Pyrrolizidine Alkaloids. Biosynthesis of Monocrotalic Acid, the Necic Acid Component of Monocrotaline

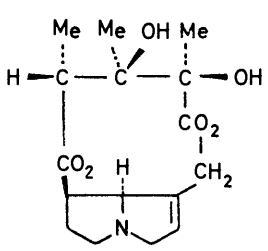
By **David J. Robins, Nicholas M. Bale,** and **David H. G. Crout,*** Department of Chemistry, University of Exeter, Stocker Road, Exeter EX4 4QD

[U-^{14}C]-L-Threonine and [U-^{14}C]-L-isoleucine were specifically incorporated into monocrotalic acid (III) the necic acid component of the pyrrolizidine alkaloid monocrotaline (I) in *Crotalaria spectabilis* and *Crotalaria retusa*.

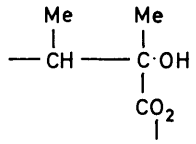
STRUCTURAL analysis of pyrrolizidine alkaloids which contain macrocyclic diester systems [*e.g.* monocrotaline (I)] has led to the conclusion that a common five-carbon unit is probably involved in the biosynthesis of many of the necic acid components of alkaloids in this series.[1] This unit is shown in its basic form in formula (II). It may be modified by further hydroxylation as in mono-

[1] D. H. G. Crout, *J. Chem. Soc.* (*C*), 1969, 1379.

crotalic acid (III) or by the introduction of unsaturation as in seneciphyllic acid (IV) where the unit (II) appears to the right of the dotted line in each formula.



(I)



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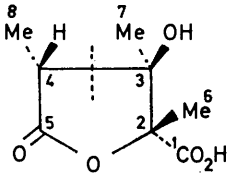
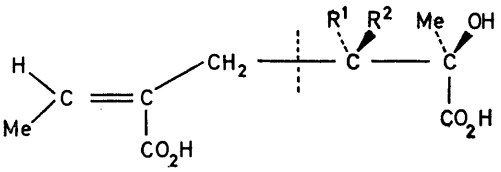
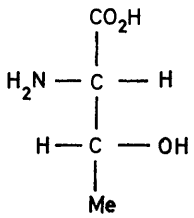


(III)



(IV) $R^1R^2 = CH_2$
(V) $R^1 = H, R^2 = Me$



(VIII)

Nowacki and Byerrum [2] observed relatively high incorporations of [1-^{14}C]acetate into monocrotaline (I) in excised young plants of *Crotalaria spectabilis*. Degradation showed that the activity was almost entirely located in the necic acid component, monocrotalic acid (III). It was concluded that acetate is a direct precursor of monocrotalic acid. However, after repeated attempts, made under a variety of conditions, we have been unable to obtain similar results in feeding experiments with *Crotalaria spectabilis* and *C. retusa*. Precursors were administered under a variety of conditions: (*a*) by absorption through the stems of excised young plants, (*b*) by the wick method in mature plants, (*c*) by absorption through the roots in young, mature, and flowering plants growing in hydroponic solution, and (*d*) by direct injection into the stems of mature plants. With [1-^{14}C]-, [2-^{14}C]-, and [^{3}H]-acetate as precursors, incorporations into monocrotaline were very low regardless of the feeding method used (Table 1). Uptake of radioactivity by the plants was rapid; the low incorporations therefore cannot be attributed to lack of absorption of the precursor. However, it was noted that the freshly isolated monocrotaline usually contained appreciable activity which gradually disappeared on repeated recrystallisation of the diluted alkaloid. The initially purified alkaloid gave only a single spot on a thin-layer chromatographic plate sprayed with the Dragendorff reagent, but when a sulphuric acid spray

TABLE 1

Incorporations of various precursors into monocrotaline in *C. retusa* and *C. spectabilis*

Experiment	Precursor	Species	Incorporation (%)
1	[1-^{14}C] Acetate (W)	R	$4{\cdot}4 \times 10^{-4}$
2	[1-^{14}C] Acetate (S)	R	$<4{\cdot}8 \times 10^{-4}$
3	[1-^{14}C] Acetate (H)	R	$<4{\cdot}6 \times 10^{-4}$
4	[1-^{14}C] Acetate (H)	R	$<4{\cdot}4 \times 10^{-4}$
5	[2-^{14}C] Acetate (H)	R	$4{\cdot}2 \times 10^{-4}$
6	[^{3}H] Acetate (S)	R	8×10^{-5}
7	[2-^{14}C] Acetate (H)	S	$<5{\cdot}6 \times 10^{-4}$
8	[^{3}H] Acetate (I)	S	$5{\cdot}1 \times 10^{-4}$
9	[U-^{14}C]-L-Threonine (H)	R	$1{\cdot}4 \times 10^{-2}$
10	[U-^{14}C]-L-Isoleucine (H)	R	$3{\cdot}2 \times 10^{-3}$
11	[U-^{14}C]-L-Threonine (H)	S	$1{\cdot}5 \times 10^{-3}$
12	[U-^{14}C]-L-Isoleucine (H)	S	$1{\cdot}8 \times 10^{-3}$
13	[1-^{14}C]-DL-Alanine (H)	S	$<1{\cdot}0 \times 10^{-4}$
14	[U-^{14}C]-L-Threonine (W)	S	5×10^{-2}
15	[U-^{14}C]-L-Threonine (W)	S	$1{\cdot}6 \times 10^{-1}$
16	[U-^{14}C]-L-Threonine (W)	S	$1{\cdot}9 \times 10^{-1}$
17	[U-^{14}C]-L-Isoleucine (W)	S	$1{\cdot}5 \times 10^{-1}$
18	[U-^{14}C]-L-Isoleucine (W)	S	$1{\cdot}1 \times 10^{-1}$

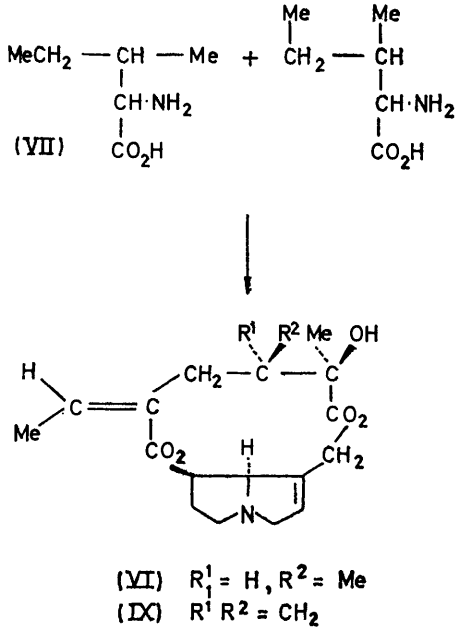
R = *Crotalaria retusa*, S = *C. spectabilis*. Feeding methods: H, hydroponic solution; I, injection; S, stem feeding in excised plants; W, wick method.

was used other compounds were revealed which, from the colours produced, appeared to be terpenoid. The difficulty in achieving radiochemical purity of the alkaloid can therefore probably be attributed to the presence of small amounts of highly labelled terpenes which accompanied the alkaloid through the extraction procedure. This interpretation is supported by the finding that when labelled amino-acid precursors were used, which were not likely to be incorporated into terpenoids, radiochemical purification of the alkaloid was achieved much more rapidly.

It has been clearly established [3] that the entire ten-carbon skeleton (including the ubiquitous C_5 component) of senecic acid (V), the necic acid component of senecionine (VI) is derived from L-isoleucine (VII) as indicated in Scheme 1. It therefore appeared probable that the five-carbon unit consisting of C-1, -2, -3, -6, and -7 in monocrotalic acid (III) was similarly derived from isoleucine (VII) and its biological precursor threonine (VIII). Accordingly, [U-^{14}C]-L-threonine and [U-^{14}C]-L-isoleucine were administered to *Crotalaria retusa* and *C. spectabilis* plants growing in hydroponic solution. Incorporations of radioactivity into monocrotaline (I) were observed at approximately ten times the rate observed for [^{14}C]-acetate. When the feeding experiments were repeated with flowering *C. spectabilis*

[2] E. Nowacki and R. U. Byerrum, *Life Sciences*, 1962, **5**, 157.
[3] D. H. G. Crout, N. M. Davies, E. H. Smith, and D. Whitehouse, *J.C.S. Perkin I*, 1972, 671.

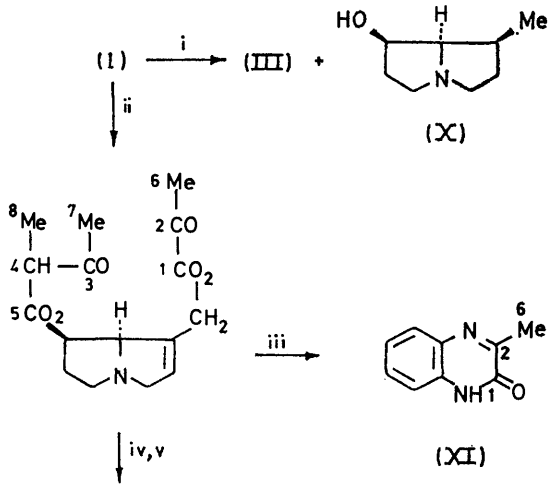
plants, the precursor being administered by the wick method, incorporations in the range 0·05—0·19% were

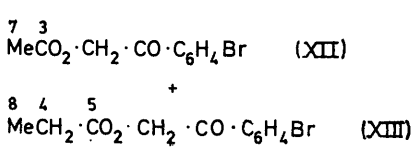


(VI) $R^1 = H, R^2 = Me$
(IX) $R^1 R^2 = CH_2$

SCHEME 1

observed (Table 1). These values are of the same order of magnitude as the incorporations of the same precursors into senecionine (VI) and seneciphylline (IX).





SCHEME 2

Reagents: i, H_2–Pt; ii, $NaIO_4$; iii, *o*-$C_6H_4(NH_2)_2$; iv, KOH; v, $BrCH_2{\cdot}CO{\cdot}C_6H_4Br$

The labelled monocrotaline from these various experiments was subjected to hydrogenolysis to give monocrotalic acid (III) and retronecanol (X) (Scheme 2). In each case the radioactivity was found to reside almost exclusively in the necic acid component; the pyrrolizidine base was labelled to a slight extent only (Table 2). It was concluded that, as in the biosynthesis

TABLE 2

Distribution of radioactivity in the necic acid and necine base components of monocrotaline (I) derived from [U-^{14}C]-L-isoleucine and [U-^{14}C]-L-threonine

	% Activity	
Experiment	Retronecanol (X)	Monocrotalic acid (III)
10	3·5	103
12	6·0	90
17	2·9	104
18	2·6	97
9	4·0	99
11	4·9	99
14	4·5	97
15	3·9	95
16	4·0	96

Experiments 10, 12, 17, 18: [U-^{14}C]-L-isoleucine feeding; experiments 9, 11, 14, 15, 16: [U-^{14}C]-L-threonine feeding.

of senecic (V) and seneciphyllic (IV) acids, L-threonine and L-isoleucine are specific precursors of the necic acid component of the alkaloid.

In order to investigate the pattern of incorporation of [U-^{14}C]-L-threonine and [U-^{14}C]-L-isoleucine in more detail, the labelled alkaloid from experiments 14 and 16—18 (Table 1) was degraded as shown in Scheme 2 to give C-1, -2, and -6 as the quinoxalone derivative (XI), C-3 and -7 as the *p*-bromophenacyl ester of acetic acid (XII) and C-4, -5, and -8 as the *p*-bromophenacyl ester of propionic acid (XIII).

The specific incorporation of a C_5 unit derived from [U-^{14}C]-L-isoleucine into the unit comprising C-1, -2, -3, -6, and -7 of monocrotalic acid (III) should lead to uniform labelling of this structural component. The observed percentage activities of the two degradation products representing C-1, -2, and -6 and C-3 and -7 are shown in Table 3. The ratios of the activities of these two fragments were 1·6 : 1 and 1·8 : 1 in experiments 17 and 18, respectively (Table 3), in reasonable agreement with the predicted ratio of 1·5 : 1. Further, the activity

TABLE 3

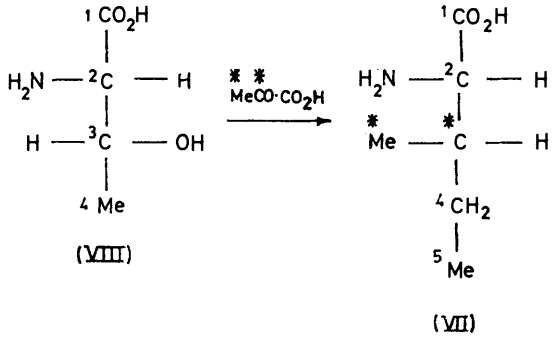
Distribution of radioactivity in monocrotalic acid (III) derived from [U-^{14}C]-L-isoleucine

	% Activity in			
Experiment	C-1, -2, and -6 (A^1)	C-3 and -7 (A^2)	C-4, -5, and -8	A^1/A^2
17	46	28	17	1·6
18	46	25	14	1·8

of the unit comprising C-4, -5, and -8 is seen to be significantly lower than the activities of the other two degradation products. These results therefore support the proposition that a five-carbon unit derived from L-isoleucine is incorporated specifically into the five-carbon unit comprising C-1, -2, -3, -6, and -7 of monocrotalic acid.

The results from the degradation of alkaloid derived

from [U-^{14}C]-L-threonine support this conclusion. Carbon atoms 1—4 of threonine (VIII) provide C-1, -2, -4, and -5 of isoleucine (VII) as indicated in Scheme 3.[3] If, by



SCHEME 3

decarboxylation, isoleucine provides the C_5 unit comprising C-1, -2, -3, -6, and -7 of monocrotalic acid (III) as in senecionine (VI)[3] and seneciphylline (IX)[4] biosynthesis, it follows that threonine should provide C-1, -3, and -7 of monocrotalic acid (III) and that these positions should be labelled equally with [U-^{14}C]-L-threonine as precursor.

The results of the degradation of monocrotaline derived from [U-^{14}C]-L-threonine are given in Table 4.

TABLE 4

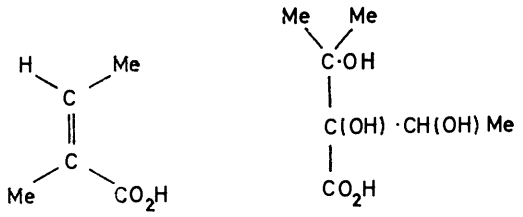
Distribution of radioactivity in monocrotalic acid (III) derived from [U-^{14}C]-L-threonine

	% Activity in			
Experiment	C-1, -2, and -6 (A^1)	C-3 and -7 (A^2)	C-4, -5, and -8	A^2/A^1
14	20	36	35	1·8
16	25	40	25	1·6

The ratio of the activities of the degradation products representing C-3 and -7 and C-1, -2, and -6 were 1·8 : 1 and 1·6 : 1 in experiments 14 and 16, respectively (Table 4), in reasonable agreement with the predicted value of 2·0 : 1.

It remains to consider the origin of the three-carbon unit comprising C-4, -5, and -8 of monocrotalic acid (III). Tables 3 and 4 show that this unit was significantly labelled with both [U-^{14}C]-L-threonine and [U-^{14}C]-L-isoleucine as precursors. On structural grounds it appears possible that this three-carbon unit might be derived from propionate; indeed, Nowacki and Byerrum reported that propionate was incorporated into the necic acid component of monocrotaline in *Crotalaria spectabilis*.[2] In this connection it is of interest that both threonine and isoleucine are potential sources of propionyl-coenzyme A. Thus threonine is converted into 2-oxobutanoate in the pathway leading to isoleucine;[5] 2-oxobutanoate could, by oxidative decarboxylation, furnish propionyl-CoA directly. The established pathway for the catabolism of isoleucine in both animal and microbiological systems leads, *via* tigloyl-CoA and 2-methyl-3-oxobutanoyl-CoA, to acetyl-CoA and propionyl-CoA.[5] The observation that radioactivity from both [U-^{14}C]-L-threonine and [U-^{14}C]-L-isoleucine is incorporated into the unit comprising C-4, -5, and -8 of monocrotalic acid (III) is therefore consistent with the proposition that this unit is furnished by propionate. [A possible precursor, L-alanine, was incorporated to a negligible extent (Table 1).] However, the available evidence[2] for the intermediacy of propionate is not conclusive and experiments are in hand to obtain further information on the specificity of propionate as a precursor of monocrotalic acid.

The observation that L-isoleucine is a specific precursor of monocrotalic acid provides the fourth example of the involvement of branched-chain amino-acids in necic acid biosynthesis. Previous work has shown that



isoleucine is the precursor of necic acids of the senecic (V) type[3,4] and of the angelic acid (XIV) component of heliosupine,[6] and that valine is a specific precursor of the echimidinic acid (XV) component of heliosupine.[7]

EXPERIMENTAL

All m.p.s are corrected. Radioactivity determinations were carried out with a Tritium Scintillation Counter model 6012 (Isotope Developments Ltd.) or with a Packard TriCarb series 2000 spectrometer. Radioactive samples were counted in either NE 220 scintillation fluid (Nuclear Enterprises Ltd.) or in B.D.H. dioxan scintillator. Sufficient counts were taken to give a standard error of not greater than 3% and usually less than 1% for each individual measurement.

Feeding Methods.—Wick method: the precursor was administered through a cotton thread inserted into the stem of the plant. Stem feeding: the precursor was administered by absorption through the stems of excised plants as described by Nowacki and Byerrum.[2] Injection method: the precursor was injected directly into the stem cavity by means of a hypodermic syringe. Hydroponic method: the plants were grown in a standard compost until required for use. The roots were then cleaned by washing with tap water and finally with distilled water. The plants were then placed in blackened beakers containing Phostrogen solution (Phostrogen Ltd.; 600 mg dm^{-3}). The precursors were added to the nutrient as aqueous solutions. The solution was continually aerated for the duration of the experiment.

Extraction of Monocrotaline.—In a typical experiment the

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

[5] V. W. Rodwell, 'Metabolic Pathways,' vol. III, 3rd edn., ed. D. M. Greenberg, Academic Press, New York and London, 1969 p. 201 *et seq.*

[6] D. H. G. Crout, *J. Chem. Soc.* (*C*), 1967, 1233.

[7] D. H. G. Crout, *J. Chem. Soc.* (*C*), 1966, 1968

plants were macerated with methanol in a Waring Blendor. The macerate was filtered and the procedure was repeated until the filtrate was colourless (three to six extractions were required). The methanol was removed on a rotary evaporator and the residue was treated with M-H_2SO_4 (20 cm^3). The acidic solution was extracted with chloroform (4 × 50 cm^3), filtered (kieselguhr), and stirred with zinc dust for 90 min. The resulting mixture was filtered and the filtrate was extracted with chloroform (4 × 50 cm^3), made strongly alkaline with ammonia, and again extracted with chloroform (4 × 50 cm^3). The latter extracts were dried ($MgSO_4$) and evaporated to give the crude alkaloid (yield *ca.* 0·15% based on the dry weight of the plant material). The crude alkaloid was diluted with a large excess of monocrotaline and recrystallised to constant activity. In some experiments, preparative t.l.c. was used to aid purification. Plates were prepared with Kieselgel G as adsorbent and developed with chloroform–methanol–ammonia (85 : 14 : 1).

In all experiments in which ^{14}C-labelled precursors were fed, uptake of radioactivity by the plants was greater than 90% and usually greater than 95%. With ^3H-labelled presursors, a considerable proportion of the label appeared as tritiated water which passed into the nutrient solution. The incorporation values are based on an assumed uptake of 100% of the precursor and are therefore subject to a 10% probable error.

Hydrogenolysis of Monocrotaline.—In a typical experiment monocrotaline (200 mg), together with platinum oxide catalyst (15 mg), in 0·1M-HCl (20 cm^3), was hydrogenated at atmospheric pressure [uptake 2·0—2·2 moles H_2 per mole (I)]. The solution was filtered and the filtrate was extracted continuously with ether for 48 h. The extract was dried ($MgSO_4$) and evaporated to give monocrotalic acid (III) (120 mg, 100%), which crystallised from acetone–light petroleum (b.p. 60—80°) as prisms, m.p. 184° (95 mg).

The aqueous residue from the ether extraction was passed through a column of Dowex 1-X8 ion-exchange resin (OH^- form; 5 g) and the column was washed with deionised water until the eluate was neutral. The eluate was extracted continuously with ether for 48 h and the extract was dried ($MgSO_4$) and evaporated to give retronecanol (X) as an oil which crystallised from light petroleum (b.p. 60—80°) as prisms, m.p. 94° (81 mg).

Degradation of Monocrotalic Acid.—Monocrotaline (370 mg, 1·1 mmol) was dissolved in periodic acid (60 cm^3; 0·1 mol dm^{-3}). The solution was left in the dark for 2 h and was then passed through a column of Dowex 1-X8 ion-exchange resin (HCO_3^- form; 25 g). The column was washed with deionised water until the eluate gave a negative test with Dragendorff's reagent. The eluate was treated with freshly crystallised *o*-phenylenediamine (160 mg) in water (4 cm^3). The solution was concentrated at room temperature until the quinoxalone (XI) precipitated. The crystalline derivative was filtered off, dried, and purified by chromatography on basic alumina with chloroform as eluant to give the derivative (86 mg) which crystallised from ethanol as needles, m.p. 247—251° (lit.,[8] 245°).

The filtrate obtained after removal of the quinoxalone was treated with one and a half times its weight of potassium hydroxide. This solution was stirred at 100° for 4 h, cooled, washed with ether (2 × 50 cm^3), and acidified (Congo Red) with 50% H_2SO_4. It was then distilled and the distillate was titrated against 0·01M-NaOH (phenolphthalein). Distillation was continued until the distillate was neutral. The neutralised distillate was concentrated to 2 cm^3, filtered, and treated with *p*-bromophenacyl bromide (625 mg) in ethanol (75 cm^3). The solution was boiled under reflux for 85 min, cooled, diluted with water (75 cm^3), and extracted with chloroform (1 × 200 cm^3; 2 × 100 cm^3). The combined extracts were dried (Na_2SO_4) and evaporated. The residue (564 mg) was purified by preparative t.l.c. on Kieselgel PF_{254} in light petroleum (b.p. 60—80°)–benzene–chloroform–ethyl acetate (5 : 3 : 2 : 1).[9]

The bands corresponding to *p*-bromophenacyl acetate (R_F 0·40) and *p*-bromophenacyl propionate (R_F 0·54) were removed and extracted with chloroform for 2 days. The *p*-bromophenacyl acetate (80 mg) crystallised from ethanol–water as prisms, m.p. 81—83° (lit.,[9] 81—83°). The *p*-bromophenacyl propionate crystallised from ethanol–water as prisms, m.p. 52—54° (lit.,[9] 52—54°).

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[4/549 *Received*, 19*th March*, 1974]

[8] O. Hinsberg, *Annalen*, 1896, **292**, 245.
[9] D. Gröger, K. Stolle, and K. Mothes, *Arch. Pharm.*, 1967, **300**, 393.