Pyrrolizidine Alkaloids. Biosynthesis of Trichodesmic Acid

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^{14}C-Labelled threonine (**3**), isoleucine (**4**), valine (**5**), and leucine (**6**) were incorporated specifically into trichodesmic acid (**12**), the necic acid component of the pyrrolizidine alkaloid trichodesmine (**2**) produced by *Crotalaria globifera*. Degradation of the ^{14}C-labelled trichodesmic acid showed that threonine and isoleucine mainly label one half of the C_{10} acid, while valine and leucine predominantly label the other half of the acid.

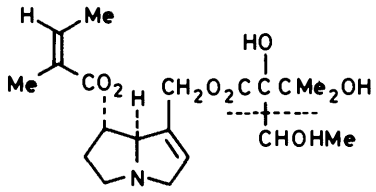
Many pyrrolizidine alkaloids are composed of a dihydroxy base portion (necine), which is esterified with two separate acids (necic acids) as in heliosupine (**1**) or with a diacid to give a macrocyclic diester as in trichodesmine (**2**).[1] Because many of the necic acids contain ten carbon atoms, they were originally believed to be of terpenoid origin. However, experiments with ^{14}C- and ^{3}H-labelled precursors have established that a number of these necic acids are formed from one or more of the common branched-chain amino-acids threonine (**3**), isoleucine (**4**), and valine (**5**).

Thus, Crout has shown that (2*S*)-isoleucine is a precursor of the angelic acid component of heliosupine (**1**)[2] and that valine is a specific precursor of part of the other (echimidinic) acid component of heliosupine.[3] Structural analysis of pyrrolizidine alkaloids which contain a macrocyclic dilactone has shown that many of the C_{10} necic acids include a similar C_5 unit (**7**). It has been clearly established by Crout and co-workers that this 'right hand' C_5 unit in senecic acid (**8**) is derived from (2*S*)-isoleucine (**4**) or its biosynthetic precursor (2*S*)-threonine (**3**).[4,5] (The remaining C_5 unit in senecic acid is also derived from isoleucine.) A similar C_5 unit in monocrotalic acid (**9**) has also been shown to be formed from (2*S*)-isoleucine, although the derivation of the remaining three carbon atoms has not been firmly established.[6]

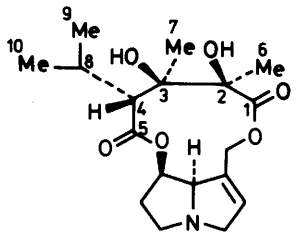
Our recent isolation of trichodesmine (**2**) and grantaline (**10**) from *Crotalaria globifera*[7] prompted us to study the biosynthesis of another C_{10} necic acid. Trichodesmic acid (**12**) also contains the C_5 unit (**7**) which is likely to be derived from threonine or isoleucine. The precursor for the 'left hand' portion might be valine (**5**) as suggested by Crout,[3] or leucine (**6**), since the biosynthesis of valine and leucine proceeds by way of the common intermediate 3-methyl-2-oxobutanoic acid.

To begin this biosynthetic study, ^{14}C-labelled acetate was tested as a precursor for trichodesmic acid, since it has been claimed[8] that high incorporations of [1-^{14}C]acetate into monocrotalic acid (**9**) were obtained. Samples of ^{14}C-labelled acetate were mixed with [1,4-^{3}H]putrescine as standard. Putrescine (tetramethylenediamine) is a known precursor of the common base portion, retronecine (**11**).[9] Greater confidence can then be placed in comparison of the ^{3}H/^{14}C values of the alkaloids obtained with those of the precursors than in measurement of incorporation rates.[9] Samples of the ^{14}C-labelled amino-acids threonine, isoleucine, valine, and leucine were also mixed with [1,4-^{3}H]putrescine and administered to *C. globifera* plants. The ^{3}H/^{14}C values were measured for the precursors and for the two pyrrolizidine alkaloids trichodesmine and grantaline which were isolated and separated. [An *X*-ray crystal structure for grantaline (**10**) has recently been reported.[10]]

From the results (Table 1) it is clear that ^{14}C-labelled acetate is more than ten times less efficient in labelling the alkaloids than the four amino-acids studied. This confirms the previous results of Robins *et al.* that acetate is not an efficient precursor



(**1**)



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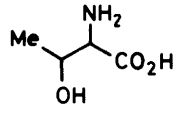

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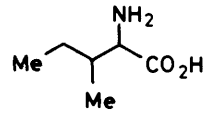

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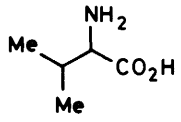

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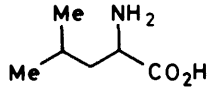


(**6**)

of necic acids.[6] By contrast the ^{14}C-labelled amino-acids were all incorporated with similar efficiency into the two alkaloids. The ^{3}H/^{14}C values changed from an initial value of 10 to a final value of 16—36 (Table 1). The similarity in values obtained in each experiment for trichodesmine and grantaline indicates a close biosynthetic relationship for these two alkaloids as would be expected from consideration of their structures. Comparison of the ratios obtained for (2*S*)-[U-^{14}C]- and (2*RS*)-[4-^{14}C]valine and for (2*S*)-[U-^{14}C]- and (2*RS*)-[2-^{14}C]-leucine in trichodesmine and grantaline suggests that only the (2*S*)-isomer is utilised in each case. This is in agreement with the finding of Crout *et al.* that only (2*S*)-isoleucine is involved in senecic acid biosynthesis.[5]

Total incorporations for all the compounds tested were

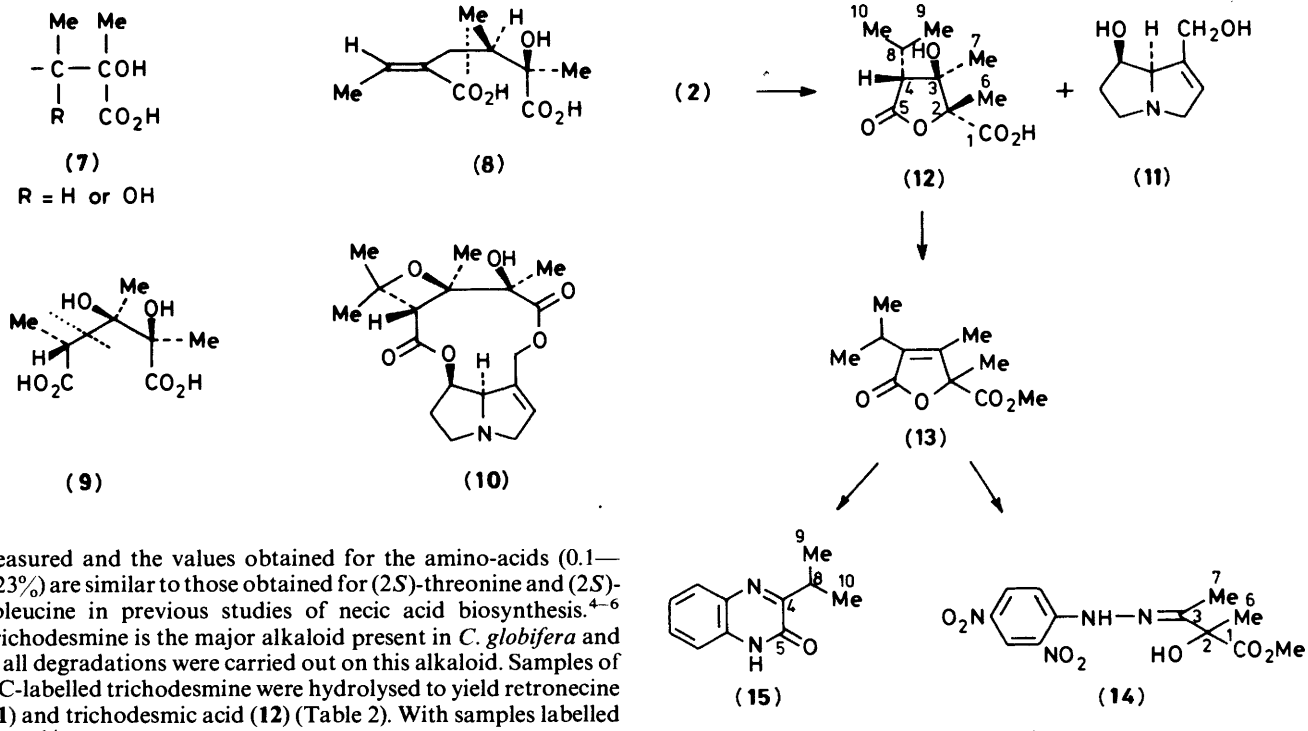
Table 1. Incorporation of various precursors into trichodesmine (**2**) and grantaline (**10**) in *Crotalaria globifera*

Experiment	Precursor	$^3H/^{14}C$ Precursor[a]	$^3H/^{14}C$ Trichodesmine (**2**)	$^3H/^{14}C$ Grantaline (**10**)
1	[1-^{14}C]acetate	10.3	382	370
2	[2-^{14}C]acetate	10.1	347	334
3	(2*S*)-[U-^{14}C]threonine	10.6	29	32
4	(2*S*)-[U-^{14}C]isoleucine	9.8	25	27
5	(2*S*)-[U-^{14}C]valine	9.3	17	16
6	(2*RS*)-[4-^{14}C]valine	10.1	27	30
7	(2*S*)-[U-^{14}C]leucine	10.0	20	19
8	(2*RS*)-[2-^{14}C]leucine	10.5	36	36
9	[1,4-$^{14}C_2$]putrescine	10.6	9.1	8.8

[a] [1,4-^{3}H]Putrescine was added to each ^{14}C-labelled precursor (5 μCi) to give an initial $^3H/^{14}C$ value of *ca.* 10.

Table 2. Incorporation of various precursors into trichodesmine (**2**) and the distribution of radioactivity in the necine and necic acid components

Experiment	Precursor	Amount fed (μCi)	Total incorporation (%)	% Activity in retronecine (**11**)	% Activity in trichodesmic acid (**12**)
10	[1-^{14}C]acetate	95	0.011	48	49
11	[2-^{14}C]acetate	95	0.014	52	45
12	(2*S*)-[U-^{14}C]threonine	45	0.10	5.1	94
13	(2*S*)-[U-^{14}C]isoleucine	45	0.12	4.0	98
14	(2*S*)-[U-^{14}C]valine	45	0.23	4.3	100
15	(2*RS*)-[4-^{14}C]valine	45	0.14	3.8	97
16	(2*S*)-[U-^{14}C]leucine	45	0.19	2.9	101
17	(2*RS*)-[2-^{14}C]leucine	45	0.12	3.5	97
18	[1,4-$^{14}C_2$]putrescine	45	0.41	97	1.5



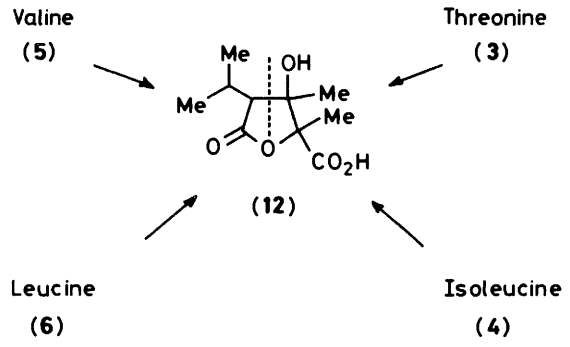
Scheme 1.

measured and the values obtained for the amino-acids (0.1—0.23%) are similar to those obtained for (2*S*)-threonine and (2*S*)-isoleucine in previous studies of necic acid biosynthesis.[4–6] Trichodesmine is the major alkaloid present in *C. globifera* and so all degradations were carried out on this alkaloid. Samples of ^{14}C-labelled trichodesmine were hydrolysed to yield retronecine (**11**) and trichodesmic acid (**12**) (Table 2). With samples labelled from ^{14}C-acetate, the radioactivity was spread between the acidic and basic portions (experiments 10 and 11) indicating that acetate is not a specific precursor for necic acids. Degradation of trichodesmine produced with ^{14}C-labelled amino-acids (experiments 12—17) showed that most of the radioactivity is in the necic acid component with very little activity in the retronecine. Radioactivity derived from the feeding experiment with [1,4-^{3}H]putrescine was located almost entirely in the pyrrolizidine base, confirming that putrescine is a specific precursor for retronecine.[9]

In order to investigate the pattern of incorporation of the amino-acids in more detail, ^{14}C-labelled trichodesmic acid (**12**)

Table 3. Distribution of radioactivity in trichodesmic acid (**12**) from feeding experiments with amino acids

Experiment	Precursor	% Activity in C-1, -2, -3, -6, and -7	% Activity in C-4, -5, -8, -9, and -10
12	(2*S*)-[U-^{14}C]threonine	73	24
13	(2*S*)-[U-^{14}C]isoleucine	81	14
14	(2*S*)-[U-^{14}C]valine	16	85
15	(2*RS*)-[4-^{14}C]valine	13	89
16	(2*S*)-[U-^{14}C]leucine	18	79
17	(2*RS*)-[2-^{14}C]leucine	14	90



Scheme 2.

obtained in experiments 12—17 was degraded as shown in Scheme 1 to give the 'right hand' half of the C_{10} acid, corresponding to C-1, -2, -3, -6, and -7, as the 2,4-dinitrophenylhydrazone (**14**) of methyl acetolactate, and the remaining half of the acid gave the quinoxalone (**15**). Measurement of the radioactivity in these derivatives (Table 3) indicated that threonine and isoleucine provide the 'right hand' C_5 unit as anticipated, while valine and leucine predominantly label the 'left hand' C_5 unit of trichodesmic acid. Further information on this point was gained by the use of specifically ^{14}C-labelled valine and leucine to obtain labelled trichodesmine (experiments 15 and 17). Most of the radioactivity of the trichodesmine was located in the quinoxalone (**15**). Degradation of this material (**15**) by Kuhn–Roth oxidation gave a small amount of acetic acid which was converted into *p*-bromophenacyl acetate. In experiment 15 using (2*RS*)-[4-^{14}C]valine, this derivative contained *ca.* 90% of the trichodesmic acid activity, while in experiment 17 using (2*RS*)-[2-^{14}C]leucine less than 2% of the trichodesmic acid activity was present in the *p*-bromophenacyl acetate.

These patterns of incorporation provide strong support for the proposed biosynthesis of trichodesmic acid (Scheme 2). The C_5 unit (**7**) is formed from threonine or isoleucine, while the remaining C_5 unit is derived from valine or leucine. The finding that (2*S*)-leucine is a specific precursor of trichodesmic acid provides another example of the involvement of branched-chain amino-acids in necic acid biosynthesis. Further study is required to establish the exact nature of the two C_5 species involved in the coupling process. The mechanism for joining the two C_5 units in necic acid biosynthesis is also not known, but Crout and co-workers[11] have investigated the stereochemistry of this process involving two isoleucine units in senecic acid (**8**) biosynthesis. The coupling takes place with retention of the C-4 *pro-R*-hydrogen and loss of the C-4 *pro-S*-hydrogen from both molecules of isoleucine.

Experimental

General.—M.p.s were measured with a Kofler hot-stage apparatus. Organic solvents were dried with anhydrous $MgSO_4$, and solvents were evaporated off under reduced pressure below 40 °C. T.l.c. was carried out on silica gel plates of 0.25 mm thickness developed with chloroform–methanol–concentrated ammonia (85:14:1), and the bases were located by oxidation with *o*-chloranil followed by treatment with Ehrlich's reagent.[12]

Radiochemical Methods.—All radiochemicals were purchased from the Radiochemical Centre, Amersham, or C.I.S. (Fluorochem. Ltd.). Activities of ^{14}C and ^{3}H were measured with a Philips liquid scintillation analyser using toluene–methanol solution. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. Radioactive samples were normally recrystallised to constant specific activity and counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for the radioscanning of t.l.c. plates.

Feeding Method.—Crotalaria globifera plants were propagated from seed obtained from Port Edward, Natal,[7] and grown in a standard compost until they flowered. For experiments 1—9 two plants were used for each experiment, and [1,4-^{3}H]putrescine was added to each ^{14}C-labelled precursor as a reference to give a ^{3}H/^{14}C value of *ca.* 10.0. This value was then measured accurately (Table 1). Aqueous solutions of these precursors were fed as described previously.[9] For experiments 10—18, twenty plants were used for each experiment. The precursors tested are listed in Table 1.

Extraction of Trichodesmine (**2**) *and Grantaline* (**10**).—Two weeks after administration of the precursor, the plants were extracted as described previously to give a mixture of pyrrolizidine alkaloids.[7] The yield varied between 0.3 and 0.6% based on the dry weight of the plant material. In each experiment, radioscans of t.l.c. plates showed two main radioactive bands, coincident with authentic unlabelled trichodesmine and grantaline at R_F 0.50 and 0.55, respectively. The alkaloids were separated by preparative t.l.c. and recrystallised to constant specific activity. Trichodesmine (60—75% of mixture) had m.p. 158—160 °C (lit.,[7] 158—160 °C), and grantaline, m.p. 217—219 °C (lit.,[7] 218—220 °C). For experiments 1—9 the ^{3}H/^{14}C values were measured for each alkaloid.

Hydrolysis of Trichodesmine (**2**).—This was carried out on *ca.* 50 mg of material as described for the hydrolysis of senecionine.[3] Trichodesmic acid (**12**) was recrystallised from acetone–light petroleum (b.p. 60—80 °C) (*ca.* 25 mg, 85%), m.p. 219—220 °C (lit.,[13] 209 °C). Retronecine (**11**) was sublimed at 90 °C and 1 mmHg and recrystallised from acetone–light petroleum (b.p. 60—80 °C) as white needles (*ca.* 14 mg, 64%), m.p. 118—119 °C (lit.,[9] 118—120 °C).

Degradation of Trichodesmic Acid (**12**).—Trichodesmic acid (*ca.* 20 mg) was converted into its methyl ester with excess of diazomethane. Methyl trichodesmate was heated under reflux at 120 °C for 2 h in pyridine (1 ml) and phosphoryl trichloride (100 mg).[3] Water (2 ml) was added to the cooled mixture, and the solution was acidified with concentrated hydrochloric acid and extracted with diethyl ether (4 × 5 ml). The extracts were dried and concentrated to a yellow oil, methyl anhydrotrichodesmate, $\lambda_{max.}$ 229 nm (ε 11 000).[14] Without purification the unsaturated ester (**13**) (16 mg) was ozonolysed in dry ethyl acetate (5 ml) for 6 h at 0 °C. Excess of ozone was removed with nitrogen, and the ozonide was decomposed by being stirred in water (5 ml) containing manganese dioxide (50 mg) for 30 min. The ethyl acetate layer was separated and the aqueous layer was

extracted with ethyl acetate (4 × 5 ml). The combined organic layers were concentrated to an oil, which was hydrolysed with 0.5M sodium hydrogen carbonate (3 ml) for 1 h. The solution was extracted with diethyl ether (10 × 3 ml). The methyl acetolactate (methyl 2-hydroxy-2-methyl-3-oxobutanoate) in the ether layers was converted into its 2,4-dinitrophenylhydrazone (**14**), orange needles (*ca.* 15 mg, 50%), m.p. 171—172 °C (lit.,[15] 173.5—174 °C), which was purified by chromatography on basic alumina with ethanol as eluant and recrystallised to constant specific activity from ethanol.

The aqueous layer obtained after removal of the methyl acetolactate was treated with a solution of freshly crystallised *o*-phenylenediamine (20 mg) in dilute acetic acid (1 ml). The solution was concentrated at room temperature until the quinoxalone (**15**) precipitated. The derivative was filtered off, dried, and purified by chromatography on basic alumina with chloroform as eluant. Recrystallisation from benzene gave the lactam (**15**) as needles (*ca.* 10 mg, 55%), m.p. 228—230 °C (lit.,[16] 233—235 °C). For experiments 14 and 17, Kuhn–Roth oxidation was carried out as described.[4] The acetic acid formed was distilled and titrated against standard sodium hydroxide. *p*-Bromophenacyl acetate was prepared and purified as described,[6] m.p. 80—82 °C (lit.,[6] 81—83 °C). The specific activities obtained were corrected by a factor of two.

Acknowledgements

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