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PYRROLIZIDINE ALKALOIDS
THE BIOSYNTHESIS OF SENECIPHYLLIC ACID

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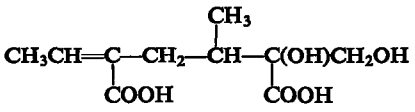
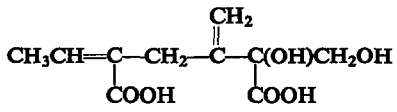
Abstract—The biosynthesis of seneciphyllic acid in *Senecio douglasii* DC. has been investigated. Incorporation studies with [^{14}C]-acetate and [2-^{14}C]-mevalonate have shown that biosynthesis of the acid does not involve either direct condensation of acetate units or participation of the acetate-mevalonate pathway. High, specific incorporations were observed with [U-^{14}C]-L-threonine and [U-^{14}C]-L-isoleucine. The results of degradative experiments indicate that L-threonine is incorporated after transformation into L-isoleucine. It is suggested that L-isoleucine furnishes a five-carbon-atom fragment from which seneciphyllic acid is derived by coupling with another five-carbon-atom unit of different origin. Incorporation experiments with [Me-^{14}C]-L-methionine and [^{14}C]-formate show that C_8 in seneciphyllic acid is provided by a one-carbon-atom donor. On the basis of the present studies, a general hypothesis is proposed for the biosynthesis of some of the acids encountered among the pyrrolizidine alkaloids.

THE occurrence among the pyrrolizidine alkaloids of acids of several structural types has been demonstrated.[1] The carbon skeleton associated with one of the larger groups is exemplified by retronecic acid (I), obtained by hydrolysis of the alkaloid retrorsine, the diester of retronecic acid with the pyrrolizidine base retronecine* (V).[2,3]

Acids in this group have an apparent isoprenoid skeleton, with, however, an unusual tail-to-middle union of the constituent C_5 units and unusual oxygenation patterns. Various



I



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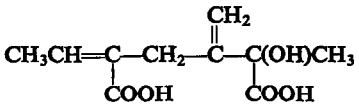

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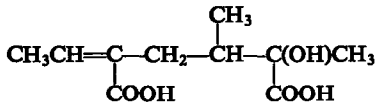

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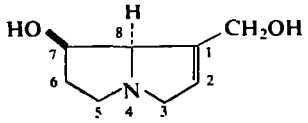


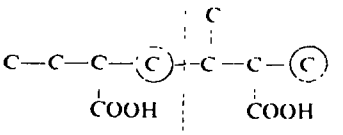
V

* The generic names "necine base" and "necic acid" are used to describe the pyrrolizidine bases and the esterifying acids, respectively, in this series.

[1] R. H. F. MANSKE (Ed.) *The Alkaloids* (a) **1**, 108 (1950); (b) **6**, 37 (1960). Academic Press, NewYork.
[2] S. M. H. CHRISTIE, M. KROPMAN, E. C. LEISEGANG and F. L. WARREN, *J. Chem. Soc.* 1700 (1949).
[3] S. M. H. CHRISTIE, M. KROPMAN, L. NOVELLIE and F. L. WARREN, *J. Chem. Soc.* 1703 (1959).

speculations have been made as to the biosynthetic origins of these compounds.[4–7] Published experimental evidence for their biosynthesis is up to the present time based solely on the investigations of Hughes and Warren, who have studied the biosynthesis of retronecic acid in *Senecio isatideus*.[8]

On the basis of acetate incorporation studies Hughes and Warren proposed a biogenetic route which involved condensation of acetate to acetoacetate, insertion of a one-carbon unit at the α-position, and, joining of two of the resulting five-carbon-atom units as indicated in VI.



VI

-- (C)— from a one-carbon donor

We have carried out related studies with *Senecio douglasii* DC., a plant common to the South-western United States. *S. douglasii* produces four closely related alkaloids, senecionine, retrorsine, seneciphylline and riddelline, which are the diesters with retronecine (V) of senecic (IV), retronecic (I), seneciphyllic (III) and riddellic (II) acids respectively.[9–11] Present investigations were based on seneciphyllic acid (III) since the parent alkaloid, seneciphylline, is the major component of the mixture, occurring to the extent of 60–65 per cent.

S. douglasii plants were grown in hydroponic solution with continuous aeration. ^{14}C-labelled precursors were administered by adding them in aqueous solution to the nutrient. After eight to ten days the alkaloids were extracted and the seneciphylline isolated and purified by chromatography and recrystallisation. Alkaline hydrolysis of the pure alkaloid gave retronecine and seneciphyllic acid. The seneciphyllic acid was degraded in a stepwise manner by the methods described below and the activities of the various degradation products determined.

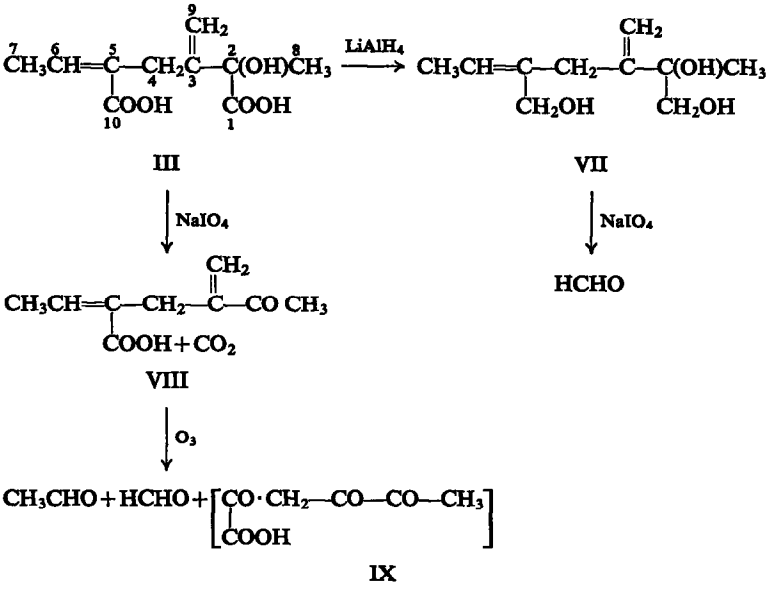
Degradation for C_1

Seneciphyllic acid was reduced with lithium aluminium hydride and the resulting 1,2-diol (VII) cleaved with sodium periodate to give C_1 as formaldehyde. This was isolated and purified as the dimedone derivative.

Degradation for C_9 and the Unit C_6—C_7

Ozonolysis of seneciphyllic acid was reported to give acetaldehyde consistently but formaldehyde only occasionally and in small amounts.[12] This finding has been confirmed in the

[4] F. L. WARREN, *Fortschr. Chem. Org. Naturstoffe* **12**, 230 (1955).
[5] F. L. WARREN, *Record Chem. Progr.* **20**, 18 (1959).
[6] R. ADAMS and M. GIANTURCO, *Festschr. A. Stoll* 72 (1957), Birkhaüser, Basel.
[7] R. ADAMS and M. GIANTURCO, *Angew. Chem.* **69**, 5 (1957).
[8] C. HUGHES and F. L. WARREN, *J. Chem. Soc.* 34 (1962).
[9] R. ADAMS and T. R. GOVINDACHARI *J. Am. Chem. Soc*, **71**, 1956 (1949).
[10] F. L WARREN, M. KROPMAN, R. ADAMS, T. R. GOVINDACHARI and J. H. LOOKER, *J Am. Chem. Soc.* **72**, 1421 (1950).
[11] R. ADAMS and J. H. LOOKER, *J. Am. Chem. Soc*, **73**, 134 (1951).
[12] R. ADAMS, T. R. GOVINDACHARI, J. H. LOOKER and J. D. EDWARDS, *J. Am. Chem. Soc.* **74**, 700 (1952).



III VII VIII IX

present work. The equivalent procedure of simultaneous hydroxylation with permanganate and periodate cleavage, either at pH 7–8 by the procedure of Lemieux and Rudloff using catalytic amounts of permanganate,[13] or under acidic conditions using stoichiometric amounts of permanganate, gave both acetaldehyde and formaldehyde in equivalent amounts. These were isolated as the dimedone derivatives. The mixture of derivatives was heated in glacial acetic acid, conditions under which the acetaldehyde derivative was converted to the neutral anhydro compound leaving the formaldehyde derivative unaffected.[14] The latter, being alkali-soluble, was readily separated from the anhydro-acetaldehyde derivative.

Degradation for C_8

Seneciphyllic acid has been oxidised with lead tetraacetate to the keto-acid (VIII).[12] This reaction is not easy to control and considerable over-oxidation occurs, making it difficult to isolate the product in a pure form. Sodium periodate has been found to oxidise seneciphyllic acid smoothly to give the keto-acid (VIII) in a high state of purity and in good yield. Under the conditions used (0·1 M initial concentration of periodate, two-times excess), oxidation was complete after 19–24 hr at room temperature. The keto-acid was isolated and characterised as the thiosemicarbazone and as the 2,4-dinitrophenylhydrazone. In degradation experiments the excess periodate was destroyed with sulphur dioxide and the solution of the keto-acid treated directly with sodium hydroxide-iodine reagent to give C_8 as iodoform.

Degradation for C_{10}

The keto-acid (VIII), obtained as above, was ozonised to give the acid (IX). This was not isolated but was reduced with lithium aluminium hydride and the product cleaved with periodate as in the isolation of C_1, to give C_{10} as formaldehyde.

[13] R. U. Lemieux and E. Von Rudloff, *Can. J. Chem.* **33**, 1701, 1710 (1955).
[14] D. Vorländer, C. Ihle and H. Volkholz, *Z. Anal. Chem.* **77**, 321 (1929).

It was anticipated that this procedure might lead to the isolation of the C_2—C_8 unit by the sequence shown in Scheme 1. However, no acetaldehyde could be detected in the final product. It is possible that reduction of the α-diketo grouping (IX) was prevented by enolisation or that it did not survive the ozonolysis step.

$$\underset{\text{IX}}{\text{R—CO—CO—CH}_3} \xrightarrow{\text{LiAlH}_4} \text{R—CH(OH)CH(OH)CH}_3 \xrightarrow{\text{NaIO}_4} \text{R—CHO} + \text{OHCCH}_3$$

SCHEME 1. (R = —CH_2—CO—COOH).

Degradation for C_7 and C_2

Kuhn-Roth oxidation of seneciphyllic acid gave consistently 2·0 moles of acetic acid. This was isolated as crystalline barium acetate (C_2-C_8, C_6-C_7) which was degraded by the Schmidt reaction to give methylamine (C_7+C_8), isolated as 5-methylamino-2,4,-dinitrotoluene, and carbon dioxide (C_2+C_6), isolated as barium carbonate. The activity of C_2 was obtained by subtracting from the barium carbonate activity, the activities of the C_6-C_7 unit and C_8. Owing to the accumulation of errors this method was not regarded as highly reliable but was used to give an approximate activity for C_2 in lieu of alternative procedures.

The activity of C_7 was obtained by subtracting the activity of C_8 from the activity of the methylamine derivative (C_7+C_8).

Because of uncertainties in absorption and metabolic utilization of administered compounds, absolute incorporation rates are not a reliable guide to the specificity of a given precursor for alkaloid biosynthesis.[15] In the present studies a more reliable criterion has been the comparison of the specific activities of retronecine and seneciphyllic acid after hydrolysis of seneciphylline. Precursors entering general metabolic pools before incorporation into the alkaloids would be expected to label the acid and base to a comparable extent. Precursors specific for seneciphyllic acid biosynthesis should be preferentially incorporated into the acid moiety. Since complete non-specificity was revealed by an equal distribution of activity between the acid and base components, the specificity of a given precursor was taken as $x-(100-x)$ where x was the percentage incorporation into seneciphyllic acid relative to seneciphylline.

The overall incorporation rates were based on the activities of the total alkaloid mixture (designated for convenience by the obsolete name "douglasiine"[16]); this procedure being justified by the close structural similarities between the component alkaloids.

RESULTS

Labelled acetate was incorporated into "douglasiine" at low rates, comparable to those observed by Hughes and Warren for retronecic acid. Typical values are given in Table 1. The acid-base activity balance indicated nearly complete randomisation, a result which is not in accord with a direct (i.e. immediate) biosynthetic route from acetate. Stepwise degradation revealed the incorporation pattern shown in scheme 2, which differs radically from that recorded for retronecic acid. The only clear point of agreement with the observations of Hughes and Warren is the very low incorporation of activity into C_8 from both [1-^{14}C] and [2-^{14}C]-acetate. Apart from this result, the distribution of activity in both cases reinforces the

[15] E. LEETE, *J. Am. Chem. Soc.* **86**, 2509 (1964).
[16] R. H. F. MANSKE (Ed.) *The Alkaloids* **1**, 109 (1950).

conclusion already arrived at from consideration of the acid-base activity balance, that acetate must be incorporated by an indirect route and not by direct condensation to immediate precursors of the necic acid.

TABLE 1. INCORPORATION OF [1-^{14}C]-ACETATE AND [2-^{14}C]-ACETATE INTO "DOUGLASIINE" AND DISTRIBUTION OF ACTIVITY IN SENECIPHYLLINE

Acetate label	Percentage incorporation into "douglasiine"	Percentage activity in	
		Seneciphyllic acid	Retronecine
1	0·036	46·4	50·4
2	0·014	38·0	58·0
2	0·025	39·5	60·5

This conclusion obtained further support from the results of experiments in which [3-^{14}C]-acetoacetate was fed. The precursor was administered both as the free acid and as the ethyl ester. In both cases neither the overall incorporation rates not the acid-base activity ratio differed significantly from the results of the feeding experiments with correspondingly labelled acetate (Table 2). If acetoacetate were a more direct precursor to seneciphyllic acid than acetate, a much higher relative incorporation into the necic acid should have been observed.

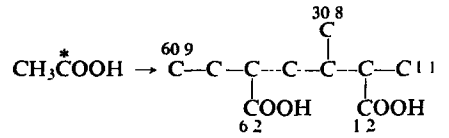
TABLE 2. INCORPORATION OF [3-^{14}C]-ACETOACETATE INTO "DOUGLASIINE" AND DISTRIBUTION OF ACTIVITY IN SENECIPHYLLINE

Precursor	Percentage incorporation into "douglasiine"	Percentage activity in	
		Seneciphyllic acid	Retronecine
Acid	0·01	47·6	43·0
Acid	0·032	41·0	63·0
Ethyl ester	0·032	36·1	67·2

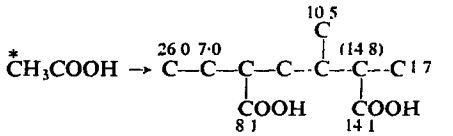
Percentage activity in C_6-C_7 unit (seneciphyllic acid = 100); 47·4 and, 42·1 (last two experiments); in C_7, 36·7 (last experiment).

The results obtained suggest that the acetoacetate was degraded to acetate before incorporation into the alkaloids. On this view, the observed incorporation of 42–47 per cent of the activity into the ethylidene group (C_6-C_7) with the predominant amount (37 per cent) in C_7, is consistent with the high incorporation of [1-^{14}C]-acetate into C_7 (Scheme 2).

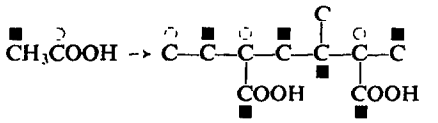
The acetate–acetoacetate direct pathway having been discarded on the basis of the foregoing evidence, attention was directed to the acetate-mevalonate pathway as an alternative route to the necic acids. On the assumption that acetate is incorporated via mevalonate, the following pattern of acetate incorporation into seneciphyllic acid would be expected (Scheme 3).



(Total activity of acid = 100)



SCHEME 2. DISTRIBUTION OF ACTIVITY IN SENECIPHYLLIC ACID FROM ACETATE FEEDING.



SCHEME 3. EXPECTED INCORPORATION PATTERN OF ACETATE INTO SENECIPHYLLIC ACID VIA THE ACETATE-MEVALONATE PATHWAY.

The results obtained (c.f. Scheme 2) do not agree with these predictions. In particular, C_8, which should have been provided by acetate-methyl was inactive in seneciphyllic acid derived from both acetate-1-C^{14} and acetate-2-C^{14}. Also, C_{10}, where exclusive incorporation of acetate-methyl would have been expected, was provided equally well by acetate-methyl or -carboxyl.

In order to gain more information, the incorporation of [2-^{14}C]-mevalonate was investigated. Overall incorporation rates for this precursor were no higher than for acetate (Table 3) although greater specificity was observed. The direct utilisation of C-2-labelled mevalonate would have been expected to provide C_1 or C_8 very efficiently. However, degradation of the [2-^{14}C]-mevalonate derived seneciphyllic acid showed that these positions carried respectively 0·35 and 1·7 per cent of the total activity. Further degradation was precluded by the very low activity of the acid obtained in this experiment but the results obtained clearly exclude the acetate-mevalonate pathway from involvement in necic acid biosynthesis.

During preliminary experiments in which various ^{14}C-labelled amino acids were screened as possible precursors it was observed that (U-^{14}C]-L-threonine was incorporated into the alkaloids at a significantly higher rate than other compounds (Table 4). This result was con-

TABLE 3. INCORPORATION OF [2-^{14}C]-DL-MEVALONATE INTO "DOUGLASINE" AND DISTRIBUTION OF ACTIVITY IN SENECIPHYLLINE

Percentage incorporation into "douglasine"	Percentage activity in	
	Seneciphyllic acid	Retronecine
0·028	85	10
0·023	70	27

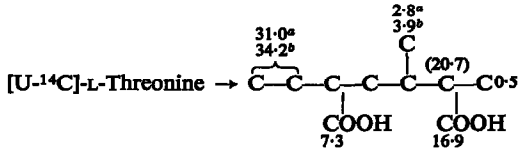
TABLE 4. INCORPORATION OF [U-^{14}C]-L-THREONINE INTO "DOUGLASIINE" AND DISTRIBUTION OF ACTIVITY IN SENECIPHYLLINE

Percentage incorporation into "douglasiine"	Percentage activity in	
	Seneciphyllic acid	Retronecine
0·42	—	—
0·15	100·0	2·8
0·35	99·1	0·7

firmed in later experiments and the high incorporation rate was found to be associated with almost complete (> 99 per cent) specificity for necic acid biosynthesis. Overall incorporation rates into seneciphyllic acid were twenty to thirty times greater than for acetate.

It was considered at the time that L-threonine might provide a four-carbon-atom unit which by insertion of a one-carbon unit at the α-position could be converted to a five-carbon intermediate. Two such five-carbon units might then be joined in the manner envisaged by Hughes and Warren for their similar acetoacetate-derived five-carbon unit.

Degradation of [U-^{14}C]-L-threonine-derived seneciphyllic acid gave the incorporation pattern shown in Scheme 4. The results show that threonine was *not* incorporated in a symmetrical manner and that the simple mode of utilisation envisaged above must therefore be incorrect.



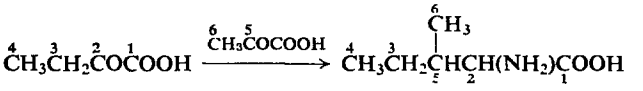
[a] Ozonolysis.
[b] Periodate/permanganate.

SCHEME 4. DISTRIBUTION OF ACTIVITY IN SENECIPHYLLIC ACID FROM [U-^{14}C]-L-THREONINE FEEDING.

A major pathway of threonine metabolism leads to α-ketobutyric acid[17–21] which is used in isoleucine biosynthesis in the manner shown in Scheme 5.[21–26] It has been reported that isoleucine is metabolised to propionyl CoA and acetyl CoA via tiglyl CoA.[27] Both tiglic acid and its isomer angelic acid occur in ester form among the pyrrolizidine alkaloids. In particular, angelic acid occurs esterified to the C_7 hydroxyl group of the necine base in several alkaloids.[1a, b]

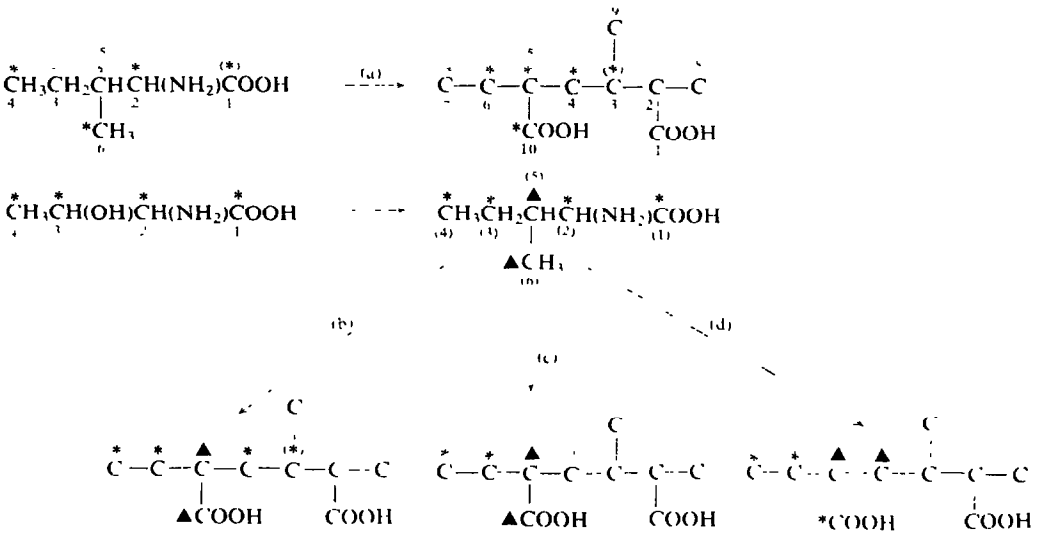
[17] H. E. UMBARGER and E. A. ADELBERG, *J. Biol. Chem.* **192**, 883 (1951).
[18] P. H. ABELSON, *J. Biol. Chem.* **206**, 335 (1954).
[19] P. H. ABELSON and H. J. VOGEL, *J. Biol. Chem*, **213**, 355 (1955).
[20] E. A. EDELBERG, *J. Biol. Chem.* **216**, 431 (1955).
[21] R. L. HERRMAN and J. L. FAIRLEY, *J. Biol. Chem.* **227**, 1109 (1957).
[22] D. M. GREENBERG, *Metabolic Pathways*, Vol. II, p. 195. Academic Press, N.Y. (1961).
[23] E. A. ADELBERG, *Amino Acid Metabolism*, p. 421. Johns Hopkins Press, Baltimore, (1955).
[24] R. L. WIXOM and R. J. HUDSON, *Plant Physiol.* **36**, 598 (1961).
[25] W. L. KRETOVICH and Z. S. KAGAN, *Nature* **195**, 81 (1962).
[26] R. L. WIXOM and M. KANAMORI, *Biochem. J.* **83**, 9P (1962).
[27] W. G. ROBINSON, B. K. BACHHAWAT and M. J. COON, *J. Biol. Chem.* **218**, 391 (1956).

These considerations prompted an investigation of isoleucine incorporation. From an examination of the known biosynthetic relationship between threonine and isoleucine it was possible to make some predictions about the mode of threonine and isoleucine incorporation into seneciphyllic acid, assuming this route to be operative. Thus in the transformation of threonine to isoleucine, C_1, C_2, C_3 and C_4 of threonine become C_1, C_2, C_3 and C_4 of isoleucine respectively (Scheme 5), the side chain C_5–C_6 being derived from pyruvate through "active acetaldehyde".[21–26]



SCHEME 5. BIOSYNTHETIC TRANSFORMATION OF α-KETOBUTYRIC ACID INTO ISOLEUCINE.

Consideration of possible ways in which isoleucine could be used in seneciphyllic acid biosynthesis suggested that it might provide either the six-carbon unit C_3-C_4-C_5-C_6-C_7-(C_{10}) of seneciphyllic acid or the corresponding five-carbon unit, omitting C_3 (Scheme 6, *pathway a*). Assuming incorporation via isoleucine, threonine should provide C_3, C_4, C_6 and C_7 of seneciphyllic acid in the first case (Scheme 6, *pathway b*), but in the second case, where two modes of incorporationof a five-carbon unit are possible, threonine could provide either C_4, C_6 and C_7 (Scheme 6, *pathway c*), or C_{10}, C_6 and C_7 (Scheme 6, *pathway d*). If *pathways b* or *c* (Scheme 6) are applicable, then in seneciphyllic acid derived from uniformly labelled threonine, the ratio of the activities of the ethylidene group, C_6–C_7 and the carboxyl group, C_{10}, should be high (>2), whereas *pathway d* should lead to labelled seneciphyllic acid in which the activity of the ethylidene group C_6–C_7 (two labelled carbon atoms) it just twice that of the carboxyl carbon C_{10} (one labelled carbon atom). Regardless of whether a five- or a six-carbon-atom unit were involved, incorporation of uniformly labelled isoleucine according to *pathway a* (Scheme 6) would lead to seneciphyllic acid in which the activity of the ethylidene group, C_6–C_7, was just twice that of the carboxyl carbon atom, C_{10}.



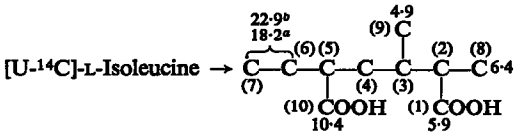
▲ Carbon atom derived from pyruvate

SCHEME 6. POSSIBLE MODES OF INCORPORATION OF [U-^{14}C]-THREONINE AND [U-^{14}C]-ISOLEUCINE INTO SENECIPHYLLIC ACID

TABLE 5. INCORPORATION OF [U-^{14}C]-L-ISOLEUCINE AND [1-^{14}C]-L-ISOLEUCINE INTO "DOUGLASIINE" AND DISTRIBUTION OF ACTIVITY IN SENECIPHYLLINE

Precursor label	Percentage incorporation into "douglasiine"	Percentage incorporation into	
		Seneciphyllic acid	Retronecine
Uniform	0·13	100·0	0·9
1-^{14}C	0·011	71·0	27·3

In the event, uniformly-labelled isoleucine was found to be incorporated at a high rate and with complete specificity, into seneciphyllic acid (Table 5). In addition, the activity of the ethylidene group C_6–C_7 was just twice that of the C_{10} carbon atom (Scheme 7).



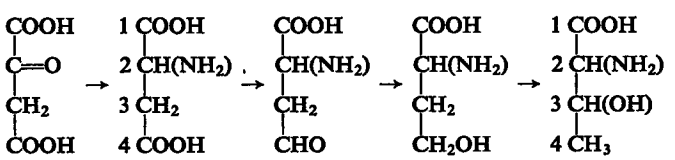
[a] Ozonolysis.
[b] Periodate/permanganate method.

SCHEME 7. DISTRIBUTION OF ACTIVITY IN SENECYPHYLLIC ACID FROM [U-^{14}C]-L-ISOLEUCINE FEEDING

Experiments with [1-^{14}C]-L-isoleucine showed that this precursor was incorporated with less than one-tenth the efficiency of [U-^{14}C]-L-isoleucine and with significantly lower specificity—42 per cent as opposed to 100 per cent (Table 5).

This result shows that C_1 of isoleucine was not incorporated and that isoleucine probably provided the C_5 fragment C_4-C_5-C_6-C_7(C_{10}) of seneciphyllic acid. In addition, uniformly-labelled threonine was incorporated into the C_6-C_7 unit more than four times better than into C_{10} (Scheme 4), indicating that C_2, C_5, C_3, C_4 and C_6 of isoleucine become C_4, C_5, C_6, C_7 and C_{10} respectively of seneciphyllic acid (Scheme 6, *pathway c*).

Support for the foregoing interpretation was found in feeding experiments with [4-^{14}C]-DL-aspartate. Aspartate has been shown to be a precursor in the biosynthesis of threonine. The pathway is discussed in greater detail below, but at present it will be sufficient to state that as a result of the various transformations, C_4 of aspartate becomes C_4 of threonine (Scheme 8). Accordingly, operation of any of the *pathways a, b, c* or *d* (Scheme 6) should give seneciphyllic acid labelled predominantly in C_7 following incorporation of [4-^{14}C]-DL-aspartate. The results of the appropriate feeding experiments are given in Tables 6A and 6B. Total incorporation of aspartate into "douglasiine" was rather low, but its specificity for seneciphyllic acid biosynthesis was quite high (80 per cent). In addition, in two separate feeding experiments, 71·1



SCHEME 8. BIOSYNTHESIS OF THREONINE.

Table 6A. Incorporation of [4-^{14}C]-DL-aspartate into "douglasiine" and distribution of activity in seneciphylline

Percentage incorporation into "douglasiine"	Percentage activity in	
	Seneciphyllic acid	Retronecine
0·030	89·9	11·6
0·005	92·2	7·3

Table 6B. Distribution of activity in seneciphyllic acid derived from [4-^{14}C]-DL-aspartate

Percentage activity in C_7+C_8	Percentage activity in C_8	Percentage activity in C_7*
72·4	1·3	71·1
75·7	1·7	74·0

* By subtraction.

and 74·0 per cent, respectively, of the total activity of the acid was located in C_7 (Table 6B), in good agreement with expectation.

In order to obtain more information on the mode of threonine utilization, the incorporation of α-aminobutyrate was studied. This can be assimilated into the threonine-isoleucine metabolic pathway by transamination to α-ketobutyrate. Since C_1 of isoleucine was not incorporated into seneciphyllic acid it was not expected that [1-^{14}C]-DL-aminobutyrate would be incorporated into the left-hand five-carbon-atom unit, but it was hoped that it might give information on threonine incorporation into the right-hand side of the molecule. Feeding experiments however, gave an ambiguous result. The overall incorporation rate was low but specificity for seneciphyllic acid biosynthesis was moderately high (80 per cent, Table 7).

Table 7. Incorporation of [1-^{14}C]-DL-α-aminobutyrate, [1-^{14}C]-DL-α-methylbutyrate and [1-^{14}C]-angelate into "douglasiine" and distribution of activity in seneciphylline

Precursor	Percentage incorporation into "douglasiine"	Percentage activity in	
		Seneciphyllic acid	Retronecine
[1-^{14}C]-DL-α-aminobutyrate	0·005	90·1	5·6
[1-^{14}C]-DL-α-methylbutyrate	0·0025	—	—
[1-^{14}C]-DL-α-methylbutyrate	0·0127	97	5
[1-^{14}C]-DL-α-methylbutyrate	0·025	80	25
[1-^{14}C]-angelate	0·012	85	17
[1-^{14}C]-angelate	0·016	80	20
[1-^{14}C]-angelate	0·020	85	8

Some additional information, but not conclusive in nature, was obtained from experiments in which [1-^{14}C]-DL-α-methylbutyric acid and [1-^{14}C]-angelic acid were fed (Table 7). These results indicate a selective incorporation of these two five-carbon-atom acids into the necic acid, but at a low level of total incorporation. Insufficient material was available for degradation studies and it is not known whether these form the left-hand (as in VI) five-carbon-atom unit, as the results of isoleucine feeding would suggest they should do. One noteworthy feature of the investigation was the low incorporation of nearly all precursors into C_8, as the following table (Table 8) shows.

TABLE 8. INCORPORATIONS INTO C_8 OF SENECIPHYLLIC ACID WITH VARIOUS PRECURSORS

Precursor	Percentage incorporation into C_8
[2-^{14}C]-DL-mevalonate	1·7
[U-^{14}C]-L-threonine	0·5
[U-^{14}C]-L-isoleucine	6·4
[2-^{14}C]-acetate	1·7
[1-^{14}C]-acetate	1·1

It appeared possible, therefore, that C_8 was derived from a one-carbon-atom donor, and an investigation of the incorporation of [Me-^{14}C]-L-methionine was undertaken. The following results were obtained (Tables 9 and 10). Despite the relatively non-specific incorporation into the necic acid, the high activity of C_8 as compared with the activity of this position in all of the other cases (compare Tables 8 and 10) suggested strongly that a one-carbon donor was the source of C_8. An experiment in which [^{14}C]-formate was fed supported this view (Tables 9 and 10). The low overall incorporation rate and lower specificity for labelling C_8 are most probably a reflection of the wide range of metabolic pathways open to formate in the plant.

An unexpected observation, illustrated by the figures in the following table (Table 11), was made during the alkaloid purification step of the experimental procedure. As a matter of routine in isolating the seneciphylline from the total alkaloid mixture, a quantity of unlabelled,

TABLE 9. INCORPORATION OF [Me-^{14}C]-L-METHIONINE AND [^{14}C]-FORMATE INTO "DOUGLASIINE" AND DISTRIBUTION OF ACTIVITY IN SENECIPHYLLINE

Precursor	Percentage incorporation into "douglasiine"	Percentage activity in	
		Seneciphyllic acid	Retronecine
[Me-^{14}C]-L-methionine	0·058	72·5	22·8
[Me-^{14}C]-L-methionine	0·01	69·6	28·5
[Me-^{14}C]-L-methionine	0·059	81·0	16·9
[Me-^{14}C]-L-methionine	0·016	81·8	17·0
[Me-^{14}C]-L-methionine	0·111	82·5	17·3
[^{14}C]-formate	0·0024	89·5	7·5

TABLE 10. INCORPORATION OF [Me-^{14}C]-L-METHIONINE AND [^{14}C]-FORMATE INTO C_8 OF SENECIPHYLLIC ACID

Precursor	Percentage incorporation into C_8
[Me-^{14}C]-L-methionine	22·0
[Me-^{14}C]-L-methionine	25·5
[Me-^{14}C]-L-methionine	23·4
[^{14}C]-formate	8·9

TABLE 11. RELATIVE ACTIVITIES OF SENECIPHYLLINE AND "DOUGLASIINE" FROM VARIOUS INCORPORATION EXPERIMENTS

Precursor	Specific activity of seneciphylline × 10^{-4}		Ratio obs./calc.
	Calculated	Observed	
[1-^{14}C]-acetate	19	21·4	1·13
[U-^{14}C]-L-threonine	43	42·4	0·99
[Me-^{14}C]-L-methionine	46	12·9	0·28
[2-^{14}C]-DL-mevalonate	29·7	1·64	0·055
[3-^{14}C]-acetoacetate	8·8	6·24	0·71
[Me-^{14}C]-L-methionine	16·8	1·71	0·102
[U-^{14}C]-L-threonine	128	144	1·13
[Me-^{14}C]-L-methionine	102	31	0·31
[U-^{14}C]-L-isoleucine	106	112	1·05
[2-^{14}C]-acetate	253	283	1·12
[2-^{14}C]-DL-mevalonate	18·1	2·7	0·15
[^{14}C]-formate	12·4	1·14	0·092
[1-^{14}C]-acetate	120	129	1·07
[U-^{14}C]-L-isoleucine	2·96	1·95	0·66
[Me-^{14}C]-L-methionine	28·3	15·5	0·55

crude alkaloid was added to the product from the radioactive precursor-fed plants, and the whole subjected to chromatographic separation. If it is assumed that for plants grown under comparable conditions the proportion of seneciphylline in "douglasiine" is reasonably constant, it is possible to calculate the activity of the final seneciphylline, knowing the activity of the crude alkaloid and the extent of dilution with "cold" material, with the assumption that all of the alkaloids in the mixture have the same specific activity. The values observed and calculated are given in Table 11. It appears that acetate, acetoacetate, threonine and isoleucine give seneciphylline with roughly the same specific activity as the "douglasiine". Methionine, formate and mevalonate, however, give seneciphylline of significantly lower specific activity than the crude alkaloid. Since the column separation is a once-through procedure that separates the seneciphylline from alkaloids bearing an additional hydroxyl group (i.e. in which C_8 is $—CH_2OH$), this indicates that methionine and formate tend to give retrorsine and riddelline with greater activity than seneciphylline. This suggests that the biosynthesis of the C_8 group proceeds via $—CH_2OH$ and that the formation of the $—CH_3$ group at C_8 is a differentiation that occurs late in the biosynthetic pathway.

The fact that mevalonate feeding experiments also show this same difference between crude and purified alkaloid has no such direct explanation.

DISCUSSION

Although it is clear from the incorporation studies that isoleucine provides the C_5-C_6-C_7-(C_{10}) unit of seneciphyllic acid and that C_1 of isoleucine is not incorporated, the activity of C_4 could not be determined and so it is not known with certainty whether this carbon atom is included in the biosynthetic intermediate derived from isoleucine. However, the high, specific incorporations of both threonine and isoleucine strongly suggest that a five rather than a four-carbon-atom unit is involved, leading to the interesting conclusion that isoleucine furnishes a five-carbon-atom biosynthetic intermediate with an isoprenoid carbon skeleton.

The asymmetry in the biosynthesis of the two halves of the seneciphyllic acid molecule is emphasised by the finding that C_8 is provided by a one-carbon-atom donor, although the unequal incorporation of methyl-labelled methionine into seneciphylline on the one hand and retrorsine and ridelline on the other indicates that the C_1 unit actually donated may be a formyl or hydroxymethyl group rather than methyl, as was suggested above.

The exact nature of the C_1-C_2-C_3-C_9 unit remains obscure. Threonine can provide C_1 and C_2 efficiently and the low incorporation of [1-^{14}C]-acetate into the carboxyl carbon, C_1, indicates that this atom is derived from C_2 or C_3 of threonine rather than C_1 or C_4 if it is assumed that acetate is incorporated via the oxaloacetate-aspartate pathway (see below).

One of the interesting features of the incorporation of labelled acetate into seneciphyllic acid is that several carbon atoms, in particular C_9 and C_7, are derived from both acetate-methyl and -carboxyl carbon atoms (Scheme 2). The high incorporation of acetate-carboxyl carbon into the terminal position, C_7, is particularly noteworthy and can be readily explained if it is assumed that acetate is incorporated via threonine and isoleucine, as the following considerations show.

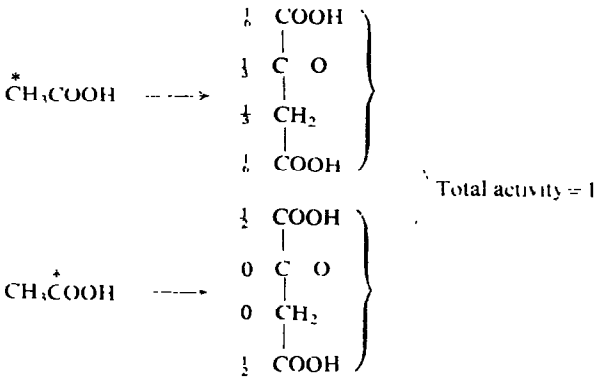
The biosynthesis of threonine has been thoroughly investigated and has been shown to follow a pathway whose major steps are illustrated above[28] (Scheme 8). Oxaloacetate is converted by transamination to aspartic acid and thence via aspartic-β-semialdehyde and homoserine to threonine. Since the carbon chain remains intact throughout these conversions, the pattern of acetate incorporation will be determined by the metabolic route from acetate to oxaloacetate which can be assumed to be mainly by way of the citric acid cycle.

It is predicted, on theoretical grounds, that acetate carboxyl should label C_1 and C_4 of oxaloacetate exclusively and that acetate methyl should be incorporated into all four positions but at twice the rate into C_2 and C_3 as into C_1 and C_4[29] (Scheme 9). These predictions have been largely borne out in practice. Ehrensvard and co-workers observed the following incorporation of doubly-labelled acetate into threonine[30] (Scheme 10). C_4 and C_5 of isoleucine are derived from pyruvate which is labelled only indirectly by acetate. The expected overall incorporation pattern of acetate into isoleucine is shown in Scheme 11.

Incorporation studies by Strassman and Weinhouse have given the following results[29] (Scheme 12). The incorporation of carboxyl-labelled acetate agrees with expectation, but the methyl carbon has evidently undergone considerable randomisation.

The incorporation of acetate into seneciphyllic acid is in good agreement with these

[28] D. M. GREENBERG, *Metabolic Pathways*, Vol. II, p. 186. Academic Press, New York (1961).
[29] M. STRASSMAN and S. WEINHOUSE, *Amino Acid Metabolism*, p. 455. Johns Hopkins Press, Baltimore (1955).
[30] G. EHRENSVÄRD, L. REID, E. SALUSTE and R. STJERNHOLM, *J. Biol. Chem*, **189**, 93 (1951).

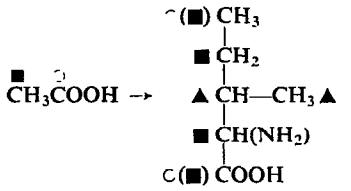


SCHEME 9. THEORETICAL DISTRIBUTION OF ACTIVITY FROM CARBOXYL AND METHYL-LABELLED ACETATE IN OXALOACETATE ASSUMING INCORPORATION VIA THE CITRIC ACID CYCLE.

$$^{13}CH_3{}^{14}COOH \rightarrow CH_3—CH(OH)—CH(NH_2)—COOH$$

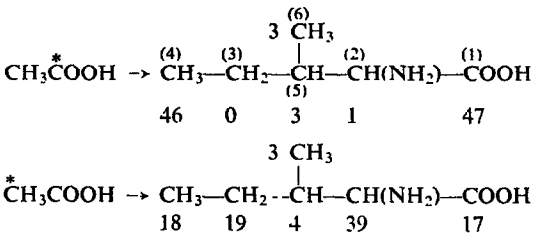
^{14}C	418	32	113	441
^{13}C	0·09	0·26	0·14	0·09

SCHEME 10. INCORPORATION OF LABELLED ACETATE INTO THREONINE.



▲: Carbon atom from pyruvate.

SCHEME 11. EXPECTED INCORPORATION PATTERN IN ISOLEUCINE DERIVED FROM LABELLED ACETATE.



SCHEME 12. INCORPORATION OF LABELLED ACETATE INTO ISOLEUCINE.

observations. Since C_1 of isoleucine (Scheme 6) is not incorporated, the methyl carbon atom (C_7 of seneciphyllic acid) is the only carbon atom on the left-hand side of the molecule which would be derived from acetate carboxyl if incorporation takes place predominantly via the threonine-isoleucine pathway. These observations explain the very high incorporation of acetate carboxyl carbon into this position and the lower but significant incorporation of acetate methyl. The incorporation of acetate carboxyl into the terminal positions of the isoleucine-derived portion of tenuazonic acid has been accounted for on a similar basis.[31,32]

The approximately equal labelling of C_{10} of seneciphyllic acid by acetate-methyl and

[31] C. E. STICKINGS and R. J. TOWNSEND, *Biochem. J.* **74**, 36P (1960).
[32] C. E. STICKINGS and R. J. TOWNSEND, *Biochem. J.* **78**, 412 (1961).

-carboxyl (Scheme 2) parallels the results described above for incorporation of acetate into the corresponding atoms (C_5 and C_6, numbered as in Scheme 5) of isoleucine (Scheme 12). This result also supports the conclusion that C_{10} of seneciphyllic acid corresponds to C_6 of isoleucine and not C_2 (Scheme 5), since in the latter case, no incorporation of acetate-carboxyl into C_{10} of seneciphyllic acid would have been expected. Thus the incorporation patterns of labelled acetate are compatible with the operation of *pathway c* (Scheme 6) rather than *pathway d*, supporting the conclusion already drawn from the feeding experiments with uniformly-labelled threonine. In the light of the considerable randomisation observed in the incorporation of acetate into C_5 and C_6 of isoleucine in the experiments quoted above, no further significance can be attached to the comparable incorporations of acetate-methyl and -carboxyl carbon atoms into C_{10} of seneciphyllic acid.

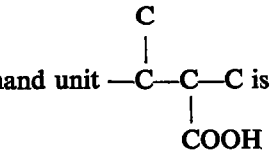
The incorporation results quoted above in connection with the biosynthesis of threonine and isoleucine, were obtained from experiments in which various microbial species were used. However, some of the later transformations in the biosynthetic pathway have been demonstrated in plants,[25,26,33] from which it appears probable that isoleucine biosynthesis follows an identical course in both types of biological system. Extrapolation of the labelling results obtained with the microbial systems to explain the results of incorporation experiments in *S. douglasii* has therefore, in this instance, some experimental justification.

In spite of the conclusion drawn from the present work, that seneciphyllic and the related C_{10} acids are not formed by a coupling of two similar five-carbon units, nevertheless a number of facts, when considered together, constitute strong arguments for retaining the view that the cyclic dibasic ester alkaloids are indeed formed by an intramolecular carbon–carbon bond formation and that a left-hand portion, joined to C_7—OH of retronecine and a right-hand portion, joined to CH_2OH of retronecine, as esters, combine to produce the final macrocyclic alkaloid.

An inspection of the structures of the acid portions of the presently known pyrrolizidine alkaloids is revealing of certain structural regularities that a theory of biosynthesis should accomodate. The following summary includes the dibasic acids, characteristic of (but not confined to) the *Senecio* alkaloids as well as the monocarboxylic acids typical of the boraginaceous alkaloids. Because of a multiplicity of minor variations in these acids in the degree of unsaturation, hydroxylation and stereochemistry, only carbon skeletons are presented in Table 12.

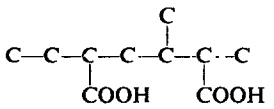
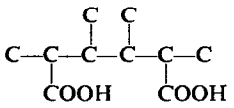
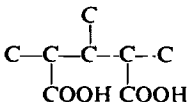
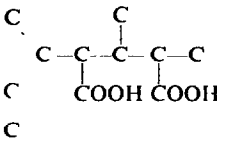
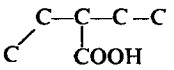
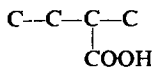
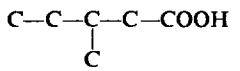
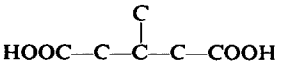
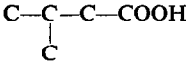
Two structural features are at once apparent: in the acids of Groups 1, 2, 3 and 4 the "right-hand" portion is constant, and consists of the five carbon atoms referred to by that term in the foregoing discussion. The left-hand portion varies, and in at least two of the cases (Groups 3 and 4) can be discerned the structural elements characteristic of common α-keto acids (from alanine in group 3, from valine in group 4). In addition, the acids of Group 5 all contain the structural element of valine attached to a two-carbon fragment.

All of these observations can be accommodated into a general scheme, in which experimental details are still lacking for Groups 2, 3, 4, 5 and 6 and are complex and often ambiguous for Group 1, but for which the results obtained in the present study are consistent and, as far as they go, persuasive.

While the nature of the right-hand unit  is not known in detail, it can be

[33] T. SATYANARAYANA and A. N. RADHAKRISHNAN, *Biochem. Biophys. Acta* **56**, 197 (1962).

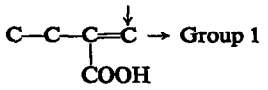
TABLE 12. VARIATION IN STRUCTURE OF THE ACIDS FROM THE PYRROLIZIDINE ALKALOIDS.

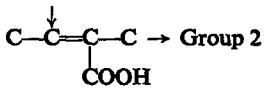
Group	Carbon skeleton	Natural acids
1.		Senecic, seneciphyllic, integerrinecic, retronecic, riddellic, isatinecic, jacobinic, jacozinic, jacolinic
2.		Sceleranecic, sceleratinic
3.		Monocrotalic
4.		Incaninic, trichodesmic, junceic, grantianic
5.		Trachelanthic, viridifloric, heliotrinic, echmidinic, macrotomic lasiocarpic
6.	'simple acids'	
		Angelic, tiglic, sarracinic
		In strigosine[34]
		Dicrotalic (β-hydroxy-β-methyl-glutaric)
		Senecioic acid

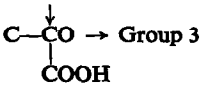
suggested that this unit acts as a nucleophilic fragment; and that the left-hand unit, which varies from one Group to another in 1, 2, 3 and 4, is an electrophilic acceptor of the attack. A generalized scheme such as the following (Scheme 13) will accommodate this hypothesis, where the arrows show the point of nucleophilic attack of the five-carbon fragment above.

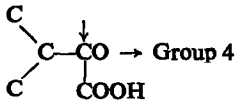
The coupling hypothesis is further strengthened by the observation, mentioned by Hughes and Warren,[8] that alkaloids having the macrocyclic diester structure all contain a necine base with the C_7 hydroxyl group in the β-configuration (C_8—H, α). No macrocyclic alkaloids are known to have the C_7-α-configuration. In the first case, where two separately esterified five-carbon acids would be directed into the fold of the pyrrolizidine nucleus, condensation is sterically favoured over the second situation where there is maximal separation of the two five-carbon units.

[34] A. R. MATTOCKS, *J Chem. Soc.* 1974 (1964).

X 

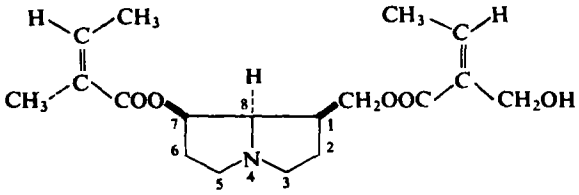
XI 

XII 

XIII 

SCHEME 13. GENERALIZED SCHEME FOR THE BIOSYNTHESIS OF SOME MAJOR GROUPS OF NECIC ACIDS.

The occurrence in pyrrolizidine alkaloids of esters of angelic and sarracinic acids, the prototypes of the electrophilic units X and XI (scheme 13), and the occurrence in these alkaloids of senecioic acid, is a reflection of the formation of the building units proposed here. Thus the alkaloid sarracine,[35] (XIV, platynecine esterified with angelic and sarracinic acids) in



XIV

which two five-carbon acids are separately esterified to the necine base, might represent an intermediate stage in the biosynthesis of the C_{10} necic acids. It is noteworthy that sarracinic acid, which is esterified to the C_1—CH_2OH of the necine, has a —CH_2OH group in a comparable position to the —CH_2OH group in retronecic (I) and riddellic (II) acids. In addition platynecine possesses the spatial requirements (C_7—OH and C_1—CH_2OH *cis*) which are an apparent prerequisite for the formation of the cyclic diester structure in alkaloids containing C_{10} dibasic acids.

EXPERIMENTAL

Counting Procedures

Radioactive samples were counted in the Nuclear Chicago Corporation Liquid Scintillation System No. 720. Colourless samples were counted in dioxan containing, per litre, naphthalene (50 g), PPO (2,5-diphenyloxazole, 7 g) and POPOP (2,2′-*p*-phenylene-bis (5-phenyloxazole), 0·5 g). Coloured samples and $BaCO_3$ were burned to CO_2 by the Van Slyke method.[36,37] The CO_2 was absorbed in a solution of ethyleneglycol monomethyl ether:

[35] T. A. GEISSMAN, *J. Org. Chem.* **26**, 3045 (1961).
[36] D. D. VAN SLYKE, J. PLAZIN and J. R. WEISIGER, *J. Biol. Chem.* **191**, 299 (1951).
[37] D. D. VAN SLYKE, R. STEELE and J. PLAZIN, *J. Biol. Chem.* **192**, 769 (1951).

ethanolamine (11:1 v/v, 6 ml).[38] For counting, a 5 ml aliquot was added to 10 ml of a solution of toluene containing 8·25 g PPO/l.

Crystalline barium acetate samples were counted either by combustion to CO_2 as described above or by direct solution in the ethanol-amine-methyl cellosolve scintillation solution. For this, the barium acetate was first dissolved in methyl cellosolve:ethanolamine (11:1 v/v, 5 ml) in the scintillation vial and the toluene-PPO solution (10 ml) added. A clear solution resulted which remained stable for at least one week.

Sufficient counts were taken to give a standard error of 3 per cent or less in the net counting rate of each sample.

Total incorporation rates and acid-base activity balances in a few experiments were measured on the Nuclear Chicago Dynacon Apparatus, Model 6000. In all cases except those of the [1-^{14}C]-angelate and [1-^{14}C]-DL-methyl butyrate feeds, the results were duplicated in later experiments in which scintillation counting was used.

Feeding Procedures

S. douglasii plants were grown from seed in a standard potting mixture. When 6 weeks to 2 months old, plants were removed from their pots and the roots cleaned by washing with distilled water. They were then transferred to 300 ml Berzelius beakers containing Hoagland's No. 2 nutrient solution.[39] The roots were shielded from direct light and a stream of scrubbed air was bubbled through the nutrient solution for the duration of the experiment.

Feeding of the radioactive precursor was begun after an interval of from 2 to 7 days after it was apparent that plants were healthy and root growth was proceeding. The precursor was administered in a single batch and the plants were harvested after 8 to 10 days.

Isolation of the Alkaloids

In a typical procedure, 4 plants were macerated with methanol in a Waring Blender and the resulting mixture filtered. The filter cake was washed with methanol and macerated again with more methanol. This procedure was repeated until the filtrate from the washings was colourless (6 to 7 extractions). The extracts were evaporated on the rotary evaporator at 30–40° and the residual tar dissolved in hot petrol (Skelly B, 100 ml). The petrol suspension was extracted with 3 N sulphuric acid in four portions, one of 40 ml, three of 20 ml.

The acid extracts were filtered through celite and the filtrate stirred with Zn dust (10 g). After 90 min, the solution was filtered and the filtrate extracted with chloroform (300 ml in 6 portions), made alkaline with ammonia and extracted again with chloroform (400 ml in 8 portions). The latter chloroform extracts were dried over $MgSO_4$ and evaporated to give the crude "douglasiine". This was dissolved in 2 N H_2SO_4 (10 ml), the solution extracted with $CHCl_3$, basified with ammonia and re-extracted with $CHCl_3$. The final chloroform extracts were dried over $MgSO_4$ and concentrated to give the pure crystalline "douglasiine". This was counted to give the overall incorporation rate.

The average yield of "douglasiine" based on the dry wt of the plants was 0·52 per cent. The average uptake of the radioactive precursors by the plants was 98·0 per cent: in all cases uptake was greater than 95 per cent. (Figures from 18 separate experiments.)

[38] H. JEFFAY and J. ALVAREZ, *Anal. Chem.* 33 (4), 613 (1961).
[39] D. R. HOAGLAND and D. I. ARNON, University of California College of Agriculture, *Circular* 347, Berkeley, Calif. (1938).

Isolation of Seneciphylline

The purified "douglasiine" was diluted with "cold" material to bring the total weight to 300–400 mg, and applied in $CHCl_3$ solution to a column of neutral alumina (15 × 1 cm) and the column eluted with $CHCl_3$. Senicephylline was eluted first with a trace of senecionine, the other alkaloids being retained at the top of the column. The seneciphylline was recrystallised from chloroform-methanol to give plates m.p. 212–214° (decomp.). The yield was 50–60% based on the recrystallised "douglasiine".

Hydrolysis of Seneciphylline

In a typical case, seneciphylline (140 mg) was boiled under reflux with $Ba(OH)_2 \cdot 8H_2O$ (300 mg) in water (10 ml) for 15 min. The mixture was then heated on the steam bath for a further 75 min. The barium was precipitated with CO_2 and the solution filtered. The filtrate was acidified with conc. HCl and extracted continuously with ether for 2 days. The ether extract was dried over $MgSO_4$ and evaporated. The residue was recrystallised from ethyl acetate-petrol (Skelly B) to give seneciphyllic acid as needles, 47 mg, m.p. 112–114°.

The residue from the ether extraction was passed through a column of Dowex 2-X8 resin (OH^- form) and the eluate collected until no longer alkaline to litmus. The eluate was evaporated on the rotary evaporator at 30° and the residue dried over P_2O_5 under vacuum. The dried residue was extracted 3 times with boiling, dry acetone. The extracts were filtered and concentrated to give retronecine, 35 mg, m.p. 117–119°. The retronecine was further purified either by recrystallization or by sublimation at 100°/3 mm pressure.

Degradation for C_8

Seneciphyllic acid (74 mg) was dissolved in water (10 ml). $NaIO_4$ (200 mg) was added, giving a solution initially 0·1 M in periodate. The mixture was allowed to stand in the dark for 22 hr. The excess periodate was reduced with SO_2 and dil. NaOH was added (1 N, 12 ml). Iodine–potassium iodide reagent (5 g, I_2, 10 g KI in 50 ml water) was added dropwise until the yellow colour persisted for more than 1 min. After 16 hr, the CHI_3 was filtered off, washed with H_2O and recrystallized from methanol-water to give 82 mg yellow hexagonal plates, m.p. 121°. The CHI_3 was further purified either by recrystallization or by sublimation at 90–100°/2–3 mm.

Degradation for C_1

Seneciphyllic acid (40·5 mg) was refluxed with $LiAlH_4$ (160 mg) in dry ether (30 ml) for 17 hours. The excess reagent was destroyed by the addition of H_2O (10 ml). The resulting mixture was warmed briefly on the steam bath to drive off the ether. The remaining slurry was filtered through celite and the filtrate stirred with Dowex 50W-X8 resin (H^+ form) until the pH had fallen to 4–5. The solution was filtered and the filtrate (volume: 35 ml) treated with $NaIO_4$ (200 mg). The mixture was allowed to stand in the dark for 20 hr and was then treated with sodium arsenite (0·5 g). After forty-five minutes, acetate buffer solution (pH 4·6, 20 ml) was added followed by 0·4% dimedone solution (30 ml). After 24 hr the crystalline precipitate was filtered off and recrystallised from water to give the derivative of formaldehyde as needles, m.p. 191° (33·7 mg).

Degradation for the Unit C_6-C_7 and for C_9

$KMnO_4$ (52 mg in 10 ml water) was added dropwise over thirty minutes to a stirred solution of seneciphyllic acid (52 mg) and HIO_4 (500 mg) in water (10 ml). The solution was stirred for

a further fifteen minutes and then filtered. The filtrate was treated with sodium arsenite (1·0 g) and, after one hour, with acetate buffer (40 ml) and dimedone solution (30 ml). After 24 hr the precipitate was filtered off, washed and dried over P_2O_5 under vacuum. The yield of mixed derivatives was 32 mg.

Alternative Procedure

A solution of seneciphyllic acid (40 mg) in water (20 ml) was brought to pH 7–8 by dropwise addition of 0·1 N K_2CO_3 solution. $NaIO_4$ solution (0·04 M, 25 ml, brought to pH 7–8 with 0·1 N K_2CO_3) was added, together with 0·005 M $KMnO_4$ (2·0 ml). The mixture was left to stand at room temperature for 2 hr. Sodium arsenite (1 g) was then added, followed by 0·4% dimedone solution. After 24 hr the mixed derivatives were filtered off, washed and dried as above.

Separation of the Derivatives

The mixed derivatives (32 mg) were dissolved in acetic acid (1 ml) and heated on the steam bath under reflux for 6 hr. The solution was then poured into water (30 ml). After 16 hr, the precipitate was filtered off, washed with water, dilute NaOH (1 N, 5 ml) and water. The alkali-insoluble material was recrystallized from ethanol–water to give the anhydro derivative of acetaldehyde as plates, m.p. 176·5–177 (11 mg). There was no depression of the mixed melting point with authentic material (m.p. 176·5–177°).

The NaOH washings were just acidified with acetic acid and acetate buffer (pH 4·6, 10 ml) was added. The resulting precipitate was filtered off after 24 hr, washed with water and recrystalized from ethanol-water to give the derivative of formaldehyde as needles, m.p. 190–191° (8·2 mg).

Degradation for C_9, C_{10} and the Unit C_6-C_7

Seneciphyllic acid (65 mg.) in water (6 ml.) was oxidized with $NaIO_4$ (130 mg.) for 27 hrs. The excess oxidant was reduced with SO_2 and the resulting solution extracted continuously with ether for 24 hr. The ether extract was dried over $MgSO_4$ evaporated down and the residue dried over P_2O_5 *in vacuo*. The dried residue was dissolved in purified ethyl acetate (6 ml) and the solution ozonized at 0° for 90 min. The resulting solution was refluxed under a stream of N_2 for 3 hr. The exit gases were passed into a solution of dimedone (40 ml) and acetate buffer solution (pH 4·6, 30 ml). After 16 hr the precipitate was filtered off and the derivatives separated as above to give the anhydro acetaldehyde derivative (30 mg) and the formaldehyde derivative (4 mg).

The residual ethyl acetate was evaporated to dryness and the residue dried over P_2O_5 *in vacuo*. The residue was dissolved in dry ether (20 ml) and refluxed with $LiAlH_4$ (150 mg) for 6 hr. The subsequent periodate oxidation was carried out as in the degradation for C_1, above, to give the dimedone derivative of formaldehyde as needles, m.p. 190–191° (24 mg). In trial experiments, none of the acetaldehyde derivative was detected.

Isolation and Identification of the keto-acid VIII

Seneciphyllic acid (50 mg) was dissolved in a solution of $NaIO_4$ (150 mg) in water (10 ml). After 24 hr the excess periodate was reduced with SO_2 and the solution added to 2,4-dinitrophenyl-hydrazine solution (0·25% in 2 N sulphuric acid, 100 ml). The mixture was heated on the steam bath for 10 min and filtered. The derivative was recrystallized from chloroform as

yellow needles, m.p. 186–188° (lit. m.p. 184°[12]) (53 mg). (Found: C, 51·35; H, 4·85; N, 15·65. Calc. for $C_{15}H_{16}N_4O_6$: C, 51·72; H, 4·63; N, 16·09%).

The thiosemicarbazone was obtained by extracting the solution from the oxidation of 100 mg seneciphyllic acid, after reduction with SO_2, continuously with ether for 26 hr. The ether extract was dried over $MgSO_4$ and evaporated. The residue was treated with a solution of thiosemicarbazide (100 mg) in water (10 ml). After 16 hr. the precipitate was filtered off and recrystallized from ethanol-water to give the derivative as plates, m.p. 161–163·5° (lit. m.p. 163–164°[12]). (Found: C, 49·81; H, 5·91. Calc. for $C_{10}H_{15}O_2N_3S$: C, 49·78; H, 6·27%).

Kuhn–Roth Oxidation of Seneciphyllic Acid

Seneciphyllic acid was oxidized by the standard procedure. The acetic acid was estimated by titration against standard $Ba(OH)_2$ solution. The neutralized solution was boiled down to 0·5 ml and filtered. The filtrate was concentrated to 0·2 ml, cooled and treated dropwise with cold ethanol until a permanent turbidity was obtained. On standing the solution deposited the barium acetate as long, silky needles.

Schmidt Degradation of Barium Acetate

Barium acetate (21·6 mg), was dissolved in 100% H_2SO_4 (0·2 ml) with warming. The solution was cooled and NaN_3 (33 mg) was added. The flask containing the mixture was connected to a N_2 train, heated to 70° and held at that temperature for 1 hr. The exit gases were passed through acidified $KMnO_4$ solution and then into 0·04 N $Ba(OH)_2$. The precipitated barium carbonate was filtered off, washed and dried; yield: 25·9 mg. (77 per cent).

The residual acid solution was made alkaline with 25% NaOH and heated under N_2 stream to 95–100°. The exit gases were passed into a solution of 2,4,5-trinitrotoluene (50 mg) in absolute ethanol (5 ml). After 6 hr the deep green-yellow solution was concentrated to half volume and left at 0° overnight. The crystalline precipitate was filtered off and the filtrate evaporated to dryness. The residue was dissolved in benzene and applied to a column (18 × 1 cm) of neutral alumina, (activity IV). The column was developed with benzene. The main yellow band was eluted with benzene, combined with the first crop of crystals and the whole rechromatographed as before. The derivative, eluted with benzene, was recrystallized from ethanol as small, yellow needles, m.p. 170–171° (8·5 mg). (Found: C, 45·70; H, 4·37; N, 19·98. Calc. for $C_8H_9N_3O_4$: C, 45·50; H, 4·30; N, 19·59%.)

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