Biosynthesis of the Necic Acids of the Pyrrolizidine Alkaloids. Further Investigations of the Formation of Senecic and Isatinecic Acids in *Senecio* Species

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Five-carbon intermediates of the pathway of isoleucine metabolism, 2-methylbutanoic acid, angelic acid (5), and 2-methyl-3-oxobutanoic acid, were not specifically incorporated into senecic acid (1), the necic acid component of senecionine (2) in *Senecio magnificus*. The mechanism of the coupling of isoleucine-derived units during necic acid biosynthesis was investigated through incorporation experiments with [6-^{3}H]- and [4-$^3H_2$]-isoleucine (3) in *S. magnificus* and *S. isatideus*. 2-Amino-3-[$^3H_2$]methylenepentanoic acid (β-[$^3H_2$]methylenenorvaline) (13) was incorporated specifically into senecic acid (1).

L-Isoleucine (3) and its biological precursor L-threonine have been shown to be incorporated specifically into senecic acid (1), the necic acid component of the pyrrolizidine alkaloid senecionine (2) (Scheme 1).[1] In seeking to elucidate the pathway by which isoleucine is converted into the necic acids and to identify the functionality introduced into the isoleucine skeleton in order to provide for the generation of the 13,14-bond in senecic acid [see (2)] between C-6 of one isoleucine-derived unit and C-4 of another, we were led to consider as possible intermediates compounds which have been suggested as products of isoleucine metabolism in micro-organisms and animals (Scheme 2).[2] Although this pathway has not been fully established in higher plants, the initial steps: isoleucine ⟶ 2-methylbutanoic acid ⟶ tiglic (angelic) acid have been demonstrated in a number of systems.[3] The occurrence of 2-hydroxy-2-methyl-3-oxobutanoic acid [(8), Scheme 2] and related compounds [4] as acylating residues in various sesquiterpenes suggests that the similarity between isoleucine metabolism in plants and that in animals and micro-organisms may be essentially complete.

2-Methyl[1-^{14}C]butanoic acid (see Scheme 2) and [1-^{14}C]angelic acid (5) were examined as precursors of seneciphyllic acid (4) in *Senecio douglasii* but incorporations were low and the distribution of activity between the necic acid and necine base components of the alkaloid showed that considerable randomisation of the label had occurred.[5] It was considered probable that both precursors were metabolised with loss of the C-1

[1] D. H. G. Crout, N. M. Davies, E. H. Smith, and D. Whitehouse, *J.C.S. Perkin I*, 1972, 671.

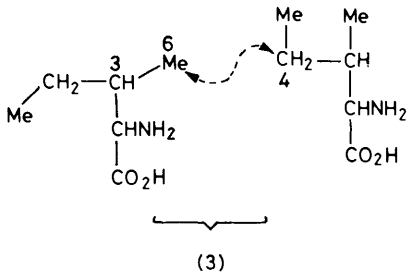
[2] V. W. Rodwell, 'Metabolic Pathways. Vol. III,' ed. D. M. Greenberg, Academic Press, London and New York, 1969, p. 191.

[3] W. C. Evans and J. G. Woolley, *J. Pharm. Pharmacol.*, 1965, **17**, Suppl. 37; E. Leete and J. B. Murrill, *Tetrahedron Letters*, 1967, 1727; D. H. G. Crout, *J. Chem. Soc.* (*C*), 1967, 1233; K. Basey and J. G. Woolley, *Phytochemistry*, 1973, **12**, 2197, 2883; E. Leete, *ibid.*, p. 2203; P. J. Beresford and J. G. Woolley, *ibid.*, 1974, **13**, 2143, 2511.

[4] W. Herz and S. V. Bhat, *J. Org. Chem.*, 1970, **35**, 2605.

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

label as a one-carbon unit which entered the one-carbon metabolic pool. The distribution of activity between



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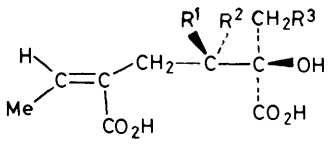
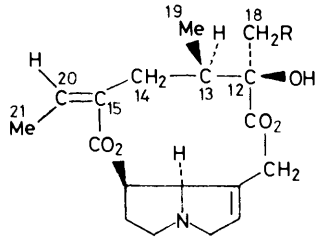


(1) R^1 = Me, R^2 = R^3 = H
(4) R^1R^2 = CH_2, R^3 = H
(9) R^1 = Me, R^2 = H, R^3 = OH

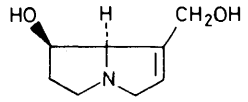
SCHEME 1

necic acid and necine base (hereinafter referred to as the 'acid–base balance') was similar to that obtained by administering [*Me*-^{14}C]methionine, the label from which probably entered the alkaloid *via* C-3 of serine and pyruvate.[1,6]

To re-examine the role of 2-methylbutanoic and angelic group. As before, angelic acid [(*Z*)-2-methylbut-2-enoic acid] (5) was chosen for examination rather than the *E*-isomer, tiglic acid, because angelic acid has the same configuration about the double bond as senecic acid (1).



(2) R = H
(3) R = OH



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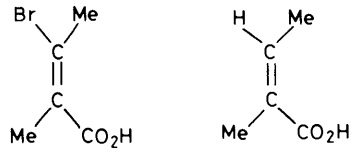
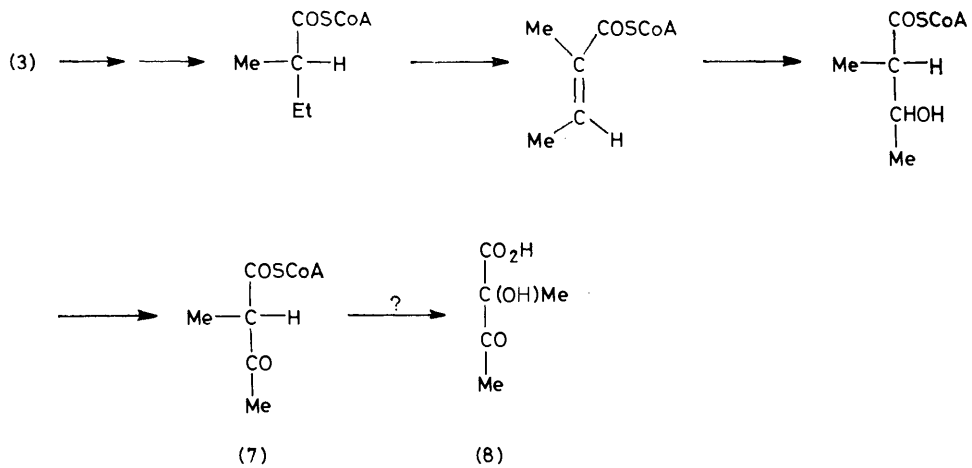


(6) (5)

(*S*)-[U-^{14}C]-2-Methylbutanoic acid was prepared from L-[U-^{14}C]isoleucine by degradation with ninhydrin to (*S*)-[U-^{14}C]-2-methylbutanal [7] followed by oxidation



SCHEME 2 CoASH = coenzyme A

acids as precursors of C_{10} necic acids it was decided to administer labelled forms of these compounds with the isotope at a less vulnerable position than the carboxy- with permanganate. (*S*)-2-Methylbutanoic acid is the logical intermediate to expect in a metabolic pathway stemming from L-isoleucine. However, to take account

[6] R. L. Kisliuk, W. Sakami, and M. V. Patwardhan, *J. Biol. Chem.*, 1956, **221**, 885.

[7] R. T. Lotfield and E. A. Eigner, *Biochim. Biophys. Acta*, 1966, **130**, 449.

of possible inversion, the corresponding racemic acid was also prepared by racemisation of (*S*)-[U-^{14}C]-2-methylbutanal in hot aqueous pyridine. [3-^{3}H]Angelic acid (5) was prepared by reduction with sodium amalgam of (*E*)-3-bromo-2-methylbut-2-enoic acid (6) [8] in the presence of ^{3}HHO. The labelled compounds were administered to *Senecio magnificus* plants growing in hydroponic solution. Incorporation of both stereochemical forms of 2-methylbutanoic acid was significant but lower than typically found for isoleucine [1] (0.1—0.4%) (Table 1). However, hydrolysis of the labelled

TABLE 1

Incorporation of (*S*)- and (*RS*)-[U-^{14}C]-2-methylbutanoate, [3-^{3}H]angelate, and methyl 2-[^{14}C]methyl-3-oxobutanoate into senecionine (2)

Precursor	% Incorporation	% Activity in senecic acid (1)	% Activity in retronecine (11)
Calcium (*S*)-[U-^{14}C]-2-methylbutanoate	0.062	37	67
Calcium (*RS*)-[U-^{14}C]-2-methylbutanoate	0.039	30	77
[3-^{3}H]Angelic acid (5)	0.0003		
Methyl 2-[^{14}C]methyl-3-oxobutanoate	8.7×10^{-5} [a]		
	0.0012 [a]		
	0.012 [b]	26	82

[a] Precursor administered through cut stems. [b] Precursor administered *via* hydroponic solution.

alkaloid to the necic acid and necine base showed that the activity had been extensively randomised with most of the label appearing in the necine base. In a parallel experiment the incorporation of [^{3}H]angelic acid (5) was so low that further degradation of the labelled alkaloid was not practicable (Table 1). Although it is possible to conceive of ways in which [^{3}H]angelic acid (5) could be specifically incorporated into senecic acid (1) with loss of the tritium label, this experiment, together with the earlier result, suggests strongly that angelic acid (5) is not a precursor of C_{10} necic acids of the senecic acid type. The randomisation of label on incorporation of 2-methylbutanoic acid also shows that this compound must be eliminated as a possible precursor, with the consequence that later intermediates in the pathway of metabolism of isoleucine (Scheme 2) must also be considered to be unlikely precursors. However, to obtain more evidence (*RS*)-2-[^{14}C]methyl-3-oxobutanoate [*cf.* (7)] was prepared by alkylation of methyl 3-diethylaminobut-2-enoate with [^{14}C]methyl iodide.[9] Because of the lability of 2-methyl-3-oxobutanoic acid, the methyl ester was fed, rather than the free acid or a salt, to *Senecio magnificus* plants. Administration of the precursor to cut stems of young plants resulted in very low incorporations (Table 1). A higher incorporation was obtained by hydroponic feeding and the activity of the alkaloid obtained from this experiment was high enough to permit determination of the acid–base balance. The results (Table 1) showed that most of the activity appeared in the necine base as in the incorporation experiments with 2-methylbutanoic acid.

The foregoing experiments pointed uniformly to the conclusion that the five-carbon intermediates of the pathway of isoleucine metabolism (Scheme 2) were not precursors of senecic acid. Accordingly, attention was redirected to obtaining evidence on the changes taking place at the coupling positions, C-6 and C-4, of isoleucine (3) during conversion into senecic acid (1) (Scheme 1). In particular, consideration was given to the possible nature of the precursor of the left-hand five-carbon unit of senecic acid (1). In this context it was clearly desirable to determine the oxidation level to which C-6 in isoleucine (3) was raised in preparation for the coupling step. Accordingly [6-^{3}H,6-^{14}C]isoleucine (3) was prepared [1] and administered to *Senecio magnificus* plants. The labelled amino-acid consisted of a mixture of DL-isoleucine and allo-DL-isoleucine in the ratio 47 : 53. The amino-acid was administered to *Senecio magnificus* plants as the mixture of stereoisomers since from previous work [10] it was known that, of the four stereoisomers, only L-isoleucine would be efficiently incorporated into senecic acid (1). It was also known that this precursor would label exclusively the necic acid component of senecionine (2), that the label would be confined to C-14 and C-18 in senecionine (2), and that the ^{14}C activity would be equally distributed between these positions.[1] The extent of tritium loss could thus be measured by comparing the ^{3}H : ^{14}C ratio in the labelled alkaloid with that in the precursor. The results of this experiment are shown in Table 2, from which it can be seen that approxi-

TABLE 2

Incorporation of [6-^{3}H,6-^{14}C]isoleucine into senecionine (2)

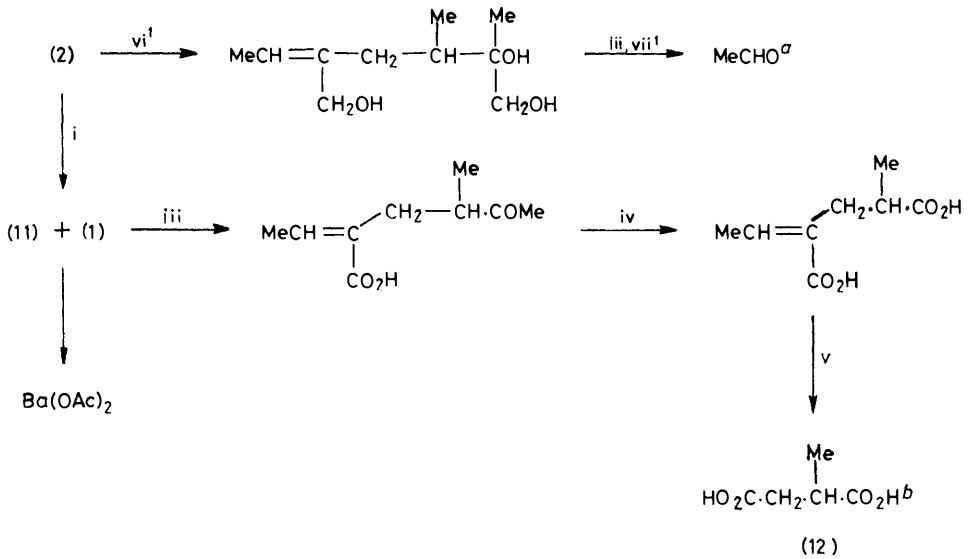
	^{3}H : ^{14}C	% Retention of ^{3}H
[6-^{3}H,6-^{14}C]Isoleucine (3)	150 : 1	
Senecionine (2)	115 : 1	77

mately five-sixths of the tritium was retained. The evidence from this experiment cannot be interpreted unambiguously because of the possible intervention of a tritium isotope effect. However, it is certain that one of the hydrogen atoms at C-6 in isoleucine (3) is retained during conversion into C-14 of senecionine (2) and it is probable that two are retained.

In a further experiment in which [6-^{3}H]isoleucine was administered, the resulting labelled senecionine (2) was degraded (Scheme 3), with the results shown in Table 3. These show that the activity of the labelled alkaloid was entirely confined to the necic acid component, that the ethylidene group (C-20 and 21) contained no activity, and that the methylsuccinic acid fragment (12) contained 37% of the activity of the necic acid. This figure agrees well with the expected activity (40%) if two molecules of isoleucine were incorporated into

[8] H. P. Kaufman and K. Kuchler, *Chem. Ber.*, 1937, **70**, 915; L. Buckles and G. V. Mock, *J. Org. Chem.*, 1950, **15**, 680.
[9] H. M. E. Cardwell, *J. Chem. Soc.*, 1949, 715.
[10] N. M. Davies and D. H. G. Crout, *J.C.S. Perkin I*, 1974, 2079.

senecic acid with loss of only one of the six hydrogen atoms of the two C-6 methyl groups. Kuhn–Roth oxidation of the senecic acid gave barium acetate containing 39% of the total activity. From the previous results a value of 60% would have been expected. The low value obtained was undoubtedly the result of proton exchange in the presumed methyl



SCHEME 3 Reagents: i, $Ba(OH)_2$; ii, CrO_3–H_2SO_4; iii, $NaIO_4$; iv, I_2–NaOH; v, $NaIO_4$–$KMnO_4$; vi, $LiAlH_4$; vii, $NaIO_4$–OsO_4
[a] Isolated as the anhydrodimedone derivative. [b] Isolated as the dicyclohexylammonium salt.

ketone intermediate, as has often been observed during Kuhn–Roth oxidation of tritiated compounds.[11]

TABLE 3

Distribution of radioactivity in senecionine (2) [a] derived from L-[6-^{3}H]isoleucine (3)

Degradation product	% Activity of senecionine (2)
Senecic acid (1)	100
Retronecine (11)	1
Anhydro-dimedone derivative of acetaldehyde (C-20, -21)	0
Barium acetate (C-12, -13, -18, -19, -20, -21)	39
Dicyclohexylammonium hydrogen succinate [*cf.* (12)] (C-12, -13, -14, -15, -19)	37

[a] 0.13% Incorporation.

A further study was carried out to determine the fate of the hydrogen atoms at C-4 in isoleucine (3) during conversion into the necic acid. For this study, *Senecio isatideus* was used. This species produces retrorsine (10) as the major alkaloid.[12] [4-$^3H_2$]Isoleucine (3), as a mixture of DL-isoleucine and allo-DL-isoleucine (45 : 55) was fed, together with L-[U-^{14}C]isoleucine as internal standard. As stated above, previous studies with *Senecio magnificus* had shown that of the four stereoisomers of isoleucine only L-isoleucine is an effective precursor of senecic acid (1).[10] It was assumed that this acid (9) and that half the tritium label was retained. It was concluded that both the C-20 vinylic hydrogen atom and the hydrogen atom at C-13 in retrorsine (10) are derived from one of the C-4 hydrogen atoms of L-isoleucine (3). This limits the oxidation level to which C-4 in the isoleucine precursor is raised during incorporation into C-13 of the alkaloid (10), to that of, for example, a carbinol or vinylic methine group. The evidence therefore eliminates the possibility that a carbonyl function is introduced to provide activation for the coupling of the two isoleucine-derived components and provides further evidence for the non-involvement of metabolities of isoleucine such as 2-methyl-3-oxobutanoate [*cf.* (7)].

TABLE 4

Incorporation of L-[4-$^3H_2$, U-^{14}C]isoleucine (3) into retrorsine (10) [a]

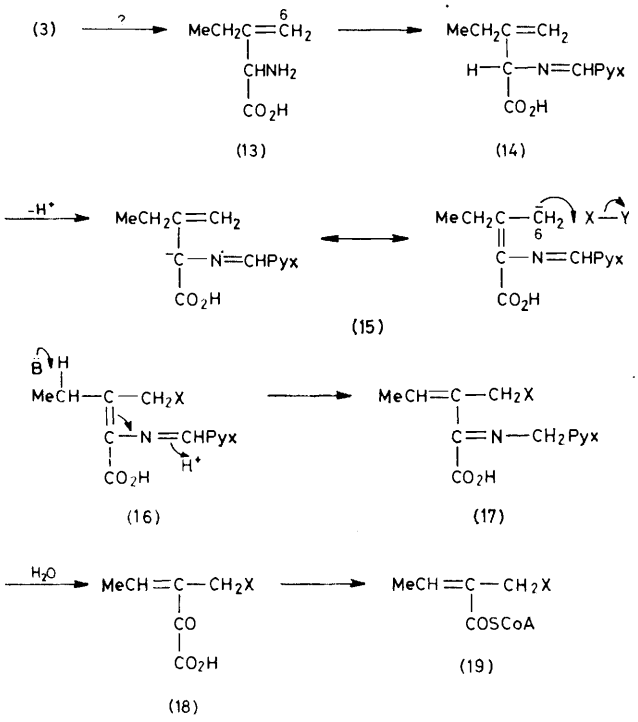
	^{3}H : ^{14}C	% Retention of ^{3}H
L-[4-$^3H_2$,U-^{14}C]Isoleucine (3)	2.36 : 1	
Retrorsine (10)	1.42 : 1	50 [b]
Isatinecic acid (9)	1.60 : 1 [c]	56 [b]

[a] 0.21% Incorporation based on ^{14}C activity. [b] Corrected for the loss of C-1 of L-isoleucine during incorporation into retrorsine (10). [c] 94% of the ^{14}C activity was contained in the necic acid component.

[11] H. Simon and H. G. Floss, 'Bestimmung der Isotopenverteilung in markierten Verbindungen,' Springer-Verlag, Berlin, Heidelberg, New York, 1967, p. 14.

[12] J. J. Blackie, *Pharm. J.*, 1937, **138**, 102; H. L. De Waal, *Onderstepoort J. Vet. Sci. Animal Ind.*, 1939, **12**, 155.

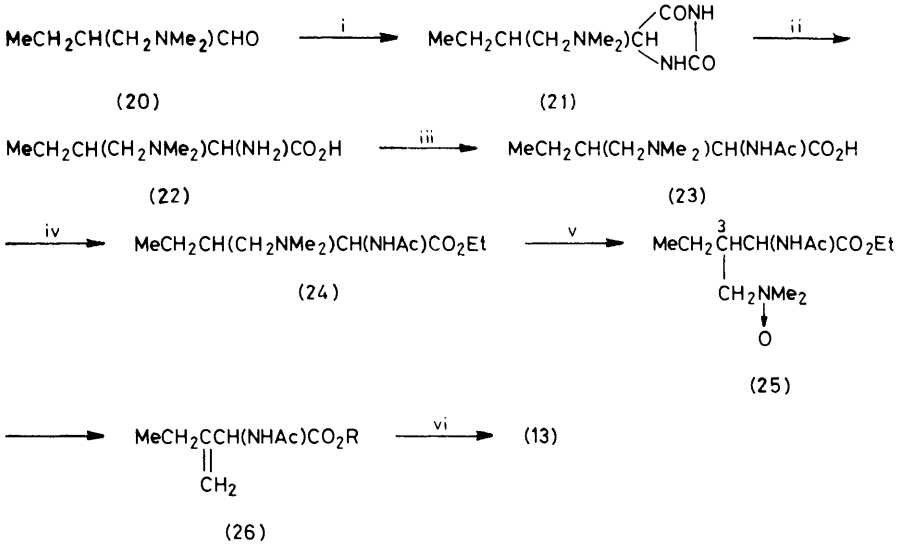
The conclusion that could be drawn from the foregoing results was that the functionality introduced into the



SCHEME 4 PyxCHO = pyridoxal phosphate

C-3,C-6 side chain of isoleucine prior to coupling might be $C{\cdot}CH_2{\cdot}OH$ or $C{:}CH_2$. The latter possibility was attractive for two reasons. First, the corresponding amino-acid, β-methylenenorvaline (2-amino-3-methylenepentanoic acid) (13) was already known in nature, as a metabolite of the fungus *Lactarius helvus*.[13] Secondly, by postulating activation of the α-hydrogen through formation of a pyridoxal phosphate Schiff's base [(14), Scheme 4] there could be readily produced a mesomeric anion (15) which would generate nucleophilic character at C-6 in isoleucine (3) (Scheme 4), thus providing a means of constructing the 13,14-bond in senecionine (2) by attack on an electrophile, schematically represented as X–Y. Such a scheme would have the additional attraction that in order to release the coenzyme, a prototropic shift with one of the γ-carbon atoms as the point of departure would be implicated, and the choice of one of the protons at C-4 would lead naturally [(16) ⟶ (17), Scheme 4] to the generation of the 16,20-double bond (or a metabolically derived function such as a 1,2-diol or epoxide) which is almost invariably present at the corresponding position in acids of the senecic acid (1) type.[14] Furthermore, hydrolysis of the isomeric Schiff's base (17) would release the intermediate as the 2-oxo-acid (18), which by oxidative decarboxylation would furnish a product (19) suitably activated, as the coenzyme A ester, for esterification to the necine base.

To elucidate the possible role of β-methylenenorvaline in senecic acid biosynthesis its synthesis was investigated. Levenberg had obtained β-methylenenorvaline (13) by a Strecker synthesis from 2-methylenebutanal [13] but in very low yield (*ca.* 0.5%), probably owing to competing lactonisation and double bond migration. It was considered necessary therefore to devise a route in which the double bond was carried through the synthesis in protected form until a late stage. The protecting method chosen was to mask the methylene group as a dimethylaminomethyl group and to generate the methylene group by conversion into the *N*-oxide followed by Cope elimination (Scheme 5). The required



SCHEME 5 Reagents: i, HCN–$(NH_4)_2CO_3$; ii, $Ba(OH)_2$; iii, Ac_2O; iv, EtOH–*NN'*-dicyclohexylcarbodi-imide; v, *m*-$ClC_6H_4{\cdot}CO_3H$; vi, HCl

[13] B. Levenberg, *J. Biol. Chem.*, 1968, **243**, 6009.
[14] L. B. Bull, C. C. J. Culvenor, and A. T. Dick, 'The Pyrrolizidine Alkaloids,' North-Holland, Amsterdam, 1968.

amino-acid (22) was prepared from the corresponding aldehyde (20) [15] *via* the hydantoin (21), by the Bucherer modification of the Strecker reaction. Before proceeding to the Cope elimination the carboxy- and primary amino-groups were protected by acetylation to give the acid (23) and conversion into the corresponding ester (24). Conversion of the protected amino-acid into the *N*-oxide (25) proceeded smoothly on oxidation with *m*-chloroperbenzoic acid.[16] The intermediates (21)—(25) were all presumed to be diastereoisomeric mixtures. However, since the asymmetry of C-3 [*cf.* (25)] was to be destroyed in a later step, no attempt was made to separate the diastereoisomers. Nevertheless the hydantoin (21) and the *N*-oxide (25) were obtained as crystalline solids for which elemental analyses could be obtained. The amino-acid (22) was obtained as a solid and the *N*-acetyl derivative (23) as a gum. The fully protected compound (24) was obtained as a distillable oil for which a satisfactory elemental analysis was obtained. A by-product from the hydrolysis of the hydantoin (21) was the hydantoic acid (27). A small quantity of this compound was carried through the next two stages when it was separated as the corresponding ethyl ester (28). The *N*-oxide (25) was pyrolysed to give the ethyl ester

$MeCH_2{\cdot}CH(CH_2NMe)_2{\cdot}CH(NHCONH_2){\cdot}CO_2R$ $MeCH{=}CMe{\cdot}CH(NH_2){\cdot}CO_2H$

(27) R = H
(28) R = Et (29)

of the *N*-acetyl derivative (26; R = Et) of β-methylenenorvaline (13) and the precursor (24) in approximately equal amounts. Alkaline hydrolysis of the ester group in the ester (26; R = Et) gave the *N*-acetyl derivative of β-methylenenorvaline in 84% yield. However, stepwise removal of the ester and *N*-protecting groups was found not to be necessary as hydrolysis of the *N*-acetyl ethyl ester (26; R = Et) with dilute hydrochloric acid gave β-methylenenorvaline (13) in 83% yield. The product as isolated was pure by amino-acid analysis.

The synthetic route thus established demonstrated the feasibility of producing β-methylenenorvaline (13) by a method which avoided problems arising from lactonisation and double bond migration. However this synthesis was too lengthy to be considered a practicable route to labelled β-methylenenorvaline (13). At this point we became aware of a direct synthesis of β-methylenenorvaline (13) from 2-methylenebutanal by a mild version of the Strecker reaction.[17] Through the courtesy of Dr. G. Dardenne we were able to examine the amino-acid produced by this method. The compound gave a single spot on paper chromatography with an R_F value identical with that of authentic β-methylenenorvaline (13) kindly provided by Dr. B. Levenberg. However the m.p. was 237—240 °C, considerably higher than that reported [13] by Levenberg (204—205 °C) and that of the amino acid produced by the synthesis described above (215—216 °C). Amino-acid analysis of the high-melting sample showed that it consisted of two components in the ratio 4 : 1. The n.m.r. spectrum showed signals expected for β-methylenenorvaline (13) but in addition a quartet at τ 5.11 and a superimposed doublet and singlet at τ 8.20 which were attributable to the double bond isomer (29) of β-methylenenorvaline (13). This isomer could not be removed by recrystallisation. However, the formation of β-methylenenorvaline (13) by a modified Strecker reaction indicated that if the conditions could be made less severe β-methylenenorvaline (13) might be obtained free from the undesired double-bond isomer. In the event, the use of a milder procedure for the hydrolysis of the intermediate aminonitrile gave β-methylenenorvaline (13) in 10% yield. In spite of the low yield the simplicity of this procedure commended itself as a means of obtaining labelled β-methylenenorvaline. Accordingly the synthesis was carried out using [^{3}H]formaldehyde. DL-β-[$^3H_2$]Methylenenorvaline (13) was obtained in 1.9% chemical yield and 0.08% radiochemical yield. The low radiochemical yield may have been due to radioautolysis of the tritiated formaldehyde. However, it is known that the equilibrium between formaldehyde, water, and formaldehyde hydrate [equation (1)] lies much further to the right for

$$HCHO + H_2O \rightleftharpoons HC(OH)_2 \quad (1)$$

[^{3}H]formaldehyde than for formaldehyde of normal isotopic composition.[18] In the Mannich reaction, therefore, unlabelled formaldehyde would be consumed preferentially and the tritiated formaldehyde would be concentrated in residual formaldehyde.

In spite of the low radiochemical yield sufficient tritiated β-methylenenorvaline (13) was obtained to permit the execution of duplicate incorporation experiments, with the results given in Table 5. Incorporations

TABLE 5

Incorporation of DL-β-[$^3H_2$]methylenenorvaline (13) into senecionine (2)

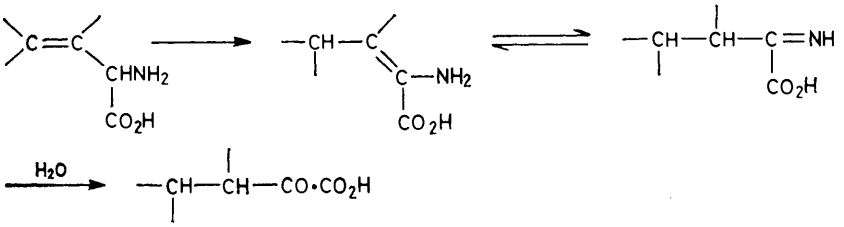
	Activity	
Labelled metabolite	Expt. 1	Expt. 1
Senecionine (2)	100 [a]	100 [b]
Senecic acid (1)	99	105
Retronecine (11)	3	0

Incorporations: [a] 0.11%; [b] 0.07% (based on DL-β-methylenenorvaline).

of the same order of magnitude as these found for L-isoleucine [1] were obtained and hydrolysis of the labelled alkaloid showed that the radioactivity was located exclusively in the necic acid. The low activity of the alkaloid precluded further degradation; therefore it is not possible to state whether β-methylenenorvaline (13)

[15] C. Mannich, B. Lesser, and F. Silten, *Chem. Ber.*, 1932, **65**, 378.
[16] J. C. Craig and K. K. Purushothaman, *J. Org. Chem.*, 1970, **35**, 1721.
[17] G. Dardenne, personal communication.
[18] H. Simon and G. Henbach, *Z. Naturforsch.*, 1963, **18b**, 160.

was incorporated into only one or into both halves of senecic acid.* Nevertheless, the results obtained provide firm evidence for the participation of β-methylenenorvaline (13) in senecic acid (1) biosynthesis. However, an alternative explanation for the specific incorporation of β-methylenenorvaline (13) is that the unsaturated amino-acid was converted adventitiously into the 2-oxo-acid analogue of isoleucine by double bond migration to give the corresponding enamine followed by enamine–imine rearrangement with subsequent hydrolysis of the imino-group (Scheme 6). Such



SCHEME 6

transformations of βγ-unsaturated amino-acids are known to be brought about by enzymes such as tryptophan synthetase [19] and serine dehydratase.[20] Transamination of the resulting oxo-acid would give isoleucine; the operation of this adventitious pathway therefore may explain, at least in part, the specific incorporation of β-methylenenorvaline (13) into senecic acid (1).

EXPERIMENTAL

All m.p.s are corrected. I.r. spectra were determined with a Hilger H900 Infrascan spectrometer and n.m.r. spectra with a Perkin-Elmer R60 or JEOL MH-100 spectrometer, for solutions in deuteriochloroform with tetramethylsilane as internal standard unless otherwise stated. Mass spectra were determined with a Perkin-Elmer–Hitachi RMU spectrometer with an electron beam energy of 80 eV. Paper chromatography of amino-acids was carried out on Whatman No. 1 paper in the solvent system butan-1-ol–acetic acid–water (37 : 9 : 25). Amino-acids were detected using ninhydrin (0.15% w/v in butan-1-ol). All radiochemicals were purchased from the Radiochemical Centre, Amersham, with the exception of [1-$^3H_2$]ethanol which was obtained from New England Nuclear Chemicals. Radioactivity measurements were made with a Packard Tri-Carb Series 2000 spectrometer in B.D.H. dioxan scintillator, Aquasol, or NE260 (Nuclear Enterprises Ltd.). *Senecio magnificus* and *S. isatideus* plants were propagated normally until required for incorporation experiments, when they were transferred to hydroponic solutions. Incorporation experiments and isolation and purification of the alkaloids were carried out as previously described.[1] Amino-acid analyses were carried out on a JEOL JAC 5AH amino-acid analyser.

(S)- *and* (RS)-[U-^{14}C]-2-*Methylbutanoic Acids*.—L-[U-^{14}C]Isoleucine (50 μCi; 10 mCi mmol^{-1}) in water (5 cm^3) was diluted with inactive L-isoleucine (100 mg). Ninhydrin (520 mg) in water (5 cm^3) was added, the solution was diluted to 50 cm^3 and distilled, with addition of water to the boiler so as to maintain a constant volume, until the distillate gave a negative result in a test for aldehyde (2,4-dinitrophenylhydrazine reagent). The distillate was divided into two equal portions. Potassium permanganate solution (0.3M; 1 cm^3) was added dropwise with vigorous stirring to one portion (7 cm^3) cooled in an ice-bath. The solution was acidified with sulphuric acid (50%) and extracted continuously with ether for 24 h. The ethereal solution was dried (Na_2SO_4), filtered, and evaporated to a clear oil. The acid was neutralised with calcium hydroxide solution [30 mg in water (50 cm^3)] with phenolphthalein as indicator. The solution was evaporated to dryness, the residue was dissolved in methanol with warming, and the methanolic solution was filtered and concentrated to give calcium (*S*)-[U-^{14}C]-2-methylbutanoate pentahydrate as prisms (24 mg, 38%; 7.4 μCi), $[\alpha]_D^{21}$ (free acid) 22.8 ± 0.8° (*c* 1.6 in H_2O) {lit.,[21] 15.58° (*c* 2.503 in H_2O); lit. (laevoratory isomer), −24° (*c* 0.9 in H_2O),[22] −25° (*c* 1 in H_2O) [23]}; radiochemical purity 100 ± 4% by dilution analysis. The remaining portion of (*S*)-[U-^{14}C]-2-methylbutanal in water (7 cm^3) was treated with pyridine (50 cm^3) and the mixture was heated to 90 °C for 8 h in a sealed container. The cooled solution was acidified (Congo Red) with sulphuric acid (50%) and distilled as above to give (*RS*)-[U-^{14}C]-2-methylbutanal, which was oxidised as before to give calcium (*RS*)-[U-^{14}C]-2-methylbutanoate pentahydrate (22 mg, 35%; 5.9 μCi), $[\alpha]_D^{21}$ (free acid) 1.4 ± 0.7° (*c* 0.8 in H_2O); radiochemical purity 97 ± 4% by dilution analysis.

[3-^{3}H]*Angelic Acid* (5).—(*E*)-3-Bromo-2-methylbut-2-enoic acid (β-bromoangelic acid) (6) (54 mg) [8] was stirred in tritiated water (0.4 cm^3; 16 mCi) over powdered sodum amalgam [sodium (108 mg) in mercury (3.46 g)] for 48 h at 5 °C under nitrogen. The solution was diluted with inactive water and decanted from the mercury, which was washed with two 1 cm^3 portions of water. The combined aqueous solutions were acidified (Congo Red) with hydrochloric acid (1 mol dm^{-3}) and extracted with light petroleum (b.p. 40—60 °C; 5 × 5 cm^3). The extracts were dried (Na_2SO_4), filtered, and evaporated to a clear oil. This was dissolved in light petroleum (b.p. 40—60 °C; 10 cm^3) and the solution was washed with water (10 cm^3). The aqueous washing was acidified (Congo Red) with sulphuric acid (0.1 mol dm^{-3}) and extracted with light petroleum (b.p. 40—60 °C; 5 × 5 cm^3). The combined organic solutions

* The apparent loss of only one of the six C-6 hydrogen atoms of the two molecules of isoleucine (3) during incorporation suggests that β-methylenenorvaline (13) is incorporated into only one of the two isoleucine-derived halves of senecic acid (1).

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were dried (Na_2SO_4) and evaporated to give angelic acid (5) (27 mg, 100%) as needles, m.p. 40—45 °C (lit.,[24] 44—46 °C); activity 39.5 μCi; radiochemical purity 95 ± 4% by dilution analysis.

Methyl 2-[^{14}C]*Methyl-3-oxobutanoate*.—[^{14}C]Methyl iodide (0.1 mCi; 55 mCi mmol^{-1}) was diluted with inactive material (284 mg) and treated with methyl 3-diethylaminobut-2-enoate (171 mg).[16] The mixture was sealed in a glass vial, left at room temperature overnight, and heated at 50—60 °C for 9 h. The cooled mixture was washed with dry ether (5 × 1 cm^3) by decantation. The viscous residue was heated with water (2 cm^3) at 60—65 °C for 30 min; the solution was then diluted with water (10 cm^3) and continuously extracted with ether for 24 h. The extract was dried and evaporated and the residue was distilled (b.p. 80—85 °C at 23 mmHg) to give the ester (113 mg, 87%), which gave a single peak on g.l.c. (Pye Argon Chromatograph; 15% silicone grease on Chromosorb P; column temperature 100 °C). Dilution analysis as 4,5-dihydro-3,4-dimethyl-5-oxopyrazole-1-carboxamide [25] indicated a radiochemical purity of 100%.

Hydrolysis of (a) *Senecionine* (2) *and* (b) *Retrorsine* (10).—(*a*) Hydrolysis of senecionine (2) to senecic acid (1) and retronecine (11) was carried out as previously described.[1]

(*b*) Retrorsine (200 mg) and barium hydroxide octahydrate (179 mg) were boiled under reflux in water (4.0 cm^3) for 2 h. The solution was cooled, treated with solid CO_2, and filtered. The filtrate (10 cm^3) was acidified (M-HCl) and extracted continuously with ether for 15 h. The extract was dried (Na_2SO_4), filtered, and evaporated. The residue was crystallised (ethyl acetate–light petroleum) to give isatinecic acid (9) (73 mg), m.p. 147—148 °C (lit.,[26] 148 °C). Retronecine (11), as the hydrochloride, was isolated as in the hydrolysis of senecionine (2).[1]

Synthesis of [6-^{14}C]*Isoleucine and* [6-^{3}H$_3$]*Isoleucine* (3).—[6-^{14}C]Isoleucine was prepared as described previously.[1] The same method was used to prepare [6-^{3}H$_3$]isoleucine from [^{3}H$_3$]methyl iodide. The product was shown by amino-acid analysis to consist of DL-isoleucine (47 ± 2%) and allo-DL-isoleucine (53 ± 2%). The radiochemical purity was found to be 100% by dilution analysis and >99% by paper chromatography with strip counting.

Degradation of Senecic Acid (1) *derived from* [6-^{3}H$_3$]-*Isoleucine*.—Senecic acid (1) (200 mg) in water (30 cm^3) was treated with sodium periodate (600 mg). After 3 days, iron(II) sulphate (900 mg) was added, the solution was filtered, and the filtrate was acidified (Congo Red) with hydrochloric acid (2 mol dm^{-3}) and extracted continuously with ether for 12 h. The extract was dried (Na_2SO_4) and evaporated. The oily product (180 mg) was dissolved in sodium hydroxide solution (M; 25 cm^3) and the solution was treated with iodine–potassium iodide solution until a brown colour persisted for 1 min. The precipitated iodoform was filtered off, and the solution was acidified (Congo Red) with hydrochloric acid (2 mol dm^{-3}) and extracted continuously with ether for 12 h. The extract was dried (Na_2SO_4) and evaporated. The residue was dissolved in ether (180 cm^3), and the solution was washed with sodium thiosulphate solution (2 × 10 cm^3) and water (2 × 10 cm^3) and evaporated. The acidic residue was dissolved in a solution of sodium periodate (1.07 g) and potassium permanganate (150 mg) in water (50 cm^3). After 6 h the solution was treated with a saturated solution of iron(II) sulphate until the permanganate colour was discharged. The mixture was filtered, the filtrate was acidified (Congo Red) with hydrochloric acid (2 mol dm^{-3}) and extracted continuously with ether for 12 h. The extract was dried (Na_2SO_4) and evaporated. The residue was freed from iodine by sublimation at 20 °C (14 mmHg). The remaining methylsuccinic acid was purified by sublimation at 95 °C (0.03 mmHg) and converted into the *dicyclohexylamine salt* (m.p. 158 °C) (Found: C, 65.3; H, 10.0; N, 5.0. $C_{17}H_{31}NO_4$ requires C, 65.1; H, 10.0; N, 4.5%).

DL-[4-^{3}H$_2$]*Isoleucine* (3).—(*a*) [1-^{3}H$_2$]Ethanol was converted into [1-^{3}H$_2$]ethyl iodide following the procedure of Murray and Ronzio [27] for the preparation of methyl iodide from methanol. In trial experiments yields of ⩾77% were obtained.

(*b*) [1-^{3}H$_2$]Ethyl iodide was converted into a mixture of allo-DL-[4-^{3}H$_2$]isoleucine and DL-[^{3}H$_2$]isoleucine as described previously,[1] to give a product of ⩾98% radiochemical purity (strip counting) as the mixture of stereoisomers.

Synthesis of β-*Methylenenorvaline* (13).—(a) 2-*Dimethylaminobutanal* (20). Butanal (78 g, 1.1 mol), dimethylammonium chloride (108 g, 1.34 mol), and aqueous formaldehyde (40% w/v; 155 cm^3, 1.29 mol) were stirred vigorously in a cold water-bath. After 3 min an exothermic reaction began and the temperature of the reaction mixture was maintained at 35—40 °C by cooling. After 5 min nearly all the butanal had dissolved and the mixture was cooled to 10 °C. Potassium hydroxide solution (50% w/v; 146 cm^3) was added with cooling to maintain the temperature below 25 °C. The resulting mixture was extracted with ether (6 × 75 cm^3), the extracts were dried ($MgSO_4$) and evaporated, and the residue was distilled at 55—61 °C and 16 mmHg (lit.,[15] 60° and 19 mmHg) to give the aldehyde (20) (66 g, 47%); $\nu_{max.}$ (neat) 2 840—2 760 (NMe_2) and 1 730 cm^{-1} (C:O); τ 0.41 (1 H, s, CHO), 7.57 (2 H, s, CH_2N), 7.79 (6 H, s, NMe_2), 8.47 (2 H, q, $MeCH_2$), and 9.08 (3 H, t, $MeCH_2$).

(b) 5-[1-(*Dimethylaminomethyl*)*propyl*]*hydantoin* (21). To dry hydrogen cyanide (8.5 g, 0.33 mol) cooled in an ice-bath was added saturated sodium carbonate solution (0.5 cm^3) followed by the aldehyde (20) (8.7 g, 0.06 mol). The mixture was allowed to warm to room temperature with stirring over 1 h. Ammonium carbonate (11.5 g, 0.12 mol) in water (30 cm^3) was added and the mixture was stirred at 50—55 °C for 2 h, during which time an oily layer separated. The temperature was raised to 82 °C over 15 min; the upper layer then dissolved to give an orange solution. The mixture was left overnight to give a yellow solid (1.9 g). Concentration of the filtrate gave more yellow solid (9.6 g). The combined solid fractions were recrystallised (ethyl acetate) to give the *hydantoin* (21) as tiny colourless needles, m.p. 138—140° (4.6 g) (Found: C, 54.55; H, 8.6; N, 21.3. $C_9H_{17}N_3O_2$ requires C, 54.25; H, 8.6; N, 21.2%); $\nu_{max.}$ 3 300—3 200 (NH) and 1 730—1 710 cm^{-1} (amide C:O); τ [$(CD_3)_2SO$] 2.14br and 2.27br (2 × 1 H, 2 × s, 2 × CONHCO), 5.76br (1 H, s, CNH*H*CO or NHC*H*CO), 5.97br (1 H, s, CHN*H*CO or NHC*H*CO), 7.43—8.25 (3 H, m, N·C*H*$_2$·C*H*), 7.86 and 7.97 (each 3 H, s, NMe_2), 8.8 (2 H, m, $MeCH_2$), and 9.09 (3 H, t, $MeCH_2$); *m/e* 199 (M^+) and 58 (base peak, CH_2NMe_2).

(c) 2-*Amino*-3-*dimethylaminomethylpentanoic acid* (22).

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The hydantoin (21) (500 mg, 2.5 mmol) and barium hydroxide octahydrate (645 mg, 3.77 mmol) were boiled under reflux in water (10 cm^3) for 90 h. The mixture was cooled, the precipitated barium carbonate was filtered off, and the filtrate was treated with ammonium carbonate (500 mg) in water (5 cm^3) and again filtered. Evaporation of the filtrate gave a white solid (450 mg) which was triturated with acetone and removed by filtration to give 2-amino-3-dimethylaminomethylpentanoic acid (22) as a solid (408 mg) which did not crystallise; τ (D_2O) 5.78 (1 H, apparent d, *J* 4 Hz, NCHCO), 6.87 (2 H, d, *J* 7 Hz, NCH_2), 7.11 (6 H, s, NMe_2), 7.8 (1 H, m, NCH_2CH), 8.65 (2 H, m, $MeCH_2$), and 9.1 (3 H, t, *J* 6 Hz, $MeCH_2$), *m/e* 174 (M^+).

Partial hydrolysis of the hydantoin (21). The hydantoin (500 mg, 2.5 mmol) and barium hydroxide octahydrate (900 mg, 5.3 mmol) were boiled under reflux in water (25 cm^3) for 24 h. The mixture was cooled, treated with a solution of ammonium carbonate (1 g) in water (10 cm^3), and filtered. The filtrate was evaporated at 40—50 °C to give a solid which was extracted with boiling ethanol (1 × 10 cm^3, 2 × 5 cm^3). The extracts were filtered and evaporated. The partially solidified residue (476 mg) was twice recrystallised (methanol–acetone) to give the *hydantoic acid* (27), m.p. 200 °C (decomp.) (Found: C, 49.7; H, 9.0; N, 18.9. $C_9H_{19}N_3O_3$ requires C, 49.8; H, 8.8; N, 19.3%); *m/e* 217 (M^+).

(d) *2-Acetylamino-3-dimethylaminomethylpentanoic acid* (23). 2-Amino-3-dimethylaminomethylpentanoic acid (22) (4 g, 23 mmol) in sodium hydroxide solution (2 mol dm^{-3}; 20 cm^3) was treated below 25 °C with acetic anhydride (6.1 cm^3, 64 mmol) in small portions. The mixture was allowed to warm to room temperature over 1.5 h and was applied to a column of Dowex 50W-X8 ion-exchange resin (H^+ form; 40 g). The column was washed with water (200 cm^3) and the amino-acid was eluted with dilute ammonia (3% w/w; 500 cm^3). Evaporation gave the *N*-acetyl derivative (23) as a gum which did not crystallise; τ 8.00 (3 H, s, MeCO).

(e) *Ethyl 2-acetylamino-3-dimethylaminomethylpentanoate* (24). The crude amino-acid (23) (3 g, 14 mmol) in dichloromethane (30 cm^3) was treated with ethanol (1 g, 21 mmol) followed by a solution of *NN'*-dicyclohexylcarbodi-imide (3.2 g, 15.5 mmol) in dichloromethane (10 cm^3). The mixture was set aside for 24 h, filtered, and evaporated to dryness, and the residue was triturated with ether (60 cm^3). The mixture was filtered and the filtrate concentrated to 20 cm^3, set aside at 3 °C overnight, and filtered, to give a solid, m.p. 133—135 °C (286 mg). This was dissolved in chloroform; the solution was filtered and evaporated and the residue recrystallised (chloroform–ether) to give the *hydantoic acid ester* (28) as needles, m.p. 144—145° (100 mg) (Found: C, 53.9; H, 10.0; N, 17.4. $C_{11}H_{23}N_3O_3$ requires C, 53.85; H, 9.45; N, 17.1%); $\nu_{max.}$ 3 480 (NH) and 1 725 cm^{-1} (ester C:O); τ (220 MHz) 5.59 (1 H, m, NHC*H*CO), 5.80 (2 H, q, *J* 7 Hz, $MeCH_2$·O·CO), 7.81 (1 H, m, N·CH_2·C*H*), 7.77 (6 H, s, Me_2N), 7.92 (2 H, m, CH_2·N), 8.72 (3 H, t, *J* Hz, $MeCH_2$·O), 8.76 (2 H, m, $MeCH_2$·CMe), and 9.10 (3 H, t, *J* 7 Hz, $MeCH_2CMe$); *m/e* 245 (M^+). After removal of the hydantoic acid ester (28) the ethereal filtrate was evaporated to give an oil (3.7 g) which was distilled at 96—106 °C and 0.01 mmHg, to give the *ester* (24) (Found: C, 59.1; H, 10.0; N, 11.5. $C_{12}H_{24}N_2O_3$ requires C, 59.0; H, 9.9; N, 11.5%); τ 1.5br (1 H, d, *J* 9 Hz, NH), 5.33 (1 H, dd, *J* 9 and 3 Hz, NHC*H*), 5.82 (2 H, q, *J* 7 Hz, $MeCH_2O$), 7.77 (6 H, s, Me_2N), 8.02 (3 H, s, MeCO), 8.75 (3 H, t, *J* 7 Hz, $MeCH_2O$), and 8.95 (3 H, m, $MeCH_2$·CH); $\nu_{max.}$ 3 280 (NH), 2 770, 2 830 (NMe_2), 1 740 (ester C:O), and 1 655 cm^{-1} (amide C:O); *m/e* 244 (M^+).

(f) N-*Oxide of ethyl 2-acetylamino-3-dimethylaminomethylpentanoate* (25). An ice-cold solution of the ester (24) (3.8 g, 15.5 mmol) in chloroform (15 cm^3) was treated dropwise with a solution of *m*-chloroperbenzoic acid (3.2 g, 18.6 mmol) in chloroform (15 cm^3). The stirred mixture was allowed to warm to room temperature over 3 h and was set aside overnight. The solution was applied to a column of alumina (Woelm basic; grade III; 65 g) and the *N*-oxide (25) was eluted with chloroform. Evaporation gave the *N*-oxide (25) as an oil which slowly crystallised as a hygroscopic solid. Recrystallisation from sodium-dried benzene–light petroleum gave the N-*oxide* (25), m.p. 97—99 °C (Found: C, 54.8; H, 9.45; N, 10.8. $C_{12}H_{24}N_2O_4$ requires C, 55.35; H, 9.3; N, 10.75%); τ −2.1br (1 H, s, NH), 5.80 (2 H, q, *J* 7 Hz, $MeCH_2O$), 6.16 (2 H, m, CH_2N^+·O^-), 6.67 and 6.88 (each 3 H, s, NMe), 7.5 (1 H, m, CH·CH_2·N^+·O^-), 8.05 (3 H, s, MeCO), 8.47 (2 H, m, $MeCH_2$·CH), 8.73 (3 H, t, *J* 7 Hz, $MeCH_2$·O), and 8.89 (3 H, m, $MeCH_2$·CH), $\nu_{max.}$ 3 200 (NH), 1 739 (ester C:O), and 1 665 cm^{-1} (amide C:O).

(g) *Ethyl 2-acetylamino-3-methylenepentanoate* (26; R = Et). The *N*-oxide (25) (3.8 g) was slowly heated to 95—100 °C *in vacuo*, at which temperature it effervesced. The temperature was maintained at 100—105 °C for 15 min (until effervescence stopped). The temperature was then raised to 135 °C to complete the reaction. A solution of the residue in ether (100 cm^3) was washed with hydrochloric acid (0.1 mol dm^{-3}) until the washings were acidic. The combined aqueous washings were made strongly acidic with hydrochloric acid (1 mol dm^{-3}) and extracted with ether (8 × 20 cm^3). The combined extracts were dried and evaporated, and the residual oil was distilled to give the *ester* (26; R = Et), b.p. 98—99 °C at 0.01 mmHg (1.3 g, 54%) (Found: C, 59.7; H, 8.4; N, 6.95. $C_{10}H_{17}NO_3$ requires C, 60.3; H, 8.6; N, 7.0%); τ 3.65br (1 H, s, NH), 4.95—5.05 (3 H, m, CH_2:C, NHC*H*), 5.82 (2 H, q, *J* 7 Hz, $MeCH_2$), 7.89 (2 H, q, *J* 7 Hz, $MeCH_2O$), 8.77 (3 H, t, *J* 7 Hz, $MeCH_2O$), and 8.95 (3 H, t, *J* 7 Hz, $MeCH_2$), $\nu_{max.}$ 3 300 (NH), 1 745 (ester C:O), and 1 655 cm^{-1} (amide C:O, C:CH_2); *m/e* 199 (M^+) and 84 (base peak).

(h) *2-Acetylamino-3-methylenepentanoic acid* (26; R = H). The ester (26; R = Et) (420 mg) in aqueous ethanol (60% v/v; 3 cm^3) was treated with sodium hydroxide (88 mg) in aqueous ethanol (60% v/v; 2 cm^3). The solution was treated with water (3 and 20 cm^3 after 24 and 48 h, respectively). The mixture was filtered and the filtrate was extracted with ether (3 × 10 cm^3), acidified (Congo Red) with hydrochloric acid, and extracted continuously with ether for 36 h. The extract was dried and evaporated to give an oil (291 mg, 82%) which slowly crystallised. The product was triturated with chloroform to give plates (222 mg), m.p. 142—144 °C. Recrystallisation (ethanol–chloroform) gave the *acid* (26; R = H), m.p. 144—145 °C (Found: C, 55.9; H, 7.8; N, 8.15. $C_8H_{13}NO_3$ requires C, 56.1; H, 7.65; N, 8.2%); τ [$(CD_3)_2SO$] 1.76 (1 H, d, *J* 8 Hz, NH), 4.98br and 5.06br (each 1 H, s, C:CH_2), 5.21 (1 H, d, *J* 8 Hz, C*H*NH), 7.89 (2 H, q, *J* 7 Hz, $MeCH_2$), 8.12 (3 H, s, MeCO), and 9.00 (3 H, t, *J* 7 Hz, $MeCH_2$); $\nu_{max.}$ 3 320 (OH), 1 715 (CO_2H), 1 645 (C:C), 1 600 (CONH), and 895 cm^{-1} (C:CH_2).

(i) β-*Methylenenorvaline* (13). The ester (26; R = Et) (96 mg) was boiled under reflux in hydrochloric acid (1 mol

dm^{-3}; 1 cm^3) for 10 h. The solution was treated with water (20 cm^3), extracted with ether (4 × 8 cm^3), and applied to a column of Dowex 50W-X8 cation-exchange resin (H^+ form; 5 g). The column was washed with water and the amino-acids were eluted with aqueous ammonia (3% w/v; 200 cm^3). The ammoniacal solution was evaporated to give a crystalline solid (50 mg, 83%), shown by paper chromatography and amino-acid analysis to consist of pure β-methylenenorvaline (13). Recrystallisation (aqueous ethanol) gave the amino-acid as flakes, m.p. 215—216 °C (Found: C, 55.4; H, 8.7; N, 10.8. Calc. for $C_6H_{11}NO_2$: C, 55.8; H, 8.6; N, 10.8%); τ ($CF_3{\cdot}CO_2H$) 2.42br (3 H, s, NH_3^+), 4.46br (2 H, s, $C{:}CH_2$), 5.13 (1 H, q, *J* 6 Hz, $CHNH_3^+$), 7.69 (2 H, q, *J* 7 Hz, $MeCH_2$), and 8.81 (3 H, t, *J* Hz, $MeCH_2$); $\nu_{max.}$ 3 360 (NH_3^+), 1 630 (C:C), 1 580 (CO_2^-), and 900 cm^{-1} ($C{:}CH_2$).

Synthesis of DL-β-[$^3H_2$]*Methylenenorvaline* (13).—[$^3H_2$]-Formaldehyde solution (1%; 0.15 cm^3; 5 mCi), inactive formaldehyde (40%; 4.65 cm^3), dimethylamine hydrochloride (4.9 g), and butanal (4.5 cm^3) were heated with stirring at 70—75 °C for 24 h. The solution was diluted with water (5 cm^3) and distilled until no more aldehyde appeared in the distillate. The organic layer was separated, the aqueous residue was extracted with ether (5 cm^3), and the organic layer and ethereal extract were combined, dried, and distilled. The entire distillate was cooled to 0 °C and was treated with an ice-cold solution of ammonium chloride (1.8 g) in water (5 cm^3) and, dropwise, with an ice-cold solution of sodium cyanide (1.5 g) in water (4 cm^3). The mixture was shaken at 4 °C for 4 h, and at room temperature for 4 h. Concentrated hydrochloric acid (6 cm^3) was added and the mixture was taken to dryness on a steam-bath. The residue was extracted with ethanol (10 cm^3), the ethanolic extract was filtered and evaporated, and the residue was applied in aqueous solution to a column of Dowex 50W-X8 ion-exchange resin (H^+; 40 g). The column was washed with water and the amino-acid was eluted with 2M-ammonium hydroxide. The eluate was evaporated to dryness and the residue was crystallised (ethanol–water) to give DL-β-[$^3H_2$]methylenenorvaline (73 mg; 4.1 μCi; 0.91% based on formaldehyde; radiochemical yield 0.082%); 100% radiochemical purity by dilution analysis.

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