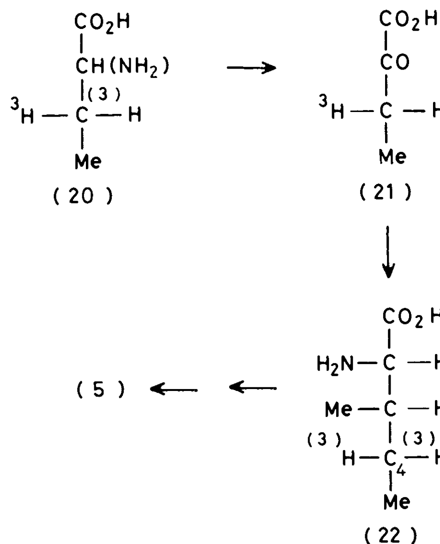
Incorporation of the tritiated amino-acids into senecionine (4) in *Senecio magnificus* proceeded with almost complete loss of the tritium label (Table) showing that during incorporation into the alkaloids it is the 4-*pro-S* hydrogen that is lost during incorporation into both halves of the necic acid. In other



Scheme 4. i *Serratia marcescens* mutant 149 [7]



Scheme 5

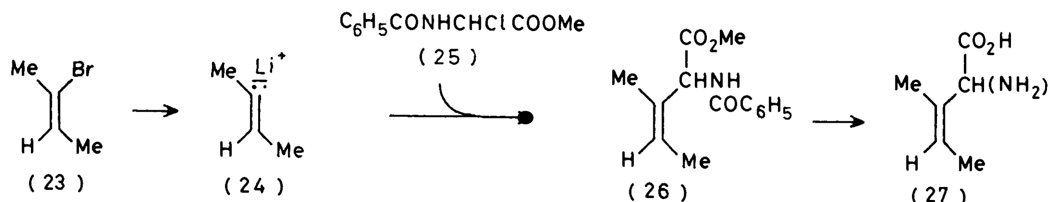


Scheme 6

words, the hydrogen atom at C-3 and the vinyl hydrogen atom of the C-15 ethylidene group in the alkaloid (4) are both derived from the 4-*pro-R* hydrogen of L-isoleucine [*cf.* (11), Scheme 3]. This conclusion was confirmed by an incorporation experiment using (2*S*,4*R*)-[4-^{3}H]isoleucine (14) produced biosynthetically from (2*R*)-[3-^{3}H]threonine (13) using *Serratia marcescens* mutant strain 149 (Scheme 4).[7] An experiment with this labelled amino-acid showed that the 4-*pro-R* hydrogen was completely retained during incorporation into retrorsine (5) (Table), precisely complementing the previous experiments with the 4-*S*-tritiated isoleucines. Further, the results show that formation of the C-13,14 bond in the necic acids [as in (4) and (5)] takes place with overall inversion of configuration at C-4 of the isoleucine molecule that furnishes the right-hand component. This conclusion depends on the assumption that the biosynthetic pathways leading to senecic (2) and isatinecic (3) acids in the closely related species *S. magnificus* and *S. isatideus*, are stereochemically congruent.

The experiments just described provided the analytical procedure necessary for a further investigation into the stereochemistry of the ethyl group migration during the rearrangement catalysed by the enzyme reductoisomerase [2,3-dihydroxy-isovalerate : NAPD$^+$ oxidoreductase (isomerizing), EC 1.1.1.86] of the isoleucine pathway of biosynthesis.[8] This enzyme catalyses the rearrangement of 2-ethyl-2-hydroxy-3-oxobutanoate (17) into 3-hydroxy-3-methyl-2-oxopentanoate (18) and reduction of the latter to 2,3-dihydroxy-3-methylpentanoate (19). The dihydroxy-acid (19) is converted into isoleucine (1) by steps that nowhere affect the transposed ethyl group (Scheme 5). The stereochemistry of this enzymatic reaction in microbial systems has been defined and has been shown to correspond to retention of configuration at the migrating centre during the rearrangement step.[7] Provided that the precursor (17) could be labelled stereospecifically at C-5, its conversion into isatinecic acid (3) would therefore provide a method for the stereochemical analysis of the intermediate isoleucine (Scheme 6), thereby defining the stereochemistry of the ethyl migration step. The immediate precursor of the substrate (17) of the reductoisomerase reaction is 2-oxobutanoate (15), which is formed in a reaction with 'active acetaldehyde' (the adduct of acetaldehyde with thiamine pyrophosphate) catalysed by the enzyme acetohydroxy-acid synthetase [acetolactate pyruvate lyase (carboxylating), EC 4.1.3.18] (Scheme 5). For the investigation in the microbial system, 2-oxobutanoate (15) was generated *in situ* in the micro-organism from stereospecifically labelled 2-aminobutanoic acid.[7] It seemed probable that in the same way stereospecifically labelled 2-aminobutanoate [as (20), Scheme 6] might also furnish 2-oxobutanoate [as (21), Scheme 6] in *Senecio* species through the action of an appropriate aminotransferase or amino-acid oxidase. Thus, if 2-aminobutanoic acid, stereospecifically labelled with tritium in the 3-*pro-S* position (20), were incorporated in the predicted manner into retrorsine (5) in *S. isatideus*, loss of the label would prove retention of configuration at the migrating centre during the rearrangement step, whereas retention of the label would prove inversion of configuration [*cf.* (20) ⟶ (22) ⟶ (5), Scheme 6].

(2*RS*)-2-Amino[3-^{3}H$_2$,3-^{14}C]butanoic acid [as (20)] was synthesised as shown in Scheme 6. The labelled amino-acid was incorporated into retrorsine (5) with very high efficiency (3.4%). Degradation of the alkaloid showed that the carbon-14 activity was entirely confined to the necic acid and that approximately half of the activity was confined to the C-6,7 unit [as in (3)] (Table) as expected for a specific conversion of 2-oxobutanoate (15) into isatinecic acid (3) *via* isoleucine (1). Furthermore, the observed retention of tritium (47% overall, 56% with respect to the C-6,7 unit), was entirely consistent with the results obtained with isoleucine correspondingly labelled at C-4 (Table). When 2-aminobutanoic acid (20), stereospecifically labelled with tritium in the 3-*pro-S* position [7] was administered, in admixture with 3-aminobutanoic acid labelled with ^{14}C at C-3 as internal standard, almost all of the tritium was lost during incorporation into isatinecic acid



Scheme 7

(Table). Since it is the 4-*pro-S* hydrogen that is lost from isoleucine during conversion into the necic acid, this result proves that the 3-*pro-S* hydrogen of 2-aminobutanoate is delivered to the 4-*pro-S* position in isoleucine (Scheme 6) and that the ethyl migration step consequently proceeds with *retention of* configuration at the migrating centre during the reductoisomerase-catalysed step.

The incorporation, with respect to ^{14}C, of 2-aminobutanoic acid was higher (3.4%) than any previously observed for a necic acid precursor. It seemed possible that this high efficiency might have been due, in part, to the utilisation of both enantiomers of the racemic precursor administered. Accordingly, (2*RS*)-amino[3-^{3}H$_2$,3-^{14}C]butanoic acid was converted into its *N*-acetyl derivative and resolved using Acylase I. The 2*S*(L)-component was incorporated into retrorsine with high efficiency (3.6%) as expected and with the same distribution of activities and retention of tritium as for the racemic material. However, an almost identical result was obtained with the 2*R*(D)-component, which was incorporated with only slightly lower (2.1%) efficiency (Table). It was therefore concluded that *S. isatideus* possesses enzymes, either aminotransferases or amino-acid oxidases, that are able to convert both 2*R*- and 2*S*-aminobutanoic acids into 2-oxobutanoate.

2-Aminobutanoic acid has been reported to occur in a number of higher plant species.[9] Of particular interest is the reported occurrence of the 2*R*-isomer in legume seeds [10] and of a 2-oxobutanoate aminotransferase in pea seedlings.[11] These observations suggest that the conversion of (2*S*)-2-aminobutanoate into 2-oxobutanoate in *Senecio* species may not represent an adventitious pathway and that a corresponding conversion of the 2*R*-isomer may be a normal metabolic process.

The conclusion concerning the stereochemistry of the ethyl migration step of the reductoisomerase reaction depended on the calibration experiment in which (2*RS*,4*RS*)-[3,4-^{3}H$_2$]isoleucine [(11) + (12), Scheme 3] was synthesised, and on the assumption of a *cis*-mode of addition of hydrogen to the (*E*)-unsaturated substrate (10). The latter was synthesised from tiglaldehyde (8) by a modified Strecker reaction (Scheme 3). In order to demonstrate that no change of configuration had taken place under the vigorous conditions of the hydrolytic step in the Strecker reaction, the corresponding (*Z*)-isodehydroisoleucine (27) was synthesised. To acquire stereochemical control over the reaction, the configurational stability of vinyl anions was employed. The species required was the vinyl-lithium derivative (24) derived from (*Z*)-2-bromobut-2-ene (23) (Scheme 7). As an electrophilic precursor of the amino-acid moiety, the 2-chlorohippurate ester (25), developed as an electrophilic amino-acid synthon by Ben-Ishai,[12] was used. Condensation of the vinyl-lithium reagent (24) with methyl 2-chlorohippurate (25) followed by deprotection, gave the (*Z*)-isodehydroisoleucine (27). Amino-acid analysis showed that the product contained less than 3% of the isomer produced by the Strecker route from tiglaldehyde. The assignment of the (*E*)-configuration to the latter isomer was thereby confirmed.

The synthesis of βγ-unsaturated amino-acids from 2-chlorohippurate esters represents a new synthetic route to unsaturated amino-acids, the development of which is currently under investigation in this laboratory.

Since the publication of our original communication, the (*Z*)-isodehydroisoleucine (27) has been reported as a constituent of the fern *Coniogramme intermedia*.[13]

Experimental

^{1}H N.m.r. spectra were determined with a JEOL MH 100 spectrometer at 100 MHz or with a Perkin-Elmer R34 spectrometer at 220 MHz by courtesy of the Physico-Chemical Measurements Unit of the S.R.C., Harwell. Amino-acid analyses were carried out using a JEOL JAC 5AH automatic amino-acid analyser. Radioactivity measurements were carried out using a Packard Tri-carb 2002 liquid scintillation counter in either a dioxan-based scintillator (B.D.H. Ltd), Aquasol (N.E.N. Ltd), or NE 260 (Nuclear Enterprises Ltd). Thin layer and preparative thin layer chromatography was carried out using Kieselgel PF$_{254}$ (Merck Ltd) plates in the solvent system butan-1-ol–acetic acid–water (4 : 1 : 1). Paper chromatography was carried out using Whatman No. 1 paper. Amino-acids were detected using ninhydrin spray reagent [0.3% (w/v) in butan-1-ol]. All radiochemicals were purchased from the Radiochemical Centre, Amersham. Acylase I from pig kidney was purchased from Koch-Light Ltd.

Synthesis of (2RS)-[4-^{3}H$_2$]*Isoleucine.—Iodo*[1-^{3}H$_2$]*ethane.* [1-^{3}H$_2$]Ethanol (2.5 mCi, *ca.* 200 mg) was distilled on a vacuum line at 0.01 mmHg, into a reaction flask-condenser unit containing hydriodic acid (55%, *d* 1.7 g cm^{-3}, 18 cm^3). The reaction flask-condenser unit was isolated from the vacuum line and the reaction mixture was boiled under reflux for 2 h. The reaction flask was again connected to the vacuum line and the iodo[1-^{3}H$_2$]ethane was transferred into a receiver cooled in liquid nitrogen *via* trains of dried sodalime and phosphorus pentaoxide; yield 400 mg (52%), pure g.l.c. (15% SE 30 on Chromosorb W, 5 ft × 1/8 in, nitrogen carrier gas, 57 °C). In subsequent experiments, the yield of iodoethane was increased to 75% by increasing the time of boiling under reflux from 2 to 4 h.

Ethyl 2-ethoxycarbonyl-3-methyl[4-^{3}H$_2$]*pentanoate.* Iodo-[1-^{3}H$_2$]ethane (400 mg) in ether (3 cm^3) was added to magnesium turnings (145 mg). When the reaction had started, ether (5 cm^3) was added. When formation of the Grignard reagent was complete, ethyl 2-ethoxycarbonylbut-2-enoate (630 mg) in ether (6 cm^3) was added during 15 min and the mixture was stirred for 1 h. The mixture was treated with hydrochloric acid (1M; 10 cm^3) and stirred until the excess of magnesium had reacted. The ether layer was separated, the aqueous layer was extracted with ether (2 × 10 cm^3), and the combined ethereal extracts were dried (MgSO$_4$) and evaporated to give the crude ethyl 2-ethoxycarbonyl-3-methyl[4-^{3}H$_2$]-pentanoate as a yellow oil (615 mg). This was used in the next stage of the synthesis without further purification.

(2RS)-[4-^{3}H$_2$]*Isoleucine.* The ester (615 mg) was stirred and

heated at 95—100 °C in a solution of potassium hydroxide (698 mg) in water (6.4 cm^3) for 5 h. Water (32 cm^3) was added and the solution was cooled to 0 °C and acidified (Congo red) with conc. hydrochloric acid, care being taken to keep the temperature below 10 °C during acidification. The solution was extracted with ether (4 × 30 cm^3), and the combined ethereal extracts were dried ($MgSO_4$), filtered, and evaporated to give crude 2-carboxy-3-methyl[4-$^3H_2$]pentanoic acid (378 mg). This was treated at 0 °C with cold concentrated sulphuric acid (5 cm^3). The mixture was allowed to warm to room temperature and was treated with sodium azide (185 mg). The solution was stirred at 60 °C for 3 h, additional portions (each 83 mg) of sodium azide were added after each of the above 3 h, and the mixture was stirred for an additional 1 h after the last addition. The mixture was cooled, poured into ice–water (40 g) and the aqueous solution was extracted with ether (3 × 15 cm^3). Barium acetate was added until the solution had been brought to pH 3. The mixture was filtered and the filtrate (containing finely divided barium sulphate) was stirred with Dowex 50W-X8 ion exchange resin (H^+, 30 g) for 30 min. The resin was filtered off, packed into a column, and washed with deionised water. The column was eluted with ammonium hydroxide (0.5M) and the eluate was evaporated to dryness; the resulting amino-acid mixture was separated by preparative t.l.c. [40 g Kieselgel PF_{254} on 20 × 20 cm plates, solvent system butan-1-ol–acetic acid–water (4 : 1 : 1)]. The bands corresponding to isoleucine were soaked in water overnight and the amino-acids extracted were purified by passage over Dowex 50W-X8 (H^+, 30 g) as before to give the mixture of isoleucine stereoisomers (5.4 mg, 15μCi), pure by t.l.c. Radiochemical purity 97% by strip counting in a liquid scintillation counter of a paper chromatogram. The mixture was shown by amino-acid analysis to consist of (2*RS*)-isoleucine : (2*RS*)-alloisoleucine (46 ± 3 : 54 ± 3). The mixture was treated with (2*S*)-[U-^{14}C]isoleucine (1.66 μCi) to give a ratio (2*S*)-[4-$^3H_2$]isoleucine : (2*S*)-[U-^{14}C]-isoleucine = 2.36 in the mixture.

(E)-(RS)-2-*Amino*-3-*methylpent*-3-*enoic Acid*.—To a solution of tiglaldehyde (25 g) in ether (10 cm^3) at 0 °C was added a cold solution of ammonium chloride (18 g) in water (50 cm^3), followed by dropwise addition of a cold solution of sodium cyanide (15 g) in water (40 cm^3). The reaction flask was stoppered and shaken for 4 h at 40 °C followed by 4 h at room temperature. Hydrochloric acid (10M; 60 cm^3) was added, the mixture was heated on a steam-bath for 12 h and evaporated to dryness under reduced pressure. The residue was extracted with ethanol (100 cm^3) and the extract was filtered; the filtrate was concentrated and the residue in aqueous solution was applied to a column of Dowex 50W-X8 ion-exchange resin (H^+ form, 200 g). The column was washed well with water and the amino-acids were eluted with ammonium hydroxide solution (2M). The ammoniacal eluate was evaporated to dryness under reduced pressure and the residue was crystallised (ethanol–water) to give the unsaturated amino-acid (9) (1.75 g) which, after recrystallisation (ethanol–water), gave colourless flakes, m.p. 257—259 °C (sublimation); δ (CF_3CO_2D) 6.02 (1 H, q, *J* 7 Hz, CH:), 4.79 [1 H, s, C*H*(NH_2)], 1.74 [3 H, presumed d (upfield component obscured by MeC: singlet), *Me*CH:], and 1.81 [3 H, s, *Me*C(CHNH$_2$)]; δ (D_2O) 5.61 (1 H, q, *J* 6 Hz, CH:), 4.04 [1 H, s, C*H*(NH_2)], 1.59 (3 H, presumed d, *Me*CH:), and 1.56 [3 H, s, *Me*C(CHNH$_2$)] (Found: C, 55.5; H, 8.8; N, 11.0. $C_6H_{11}NO_2$ requires C, 55.8; H, 8.6; N, 10.8%).

(E)-(RS)-2-*Acetylamino*-3-*methylpent*-3-*enoic Acid* (10).—The amino-acid (9) (392 mg) in sodium hydroxide solution (0.5M; 4 cm^3) was cooled to 0 °C, stirred, and treated dropwise alternately with solutions of acetic anhydride in ether [10% (v/v) 6.9 cm^3] and sodium hydroxide solution (0.5M) during 12 min. The solution was brought to pH 1 (10M-HCl) and extracted continuously with ether for 69 h. The ethereal solution was dried ($MgSO_4$) and evaporated to give the *N*-acetyl derivative (10) which, after crystallisation (H_2O) had m.p. 163—166 °C, δ (C_5H_5N) 8.98 (1 H, m, NH), 5.98 (1 H, q, *J* 7 Hz, CH:), 5.70 [1 H, d, *J* 8 Hz, C*H*(NH_2)], 2.19 (3 H, s, MeCO), 1.87 [3 H, s, *Me*C(CHNHAc)], and 2.55 (3 H, d, *J* 7 Hz, *Me*CH:) (Found: C, 56.0; H, 7.3; N, 8.0. $C_8H_{13}NO_3$ requires C, 56.1; H, 7.65; N, 8.2%). The dicyclohexylamine salt crystallised (acetone) with m.p. 176—178 °C (Found: C, 67.7; H, 10.3; N, 7.9. $C_{20}H_{36}N_2O_3$ requires C, 68.1; H, 10.3; N, 7.9%).

Di-imide Reduction of (E)-2-*Acetylamino*-3-*methylpent*-3-*enoic Acid* (10).—The acid (10) (171 mg) in dry pyridine (5 cm^3) was added to a stirred slurry of potassium azodicarboxylate (13.0 g) in pyridine (25 cm^3) in an atmosphere of nitrogen. With rapid stirring, glacial acetic acid (6.5 cm^3) was added dropwise during 90 min. The mixture was stirred for a further 5 h, after which most of the pyridine was removed under reduced pressure; the residue was treated with dilute sulphuric acid (2M) until acid (Congo red) and then extracted continuously with diethyl ether for 48 h. The ethereal extract was dried (Na_2SO_4), evaporated, and the residue boiled under reflux with hydrochloric acid (2M; 20 cm^3) for 2 h. The solution was evaporated to dryness under reduced pressure and the residue dissolved in water and applied to a column of Dowex 50W-X8 ion-exchange resin (H^+ form; 10 g). The column was washed with water and the amino-acids were eluted with aqueous ammonia (2M). The ammoniacal eluate was evaporated to give a white solid (120 mg) which was shown by amino-acid analysis to consist of (*E*)-2-amino-3-methylpent-3-enoic acid, allisoleucine, an unknown amino-acid following alloisoleucine, and isoleucine in the ratio 62 : 8 : 6 : 24.

Hydrogenation of (E)-2-*Acetylamino*-3-*methylpent*-3-*enoic Acid*.—The acid (10) (85 mg) in acetic acid (30 cm^3) was hydrogenated at room temperature and 1 atm over 10% Pd-C catalyst. When 1 mol equiv. of hydrogen had been absorbed, the suspension was filtered, evaporated to dryness under reduced pressure, and the residue boiled under reflux with hydrochloric acid (2M; 8 cm^3) for 2 h. The hydrochloric acid was evaporated under reduced pressure and the amino-acids were isolated using Dowex 50W-X8 ion-exchange resin (10 g) as above. The product was shown by amino-acid analysis to consist of a mixture of alloisoleucine and isoleucine in the ratio 51 : 49.

Synthesis of (2RS,3RS,4RS)-[3,4-$^2H_2$]*Isoleucine*.—(*E*)-2-Acetylamino-3-methylpent-3-enoic acid (10) (171 mg, 1 mmol) was dissolved in D_2O (99.8 atom%) the solution evaporated to dryness under reduced pressure; the procedure was repeated once. The residue was then dissolved in dry pyridine (40 cm^3) and stirred under nitrogen; potassium azodicarboxylate (11.6 g, 60 mmol) was added to it. To the vigorously stirred solution CH_3CO_2D [60 mmol, prepared from freshly distilled acetic anhydride (6.12 g) and D_2O (1.2 g)] was then added dropwise during 2 h. The mixture was stirred for a further 6 h after which the white slurry was acidified (Congo red) with 6M-HCl and extracted continuously with diethyl ether for 48 h. The ethereal extract was evaporated and the residue was boiled under reflux in dilute hydrochloric acid (2M; 17 cm^3) for 2 h. The solution was evaporated to dryness under reduced pressure and the amino-acids were isolated by passage through a column of Dowex 50W-X8 ion-exchange resin as before.

The mixture of amino-acids was obtained as a colourless solid (98 mg). Amino-acid analysis showed this to have a composition similar to that obtained from the reduction with di-imide of normal isotopic composition. For the separation of the labelled amino-acids, an ion-exchange column was prepared, essentially as described by Liebster *et al.*,[14] except that the resin used was Amberlite CG-120 (type II, 200 mesh) instead of Dowex 50-X4 (300—400 mesh). In trial experiments it was found that baseline separation of alloisoleucine from isoleucine could be achieved readily using this column. The combined amino-acids (191 mg) from two reductions of the acid (10) with [$^2H_2$]di-imide were applied to this column, which was eluted with pyridine–formate buffer as described.[12] Fractions (10 cm^3) were collected and the amino-acid concentration in alternate fractions was determined using the ninhydrin method described by Lie.[15] Isoleucine was detected in fractions 168—178. These were combined and evaporated under reduced pressure. The amino-acid was purified by passage through Dowex 50W-X8 ion-exchange resin (0.5 g) as before, to give (2*RS*,3*RS*,4*RS*)-[3,4-$^2H_2$]isoleucine (4 mg).

Synthesis of (2RS,3RS,4RS)-[3,4-$^3H_2$]*Isoleucine*.—To the acid (10) (185 mg) in dry pyridine (1 cm^3) was added a slurry of potassium azodicarboxylate (2.15 g) in dry pyridine (7 cm^3). To the vigorously stirred mixture was added a solution of $CH_3CO_2{}^3H$ (prepared from acetic anhydride (1.13 g) and tritiated water (0.2 cm^3, 1Ci)) in pyridine (2 cm^3) during 2 h. The mixture was stirred overnight after which the excess of potassium azodicarboxylate was decomposed by the addition of water; the solution was then acidified (Congo red) with hydrochloric acid (6M) and extracted continuously with diethyl ether for 24 h. The ethereal extract was evaporated to dryness and the residue was boiled under reflux with hydrochloric acid (2M; 18 cm^3) for 2 h. The solution was evaporated to dryness under reduced pressure and the amino-acids were isolated by passage through Dowex 50W-X8 ion-exchange resin as before. The amino-acids were separated by ion-exchange chromatography as described above to give the tritiated isoleucine (20.0 mg, 50.6 μCi) pure by amino-acid analysis; radiochemical purity >98% by dilution analysis, 100% by paper chromatography with strip counting in the liquid scintillation counter.

Resolution of (2SR,3SR,4SR)-[3,4-$^3H_2$]*Isoleucine*.—The (2*SR*,3*SR*,4*SR*)-[3,4-$^3H_2$]isoleucine, above (20 mg, 20 μCi, after dilution with inactive material), was dissolved in a solution of sodium carbonate (100 mg) in water (4 cm^3). To the stirred solution cooled to 0 °C was added dropwise a solution of acetic anhydride (50 mg) in diethyl ether (5 cm^3). The mixture was stirred for 2 h after which the ethereal layer was separated and the aqueous layer was extracted with ether. The aqueous layer was acidified (Congo red) with 6M-HCl and the solution was continuously extracted with diethyl ether for 24 h. The ethereal extract was dried (Na_2SO_4) and evaporated. The *N*-acetyl derivative was dissolved in water (7 cm^3) and the solution was brought to pH 7.0 with sodium hydroxide solution (0.1M). Acylase I from pig kidney (10 mg) was added and the mixture was incubated at 38 °C. At intervals, aliquots (0.1 cm^3) were withdrawn and passed down a column of Dowex 50W-X8 ion exchange resin (2 g) and the radioactivity of the eluate was determined. The incubation was continued until the eluted activity reached a constant value equal to half the initial activity eluted at the beginning of the incubation. The incubation solution was applied to a column of Dowex 50W-X8 ion-exchange resin (H^+ form, 10 g) and the column was eluted with water (150 cm^3). The tritiated L-isoleucine was then eluted with ammonium hydroxide solution (2M). The ammoniacal eluate was evaporated under reduced pressure to give (2*S*,3*S*,4*S*)-[3,4-$^3H_2$]isoleucine (10 mg, 7.6 μCi).

(2RS)-[3-^{3}H]*Threonine*.—This compound was prepared as previously described[7] for the correspondingly deuteriated compound. Starting from butane-2,3-dione (12 mmol) and from sodium [^{3}H]borohydride (100 mCi), (2*RS*)-[3-^{3}H]-threonine (441 mg, 27 μCi mg^{-1}) was obtained, after purification by preparative paper chromatography, as described.[7]

(2S,4R)-[4-^{3}H]*Isoleucine*.—(2*RS*)-[3-^{3}H]Threonine (30 mg, 27 μCi mg^{-1}) was diluted with inactive (2*RS*)-threonine (170 mg) and converted into L-isoleucine using *Serratia marcescens* mutant strain 149.[7] (Only D-threonine is converted into L-isoleucine by this strain.) The lyophilised culture medium was freed from protein by absorption of the free amino-acids on a column of Dowex 50W-X8 ion-exchange resin (H^+, 20—50 mesh, 20 g) and elution with ammonium hydroxide solution (2M). The free amino-acids were then separated by ion-exchange chromatography as described above. Radioactivity measurements on the fractions collected showed that >95% of the radioactivity of the mixture applied was contained in the fractions corresponding to L-isoleucine. The fractions containing pure L-isoleucine were combined, evaporated, and purified by passage over Dowex 50W-X8 ion-exchange resin as before, to give (2*S*,4*R*)-[4-^{3}H]isoleucine (42 mg, 110.4 μCi).

Synthesis of (RS)-2-*Amino*[3-$^3H_2$,3-^{14}C]*butanoic Acid*.—[1-^{3}H]Iodoethane was prepared as described above from [1-^{3}H]ethanol in 75% yield. Using a vacuum line, iodo[1-^{3}H]-ethane (2.5 mCi, *ca.* 200 mg) was distilled into a nitrogen-cooled trap containing iodo[1-^{14}C]ethane (100 Ci, 30 mg). The mixture was then allowed to distil into a reaction vessel-condenser unit containing sodium (62 mg) dissolved in dry ethanol (7.5 cm^3) to which diethyl acetamidomalonate (582 mg) had been added. The reaction flask-condenser unit was isolated from the vacuum line and its contents were heated under reflux for 8 h at 70 °C. The solution was evaporated and the residue was boiled under reflux with hydrobromic acid (47%; 14 cm^3) for 6 h. The solution was diluted with water, evaporated under reduced pressure, diluted with water, and again concentrated; it was then brought to pH 7 by the addition of concentrated ammonium hydroxide solution and applied to a column of Dowex 50W-X8 ion exchange resin (H^+, 30 g). The amino-acids were eluted, and purified by preparative t.l.c. as before to give (*RS*)-2-amino[3-$^3H_2$,3-^{14}C]-butanoic acid (67 mg; 48 μCi ^{14}C, 397 μCi ^{3}H). Radiochemical purity >99% by strip counting of a paper chromatogram.

Resolution of (RS)-2-*Amino*[3-$^3H_2$,3-^{14}C]*butanoic Acid*.—A solution of the labelled amino-acid (25 mg) in water (5 cm^3) containing potassium carbonate (172 mg) was treated dropwise, with stirring, with a solution of acetic anhydride in ether [10% (v/v) 1.1 cm^3] during 2 min. The mixture was stirred at 0 °C for 45 min and allowed to warm to room temperature. The solution was applied to a column of Dowex 50W-X8 ion-exchange resin (H^+ form, 2.5 g) and the column was eluted with water. The eluate was evaporated to dryness to give essentially pure (*RS*)-2-acetylamino[3-$^3H_2$,3-^{14}C]-butanoic acid. In trial experiments with inactive material, the acetyl derivative was obtained after crystallisation (chloroform–acetone) in 84% yield (122 mg), m.p. 131.5—132.5 °C (lit.,[16] m.p. 128—130 °C). The radioactive derivative, above, was dissolved in water (12 cm^3) and the solution was adjusted to pH 7; Acylase I (4 mg) was added and the mixture was allowed to stand until analysis, as described for the corresponding resolution of *N*-acetylisoleucine, above, showed that

hydrolysis of the *S*-component was complete. (Similar analysis of the starting solution showed less than 1% free amino-acid to be present.) The amino-acid was purified using Dowex 50W-X8 ion-exchange resin (5 g) as before to give (*S*)-2-amino[3-$^3H_2$,3-^{14}C]butanoic acid (8 mg; 3.0 μCi ^{14}C, 8.2 μCi ^3H). The aqueous eluate was evaporated to dryness and the residue was boiled under reflux with hydrochloric acid (1M; 2.5 cm^3) for 12 h; the solution was evaporated to dryness and the amino-acid was purified by ion-exchange treatment as before to give (*R*)-2-amino[3-$^3H_2$,3-^{14}C]butanoic acid (10 mg; 3.7 μCi ^{14}C, 9.5 μCi^3H). Both amino-acid samples were shown to be of greater than 97% radiochemical purity by strip counting of paper chromatograms.

Synthesis of (Z)-2-*Amino*-3-*methylpent*-3-*enoic Acid.*—Lithium wire (280 mg, 40 mmol) in sodium-dried ether (15 cm^3) was stirred vigorously at room temperature. (*Z*)-2-Bromobut-2-ene (2.7 g, 20 mmol) in ether (10 cm^3) was added dropwise until the reaction was seen to begin. The reaction vessel was immersed in an ice–salt bath at −15 °C and the remaining bromoalkene was added dropwise during 1 h. The mixture was allowed to warm to room temperature and the base content of the solution was determined by titration (0.1M-HCl). A portion of the solution of the lithio-derivative (10 mmol) in ether (17 cm^3) was cooled to 0 °C and treated with copper(I) iodide. The solution was stirred for 5 min after which the solid was allowed to settle; the supernatant liquid was then added dropwise during 30 min to a stirred solution of methyl 2-chlorohippurate (25) (1.14 g, 5 mmol) in dry ether (30 cm^3) cooled to −78 °C. The mixture was stirred at −60 °C for 16 h and allowed to warm to room temperature. Hydrochloric acid (1M; 20 cm^3) was added with stirring and the organic layer was separated; the aqueous layer was then extracted with ether (2 × 20 cm^3). The combined organic extracts were dried ($MgSO_4$), filtered, and evaporated. The residue was boiled under reflux with hydrochloric acid (1M; 25 cm^3) for 16 h after which the solution was extracted with chloroform (3 × 20 cm^3); it was then made alkaline with concentrated ammonium hydroxide, extracted with chloroform (3 × 20 cm^3), and evaporated nearly to dryness. The residue, in aqueous solution, was applied to a column of Dowex 50W-X8 ion-exchange resin (H^+ form, 20 g) which was washed with water (200 cm^3) followed by ammonium hydroxide solution (2M; 200 cm^3). The ammoniacal eluate was evaporated to dryness to give a mixture of amino-acids (122 mg) which showed a major component on t.l.c., R_F 0.28 (ninhydrin yellow turning to purple) and two minor components (R_F 0.13 and 0.10). Amino-acid analysis showed these components to be present in the ratio of approximately 6 : 1 : 1. A portion of the mixture (110 mg) was purified by preparative t.l.c. to give (*Z*)-2-amino-3-methylpent-3-enoic acid (27) (74 mg, 12%), which after recrystallisation (ethanol–water) had m.p. 162 °C, δ (D_2O, 220 MHz) 5.79 (1 H, q, *J* 6.4 Hz, CH:), 4.20 [1 H, s, C*H*(ND$_2$)], 1.67 [3 H, d, *J* 6.4 Hz (presumed), *Me*CH:], and 1.65 (3 H, s, MeC:) (Found: C, 55.65; H, 8.4; N, 10.65. $C_6H_{11}NO_2$ requires C, 55.81; H, 8.53; N, 10.85%). The component of R_F 0.1 was identified as glycine by comparison of the m.p., n.m.r. spectrum, R_F value, and amino-acid analysis with the corresponding properties of authentic material.

Incorporation Experiments with S.magnificus *and* S.isatideus.—These experiments were carried out using plants (*ca.* 6 months old) growing in hydroponic solution, as previously described.[1a,1b]

Isolation of Senecionine (4).—This operation was carried out as previously described.[1b]

Isolation of Retrorsine (5).—The plants were repeatedly macerated with methanol in a Waring Blendor until the filtrate was no longer green. The combined filtrates were evaporated, dissolved in sulphuric acid (1M; 30 cm^3), and extracted with chloroform (6 × 30 cm^3). The residual aqueous layer was stirred with zinc dust for 90 min, filtered (Kieselguhr), extracted with chloroform (4 × 30 cm^3), made alkaline with concentrated ammonium hydroxide solution, and again extracted with chloroform (6 × 30 cm^3). The latter extracts were combined, dried ($MgSO_4$), and evaporated to give the crude retrorsine (5). In a typical experiment six plants gave 1.26 g of crude alkaloid. This was dissolved in sulphuric acid (1M; 30 cm^3) and extracted with chloroform (4 × 30 cm^3). The aqueous solution was made alkaline with concentrated ammonium hydroxide solution and extracted with chloroform (6 × 30 cm^3). The combined chloroform extracts were dried ($MgSO_4$) and evaporated to give retrorsine (5) (1.1 g) which was recrystallised (aqueous acetone) to constant activity.

Hydrolysis of Retrorsine.—In a typical experiment, a mixture of retrorsine (5) (312 mg, 0.89 mmol) and barium hydroxide octahydrate (280 mg, 0.89 mmol) in water (6.25 cm^3) was boiled under reflux for 2 h. The solution was treated with carbon dioxide, filtered (Kieselguhr), acidified (Congo red) with dilute hydrochloric acid, and extracted continuously with diethyl ether for 48 h. The ethereal extract was dried ($MgSO_4$) and evaporated to give isatinecic acid (3) (176 mg, 85%) which was recrystallised [ethyl acetate–light petroleum (b.p. 60—80 °C) to constant activity.

Ozonolysis of Isatinecic Acid (3).—In a typical experiment isatinecic acid (3) (200 mg) in ethyl acetate (20 cm^3, previously distilled over calcium hydride, stored over 3A molecular sieves, and re-distilled just before use), was ozonised at −5 °C for 3 h. The exit gases were collected in a trap at −78 °C. The combined solutions were boiled under reflux with zinc powder (*ca.* 2 g) in aqueous acetic acid [50% (v/v), 6.5 cm^3], with a stream of nitrogen passing through the solution. The exit gases were passed into a solution of dimedone (0.4%, 80 cm^3) and acetate buffer (pH 4.5, 40 cm^3). The mixture was allowed to stand overnight after which the dimedone derivative of acetaldehyde was filtered off and recrystallised (ethanol–water) to constant activity. In a typical experiment the yield of derivative after two such crystallisations was 77 mg (44%).

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