Pyrrolizidine Alkaloid Biosynthesis. Relative Rates of Incorporation of the Isomers of Isoleucine into the Necic Acid Component of Senecionine

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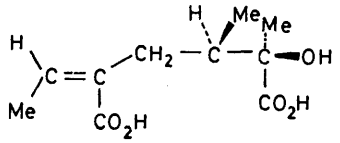
Of the four stereoisomers of isoleucine, only L-isoleucine is efficiently incorporated into senecic acid, the necic acid component of the pyrrolizidine alkaloid senecionine.

THE C_{10} necic acids of the senecic acid (I) type, which occur as diesters of pyrrolizidine bases in alkaloids such



(I)

as senecionine (II), have been shown to be derived from isoleucine (III) as illustrated in Scheme 1.[1]

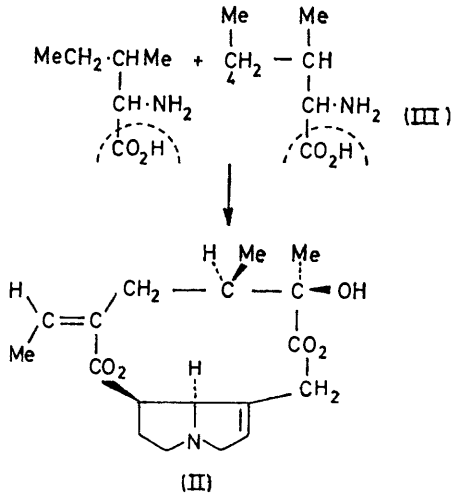
Certain microbial species can utilise all four stereoisomers of isoleucine for growth.[2] The incorporation of L-isoleucine into D-alloisoleucine residues in microbial peptides is well established [3] and recently the incorporation of L-isoleucine into the D-isoleucine residue of the peptide antibiotic monamycin has been observed.[4] Similarly, all four isomers of isoleucine were shown to stimulate the production (in *Streptomyces antibioticus* and *S. chrysomallus*) of actinomycins containing D-isoleucine and *N*-methyl-L-alloisoleucine residues.[5] It is thus clear that microbial species are capable of inverting the configurations of both chiral centres in L-isoleucine (III).

We have determined the relative rates of incorporation into senecionine (II) of the four stereoisomers of isoleucine in order to investigate the possibility that more than one isomer might be used for senecic acid (I) biosynthesis. The present results, however, demonstrate

[1] D. H. G. Crout, N. M. Davies, E. H. Smith, and D. Whitehouse, *J.C.S. Perkin I*, 1972, 671.
[2] I. Chibata, S. Yamada, H. Ito, and S. Ishikawa, *Appl. Microbiol.*, 1965, **13**, 680.
[3] M. Bodanszky and D. Perlman, *Nature*, 1968, **218**, 291.
[4] J. S. Davies, C. H. Hassall, and V. Arroyo, *J.C.S. Chem. Comm.*, 1973, 782.
[5] T. Yajima, M. A. Grigg, and E. Katz, *Arch. Biochem. Biophys.*, 1972, **151**, 565.

that L-isoleucine is a very specific precursor of senecic acid, as assumed in previous studies of necic acid biosynthesis.[1]

In order to make the most efficient use of the labelled isoleucine isomers available for this study, incorporations



(III)

(II)

SCHEME 1

of individual isomers were not compared with that of L-isoleucine; instead, the incorporations of mixtures of tritiated (L-isoleucine + L-alloisoleucine), DL-alloisoleucine and (D-isoleucine + D-alloisoleucine) were compared with that of ^{14}C-labelled L-isoleucine. These three experiments gave the information necessary for the determination of the relative incorporations of all four stereoisomers of isoleucine.

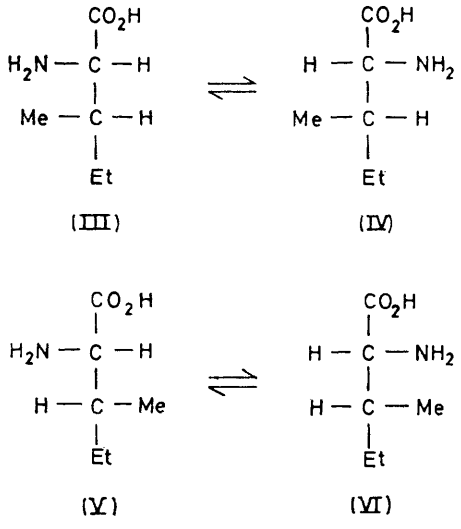
A 1 : 1 mixture of DL-isoleucine and DL-alloisoleucine was acetylated under epimerising conditions. According to Greenstein and Winitz [6] one recrystallisation of this mixture from water–acetic acid is sufficient to give DL-acetylalloisoleucine free from DL-acetylisoleucine. However, it was found that eight recrystallisations were necessary in order to remove all the DL-acetylisoleucine. The purification was monitored by hydrolysing samples from successive recrystallisations under non-epimerising conditions and subjecting the hydrolysate to amino-acid analysis. Our findings are in agreement with a recent report.[7]

Isoleucine, generally labelled with tritium, was similarly acetylated to give a mixture of [G-^3H]-DL-acetylisoleucine and [G-^3H]-DL-acetylalloisoleucine. The mixture was crystallised once to give [G-^3H]acetylalloisoleucine low in [G-^3H]acetylisoleucine. This was diluted with pure, inactive DL-acetylalloisoleucine and the resulting mixture was recrystallised to constant activity. The product was hydrolysed to give [G-^3H]-DL-alloisoleucine.

The mother liquor from the recrystallisation of the mixed labelled acetyl derivatives, which was shown by hydrolysis of a sample followed by amino-acid analysis to consist of 58% DL-acetylisoleucine and 42% DL-acetylalloisoleucine, was diluted with inactive material with the same isomer composition. The resulting mixture was hydrolysed, with acylase I, to give L-isoleucine + L-alloisoleucine (58 : 42) and D-acetylisoleucine + D-acetylalloisoleucine (58 : 42). The latter mixture was hydrolysed to give the mixture of free amino-acids.

It was necessary to check the radiochemical composition of the various isomer mixtures, since the starting labelled amino-acid, although described as DL-isoleucine, was of unknown isomer composition.

Under α-epimerising conditions, L-isoleucine (III) is in equilibrium with D-alloisoleucine (IV) and L-alloisoleucine (V) is in equilibrium with D-isoleucine (VI) (Scheme 2). In order to be sure that the L-isoleucine + L-alloisoleucine and D-isoleucine + D-alloisoleucine mixtures described above had the expected radiochemical compositions (58 : 42), it was therefore necessary to show that the L-isoleucine + D-alloisoleucine content of the original labelled amino-acid was equal to the L-alloisoleucine + D-isoleucine content. Since the conditions used for acetylation produced α-epimerisation, the acetyl derivatives of L-isoleucine and D-alloisoleucine were known to be present in the acetylated mixture in equal amounts, as were the derivatives of L-alloisoleucine and D-isoleucine. It did not follow, however, that the former pair of isomers was present in the same amount as the latter pair. In order to investigate this question, a sample of



(III) (IV)

(V) (VI)

SCHEME 2

the pure [G-^3H]-DL-acetylalloisoleucine, produced as described above, was incubated with acylase I. The percentage of the initial activity of samples of the reaction mixtures retained by a cation-exchange resin in the protonated form were determined at intervals. The activity retained reached a limiting value of 50% of the initial activity. It was concluded that the original acetylated amino-acid mixture before recrystallisation consisted of equal quantities of the derivatives of all four isomers.

[6] J. P. Greenstein and M. Winitz, 'Chemistry of the Amino Acids,' Wiley, New York, 1961, vol. 3, p. 2067.

[7] W. F. Laird, S. Matai, and L. M. Synge, *Biochem. J.*, 1970, **116**, 911.

The various mixtures of generally-labelled isomers were respectively combined with [U-^{14}C]-L-isoleucine and administered hydroponically to *Senecio magnificus* plants. After 8—10 days the labelled senecionine was isolated, diluted with inactive alkaloid, and recrystallised to constant activity. The ^{3}H : ^{14}C ratio of the amino-acids fed and of the isolated senecionine and the percentage retention of tritium are given in the Table. Only the mixture containing 58% L-isoleucine gave a high incorporation of tritium. Since in previous studies it had been shown that L-isoleucine is incorporated exclusively into senecic acid,[1] the activities given in the Table are those of the total alkaloid; these are assumed to be equal to the activities of the corresponding necic acid components.

Relative retentions of ^{3}H and ^{14}C from isoleucine isomers on incorporation into senecionine (II)

Experiment	Mixture fed	^{3}H : ^{14}C Ratio		Retention of ^{3}H relative to ^{14}C (%)
		Mixture fed	Senecionine isolated	
1	[^{3}H]-(L-isoleucine + L-alloisoleucine) (58 : 42) + [^{14}C]-L-isoleucine	61·2	39·9	65·2
2	[^{3}H]-DL-alloisoleucine + [^{14}C]-L-isoleucine	22·7	1·9	8·6
3		60·7	3·2	5·3
4	[^{3}H]-(D-isoleucine + D-alloisoleucine) (58 : 42) + [^{14}C]-L-isoleucine	70·7	1·4	2·0

The retention of tritium from the mixture of D-amino-acids was 3% of that of the mixture of L-amino-acids and was at a level expected from degradation of the precursor to low molecular weight intermediates and re-incorporation of these into isoleucine *via* the appropriate metabolic pathways. However, the retention of tritium from [G-^{3}H]-DL-alloisoleucine was significantly higher. In order to make sure that the retention observed was not due to the presence of contaminating [^{3}H]-L-isoleucine in the material fed, a sample of the [G-^{3}H]-DL-alloisoleucine was subjected to dilution analysis with DL-isoleucine. By this means it was shown that the mixture fed contained not more than 1·3% of L-isoleucine. If 1·3% of L-isoleucine impurity is assumed in experiment 2, and if it is assumed that the D-amino-acids are not incorporated, the data from experiments 1 and 2 can be used to set up two simultaneous equations, the solution of which shows that the retention (relative to ^{14}C) of tritium from L-isoleucine was 104% and from L-alloisoleucine, 14·5%. The DL-alloisoleucine experiment was repeated to give the results shown in the Table (experiment 3). In this case, dilution analysis of the material fed was carried out by hydrolysing the DL-acetylalloisoleucine with acylase I and analysing the [^{3}H]-L-alloisoleucine produced with inactive L-isoleucine. By this means it was shown that the L-isoleucine content of the DL-alloisoleucine fed was not greater than 1·7%. Combination of the data from experiments 1 and 3 as before gave values of 108% for the relative retention of tritium from L-isoleucine and 6·8% for the retention of tritium from L-alloisoleucine.

These results show that only L-isoleucine is an efficient precursor of senecic acid. The results from experiments 1—3 (Table) show that the incorporation of L-alloisoleucine was respectively 14·0 and 6·3% of that of L-isoleucine. It remains to be shown whether or not these incorporations of L-alloisoleucine into senecic acid are specific or whether the relatively high rate of the L-alloisomer as compared with the D-isomers is due simply to a higher rate of metabolism of the L- over the D-forms and incorporation of low molecular weight metabolic products into senecic acid *via* primary metabolic pathways leading to L-isoleucine. It is clear, however, that the incorporations of D-isoleucine, L-alloisoleucine, and D-alloisoleucine are of the same order ($>$0·01%) as the incorporations of non-specific precursors of the C_{10} necic acids such as acetate.[8]

A small error might have been introduced into the comparative values of tritium retention from L-isoleucine and L-alloisoleucine by the assumption that if the incorporation of L-alloisoleucine were stereospecific, the pattern of tritium loss would be the same from both isomers. This assumption is likely to lead to the introduction of errors only with respect to stereospecific tritium loss from C-4 of isoleucine [(III), Scheme 1]. In such a case, unequal labelling of the diastereotopic protons at C-4 in the original generally labelled mixture of isoleucine isomers, would lead to unequal tritium loss from C-4 in stereospecific conversions of L-isoleucine and L-alloisoleucine, respectively, into senecic acid. Although it is highly probable that proton loss from C-4 is indeed stereospecific, it is unlikely that there was a significant difference in the labelling of the prochiral C-4 protons in the original labelled amino-acid mixture, since this was prepared by the Wilzbach method which would not be expected to result in a large difference in the relative rates of exchange at these two positions.

A more probable source of variation in observed relative retentions of tritium might have been the variation in the relative amounts of labelled isomers fed to the plants. The quantities fed to the plants were chosen so as to lie in the range over which incorporations had been found to be sensibly constant in previous studies. However, even with this precaution it is probable that the various isomers were absorbed and metabolised at different relative rates in different experiments. The variation in the relative incorporations of L-alloisoleucine and L-isoleucine in experiments 2 and 3 can probably be attributed to this phenomenon.

EXPERIMENTAL

All radioactivity measurements were carried out with a Packard TriCarb series 2000 scintillation counter. Alkaloid

[8] D. H. G. Crout, M. H. Benn, H. Imaseki, and T. A. Geissman, *Phytochemistry*, 1966, **5**, 1.

samples and acetylamino-acids were counted in NE 220 scintillation fluid (Nuclear Enterprises Ltd.). Labelled isomers were counted in either a mixture of methoxyethanol–ethanolamine (11 : 1; 5 cm^3) and toluene containing 2,5-diphenyloxazole (PPO) (8·25 g dm^{-3}) (10 cm^3) or in a mixture of hyamine hydroxide (1 mol dm^{-3}) in methanol (1 cm^3) and toluene–PPO (11 cm^3). Sufficient counts were taken to give a statistical error of $<1\%$ for each determination. The overall errors for single determinations of ^3H- and ^{14}C-labelled samples was estimated to be $\gtrsim 5\%$. Radiochemicals were purchased from the Radiochemical Centre, Amersham, Bucks. Amino-acid analyses were carried out on a Technicon Amino Acid Analyser. The radiochemical purity of amino-acid samples was determined by strip counting of paper chromatograms.[1] Feeding experiments with *Senecio magnificus* plants and the isolation and purification of labelled senecionine were carried out as previously described.[1]

DL-*Acetylalloisoleucine*.—A mixture of DL-isoleucine and DL-alloisoleucine (1 : 1; 5 g) was boiled gently under reflux with acetic anhydride (7·7 g) in acetic acid (57 cm^3). The solution was cooled, the excess of reagent was evaporated off, and the mixture of acetyl derivatives was recrystallised eight times from 50% acetic acid to give DL-acetylalloisoleucine (800 mg), m.p. 170·5° (lit., 168°). A sample of the derivative (10 mg) was hydrolysed in boiling HCl (2 mol dm^{-3}; 1 cm^3) for 2 h. The solution was evaporated and the residue was found, by amino-acid analysis, to consist solely of alloisoleucine with no detectable isoleucine.

[^3H]-DL-*Acetylalloisoleucine*.—Generally labelled '[^3H]-DL-isoleucine' (5·24 mCi $mmol^{-1}$; 2·5 mCi) was diluted with a mixture of inactive DL-isoleucine and DL-alloisoleucine (1 : 1; 30 mg). The resulting mixture was boiled under reflux with acetic anhydride (0·3 cm^3) in acetic acid (2 cm^3) for 10 min. The solution was cooled and evaporated. The residue was recrystallised once from 50% acetic acid to give 15 mg of mixed derivatives rich in DL-acetylalloisoleucine. The material in the mother liquor was used for the preparation of the mixtures [^3H]-(L-isoleucine + L-alloisoleucine) and [^3H]-(D-isoleucine + D-alloisoleucine) (see below). The derivative mixture (15 mg) was diluted with pure, inactive DL-acetylalloisoleucine (199 mg) and the resulting mixture was recrystallised to constant activity (84 mg; 425 μCi $mmol^{-1}$).

[^3H]-DL-*Alloisoleucine*.—The acetyl derivative (84 mg) was boiled under reflux in hydrochloric acid (2 mol dm^{-3}; 20 cm^3) for 2 h. The solution was evaporated and water (20 cm^3) was twice added and evaporated off to give [^3H]-DL-alloisoleucine. The product was found to have a radiochemical purity of $>98\%$ by paper chromatography with liquid scintillation scanning. Dilution analysis showed that not more than 1·3% of L-isoleucine was present in the material used in experiment 2 and not more than 1·7% in that for experiment 3 (Table).

[^3H]-L-*Isoleucine* + [^3H]-L-*Alloisoleucine*.—The mother liquor from the recrystallisation of the tritiated DL-acetylisoleucine–DL-acetylalloisoleucine mixture was evaporated to dryness. A small quantity of the mixture was hydrolysed with 2M-hydrochloric acid. Analysis of the resulting mixture of amino-acids showed it to consist of isoleucine (58%) and alloisoleucine (42%). The remaining mixture of acetyl derivatives was diluted with inactive material having the same isomer composition (200 mg). A portion of the diluted mixture (97 mg) was dissolved in water (15 cm^3). The solution was brought to pH 7 with 0·01M-sodium hydroxide. Hog kidney acylase I (3 mg) was added and the mixture was incubated at 38°. The progress of the hydrolysis was followed by determining the retention of activity by a column of Dowex 50W-X8 cation-exchange resin (H^+; 1 g) when 0·1 cm^3 samples were removed from the solution and passed through the column at 4°. After 96 h the activity retained by the column had reached a constant value of $53 \pm 3\%$. The solution was passed through a column of Dowex 50W-X8 (H^+; 20 g) maintained at 4°. The eluate was evaporated to give the mixture of [^3H]-D-acetylisoleucine and D-acetylalloisoleucine. This mixture was hydrolysed to the mixture of D-isoleucine and D-alloisoleucine (58 : 42) with 2M-hydrochloric acid as described above. The column was eluted with 2M-ammonia (180 cm^3). The eluate was evaporated to give the mixture of [^3H]-L-isoleucine and L-alloisoleucine (58 : 42).

Determination of the L-*Alloisoleucine* : D-*Alloisoleucine Ratio in the* [^3H]-*Labelled Mixture used in Incorporation Experiments* 2 *and* 3.—The mixture was analysed by using acylase I as described above. By this means the mixture was shown to consist of L-alloisoleucine (50%) and D-alloisoleucine (50%).

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