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INCORPORATION OF ASSIMILATED CARBON INTO AMINOALCOHOLS OF *HELIOTROPIUM SPATHULATUM**

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Key Word Index—*Heliotropium spathulatum*; Boraginaceae; aminoalcohol; pyrrolizidine alkaloid; biosynthesis; trachelanthamidine; supinidine; retronecine.

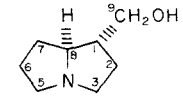
Abstract—In young plants of *Heliotropium spathulatum* exposed to pulse labelling with $^{14}CO_2$, leaves were the main, if not exclusive, organs in which necine biosynthesis took place. Removal of roots did not affect this process. The incorporation of assimilated carbon into the aminoalcohols was not light dependent; the total radioactivity found in trachelanthamidine, supinidine, and retronecine after the initial 12 hr light period was *ca* 40% of that found after the following 12 hr darkness. Even at the end of the 48 hr period, when the degree of ^{14}C incorporation into the necines was the highest, the average specific activity of carbon in the aminoalcohols was *ca* 7-, 20- and 30-fold lower than that of total carbon, in leaves, stems and roots, respectively. Trachelanthamidine exhibited the highest specific radioactivity. Molar absorptivities for trachelanthamidine, lindelofidine, supinidine, retronecine, and heliotridine, obtained by the methyl orange method, are reported; the method is more sensitive for the monoalcohols than for diols.

INTRODUCTION

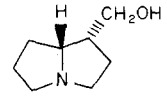
The number of plants which may contain pyrrolizidine alkaloids (PA's) is estimated to be as high as 8000 species, i.e., *ca* 3% of the world's flowering plants[1]. Among the 14 families in which PA's have been found[2–4], the Compositae, Leguminosae, and Boraginaceae have been the most examined. In the Compositae (tribes Senecioneae and Eupatorieae) PA's were found in 156 species, including 124 containing toxic PA's; in the Leguminosae and Boraginaceae the corresponding numbers are 60 and 46; and 75 and 65, respectively[5]. In the Leguminosae PA's were found only in the genus *Crotalaria*, whereas in the Boraginaceae they occur in all genera examined. Among over 200 known species of the genus *Heliotropium* (Boraginaceae), only 20 have been tested in detail [5, 6] and 19 shown to contain unsaturated toxic PA's. The presence of unsaturated PA's was established in an additional 24 species collected in Mexico and adjacent U.S.A.[7].

PA-bearing plants are geographically widespread and may be present in most environments. The toxic, including carcinogenic, effects of PA's with an unsaturated pyrrolizidine nucleus are of a chronic and progressive character resembling the effects of mytotoxins rather than those of most known alkaloids[5, 8]. PA poisoning of animals has been of serious concern for many years[9] and lately, the danger of human exposure to toxic PA's has also been recognized [5, 10–15]. PA-bearing plants have been widely used in both hemispheres in indigenous medicine for the treatment of a great variety of diseases. Many of their PA's or PA derivatives have revealed high pharmacological, including anti-tumor, activities [7, 16–22]. Plants of the genus *Heliotropium*, among others, have been described in folklore as remedies for cancer [23]. Various PA's, in particular those occurring in *Heliotropium*, may play a significant role as deterrents against vertebrate predators as well as precursors of aphrodisiacs in herbivore insects [7, 24–26]. Although the known biological effects of PA's, especially of the toxic ones, depend on both the aminoalcohol (necine) and the esterifying acid(s), the character of the necine moiety is of foremost importance [27, 28].

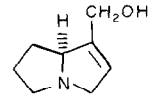
Biosynthetic studies on necines have been restricted to the most common one, namely retronecine (**4**) in *Crotalaria spectabilis* Roth[29], *Senecio douglasii* DC.[30], *S. isatideus* DC. [31–34]



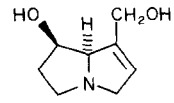
1 (−)-Trachelanthamidine



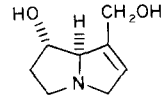
2 (+)-Lindelofidine



3 (−)-Supinidine



4 (+)-Retronecine



5 (+)-Heliotridine

*Part 1 in the series "Aminoalcohols of Pyrrolizidine Alkaloids in *Heliotropium* Species".

and S. *magnificus* F. Muell[35]. In all these species retronecine was the only necine found. Labelled precursors were introduced via roots, cut stems or by injection into the stem xylem and the aerial parts analysed after 3–11 days. Putrescine, as well as its precursors ornithine and arginine, have been shown to be specific precursors of retronecine; the polyamines spermidine and spermine were at least as efficient precursors as was putrescine[32]. The complete labelling pattern in retronecine, obtained using [^{13}C]putrescine[33], and the evidence for homospermidine as an intermediate in the necine biosynthesis[34] confirmed the classical hypothesis[36], i.e. that two putrescine molecules combine to form the necine via a symmetric intermediate C_4–N–C_4, homospermidine apparently being the normal intermediate in this process[34]. However, no publications related to biosynthesis site(s) of necines, their interconversion, or further metabolism could be found.

This study presents the incorporation of assimilated carbon into aminoalcohols of PA's in intact and de-rooted plants of *Heliotropium spathulatum* after exposure to $^{14}CO_2$. *Heliotropium spathulatum* has been chosen for several reasons. Besides retronecine, it contains (−)-trachelanthamidine (**1**) and (−)-supinidine (**3**) in measurable amounts; a fourth necine, apparently lindelofidine (**2**) has been found in traces only, and heliotridine (**5**) could not be detected[37]. Many plants of this species can be easily propagated from roots of a single plant, thus assuring a homogeneous population. Plants collected in various locations in Mexico and the U.S.A. revealed the same qualitative composition of the necines as did those grown in the greenhouse[6]; and finally, the latter accumulate relatively very high amounts of PA's[37].

RESULTS AND DISCUSSION

The total radioactivity of all plants exposed to labelled CO_2 summed up to 3.43 mCi, i.e. *ca* 98% of the activity applied; 17 μCi were found in CO_2 flushed out after the first 12 hr light exposure. The corresponding values for CO_2 flushed out after the following 12 hr dark and 24 hr light/dark periods were 35 and 8 μCi, respectively, thus indicating that the labelled CO_2 was mainly assimilated during the first 2–4 hr exposure. After 12 hr exposure only *ca* 50% of the total activity was found in the ethanol-soluble

Table 1. Weight and radioactivity of plants and their alkaloids (per plant)

Time of sampling (hr)	Plant	Organ	Plant: Dry wt* (mg)	Plant: Activity (10^{-6} × cpm) Total	Plant: Activity (10^{-6} × cpm) EtOH soluble	Alkaloids: Contents† % dry wt	Alkaloids: Contents† mg	Alkaloids: Activity (10^{-3} × cpm)
12	Intact	Leaves	357	123	60	2.18	7.8	1020
		Stem	284	59	25	1.83	5.2	181
		Roots	181	13	8	2.80	5.0	59
		Total	822	195	93	—	18.0	—
	Derooted	Leaves	345	137	75	2.09	7.2	1059
		Stem	251	63	30	1.90	4.8	303
		Total	596	200	105	—	—	—
24	Intact	Leaves	352	107	48	2.01	7.4	1383
		Stem	255	62	20	1.79	4.6	348
		Roots	175	15	6	2.69	4.7	64
		Total	782	184	74	—	16.7	—
48	Intact	Leaves	363	102	47	2.12	7.7	1424
		Stem	268	69	22	1.86	5.0	663
		Roots	189	16	5	2.95	5.6	115
		Total	820	187	74	—	18.3	—
	Derooted	Leaves	349	117	53	2.20	7.7	1397
		Stem	266	84	31	1.87	5.0	641
		Total	615	201	84	—	—	—

*The dry wt of radioactive fresh organs was calculated on the basis of percent dry matter in control leaves, stems and roots, sampled at time zero: 8.10; 6.65; and 8.63%, respectively.

†Calculated as monocrotaline, MW 325.

Table 2. Content and radioactivity of aminoalcohols (per plant)

Time of sampling (hr)	Plant	Organ	Content (μmol)*				Activity (10^{-3} × cpm)							^{14}C incorporation (% total)
							Specific			Total			Total	
			T	S	R	Total	T	S	R	T	S	R		
12	Intact	Leaves	9.8	3.7	10.5	24.0	4.4	1.5	1.1	43.1	5.6	11.5	60.2	0.049
		Stem	5.5	2.5	8.0	16.0	2.1	1.5	0.6	11.6	3.7	4.8	20.1	0.035
		Roots	3.1	1.5	10.8	15.4	0.54	0.46	0.15	1.6	0.7	1.7	4.0	0.030
		Total	18.4	7.7	29.3	55.4	—	—	—	56.3	10.0	18.0	84.3	0.042
	Derooted	Leaves	8.8	3.4	10.0	22.2	4.8	†	1.5	42.2		15.0		
		Stem	4.9	2.1	7.4	14.4	2.4	2.0	0.8	12.0	4.3	5.8	22.1	0.035
24	Intact	Leaves	9.4	3.1	10.1	22.6	13.5	5.9	4.3	126.9	18.3	43.4	188.6	0.176
		Stem	4.9	2.2	7.1	14.2	3.4	2.9	1.2	16.6	6.3	8.2	31.1	0.050
		Roots	2.8	1.5	9.9	14.2	0.56	0.47	0.36	1.6	0.7	3.1	5.4	0.036
		Total	17.1	6.8	27.1	51.0	—	—	—	145.1	25.3	54.7	225.1	0.122
48	Intact	Leaves	9.5	3.4	10.8	23.7	11.3	9.1	6.6	107.3	30.9	71.3	209.5	0.204
		Stem	5.0	2.2	7.6	14.8	3.5	3.1	1.6	17.5	6.8	12.1	36.4	0.053
		Roots	3.4	1.8	12.3	17.5	0.70	0.66	0.58	2.4	1.2	7.2	10.8	0.072
		Total	17.9	7.4	30.7	56.0	—	—	—	127.2	38.9	90.6	256.7	0.137
	Derooted	Leaves	9.9	3.3	10.5	23.7	10.8	8.9	7.0	106.9	29.4	73.5	209.8	0.180
		Stem	5.2	2.2	8.0	15.4	4.7	3.9	3.5	24.4	8.6	28.0	61.0	0.073

*(−)-Trachelanthamidine, T (including traces of a necine with a mass spectrum, R_f, and R_f of lindelofidine); (−)-supinidine, S; retronecine, R.
†Accidental radioactive contamination.

fraction and the translocation of the label to the stem and roots was almost completed (Table 1). Assimilation of the labelled CO_2 by de-rooted plants did not differ from that by intact ones (the radioactivity of water from all derooted plants was *ca* 1.6 μCi). The differences between replicates in the weight and radioactivity of organs, alkaloid content and their activity were very small, not exceeding 11%.

The total PA content of the leaves, stems and roots was *ca* 2.1, 1.8 and 2.7% dry wt, respectively. The activity of the alkaloid fraction prior to hydrolysis was the highest in the leaves, but not exceeding 1.4% of their total activity after 48 hr. The purified necines accounted for 5–15% of the radioactivity found before hydrolysis (Table 2). The ether-soluble compounds accounted for *ca* 60%, a great portion of it being probably related to the released necic acids.

The ratios between individual necines in a given organ varied very little. The share of trachelanthamidine was *ca* 40, 34 and 20% of the total in leaves, stems and roots, respectively, and that of supinidine was *ca* 15% in leaves and stems, and 10% in roots. Retronecine was the dominant base, especially in roots.

The highest ^{14}C incorporation into necines (in absolute and relative values) was found in the leaves. However, the results indicate that their synthesis is not located in chloroplasts and/or light dependent as it is in the case with lysine-derived quinolizidine alkaloids [38–41]. The greatest increase in the total necine activity occurred in the dark between 12 and 24 hr; it was very small during the following 24 hr. If compared with the specific radioactivity of carbon in the total CO_2 present in the air of the chamber during the first 2 hr of light exposure (amounting to *ca* 2.9×10^8 cpm/mg carbon) the percentage specific incorporation of carbon in the necines of leaves after 12 hr was *ca* 0.009%. The percentage specific incorporation of labelled CO_2 in nicotine isolated from aerial parts of *Nicotiana tabacum* was, as reported, *ca* 0.1% after 4 hr exposure [42].

A comparison of the average specific activities of carbon in necines with those of carbon in plant dry matter (Table 3) indicates that leaves are the main, if not the only, organ responsible for necine synthesis. The significant rise in the specific radioactivity of necines, especially that of retronecine, in the roots between 24 and 48 hr seems to be due to an influx from the aerial parts rather than to synthesis *in situ*. However, even in leaves after 48 hr the average specific activity of carbon in necines was *ca* sevenfold lower than that of total carbon in the dry matter.

Synthesis of glutamate is supposed to occur mainly in chloroplasts, its carbon skeleton being, however, formed outside the chloroplasts from photosynthates leaving the latter probably at the three-carbon level [43]. Ornithine and its derivative, arginine, can be formed from glutamate via *N*-acetylglutamate or via glutamyl-γ-phosphate, the latter being a shorter pathway. Putrescine can derive directly from ornithine by decarboxylation of the latter, or from arginine via agmatine. Our results indicate that the pathway from photosynthates to necines is rather long leading slowly via diluting pools of intermediates.

The results obtained clearly indicate that removal of roots did not affect biosynthesis of the necines as is the case with biosynthesis of nicotine in tobacco plants in which *N*-methyl putrescine oxidase has been found only in roots [44]. Changes in the total and specific activities of the necines (especially in view of their accumulation in plants) suggest that their transformation into non-alkaloidal compounds may be rather slow, in particular when compared with turnover rates reported for a series of other alkaloids [45–47].

In all organs the specific activities of trachelanthamidine were the highest and those of retronecine the lowest; a very significant increase in the specific activities of supinidine and retronecine in the leaves was observed even when that of trachelanthamidine decreased. These results suggest that in *Heliotropium*

Table 3. Average specific radioactivity of carbon in intact plants and their aminoalcohols

Organ	Time of sampling (hr)	Specific radioactivity of carbon: Organ* ($10^{-5} \times$ cpm)	Specific radioactivity of carbon: Amino alcohols ($10^{-4} \times$ cpm)	Difference factors
Leaves	12	7.6	2.6	30
	24	6.8	8.4	8
	48	6.3	9.3	7
Stem	12	4.6	1.3	36
	24	5.4	2.3	23
	48	5.7	2.5	22
Roots	12	1.6	0.27	59
	24	1.9	0.39	49
	48	1.9	0.61	31

*Assuming 45% carbon in the dry matter of the organ.

Table 4. Calibration data for aminoalcohols

Aminoalcohol	MW	$A_{525\,nm}$/ $\mu g \cdot ml$	Molar absorptivity*
Trachelanthamidine	141	0.275	38775
Lindelofidine	141	0.248	34968
Supinidine	139	0.252	35028
Retronecine	155	0.078	12090
Heliotridine	155	0.053	8215

*The molar absorptivity of monocrotaline was 40975 when standardized simultaneously with the bases.

spathulatum trachelanthamidine might be the first synthesized necine and that it might convert into retronecine via supinidine.

However, it is difficult to draw definite conclusions due to many unknown factors that could have affected our data. The possible subcellular compartmentation of newly formed and stored necines and their esters; enzymes involved in necine transformation and their subcellular location; and the form (free, esterified, *N*-oxide) in which the aminoalcohols undergo transformation, perhaps, are the most important unknowns. Small amounts of free trachelanthamidine and retronecine were found in PA extracts of *Heliotropium spathulatum* plants prior to hydrolysis[37]; they were not analysed separately in this study. Neither could we isolate lindelofidine in measurable amounts.

In order to minimize the amounts of necines required for monitoring the completeness of their extraction and above all for their accurate quantitation, an attempt was made to use the methyl orange method [48] without further modification. Previous trials to assay heliotridine with a similar sensitivity as that found for esterified amino alcohols were unsuccessful. A linear relationship between absorbance and concentration of the three monoalcohols, retronecine, and heliotridine was obtained at concentrations above 1, 4, and 5.5 μg/ml chloroform, respectively; the molar absorptivities were lower than those found for esterified necines (Table 4). The method showed the lowest sensitivity for retronecine and heliotridine. However, when compared with titration, it proved to be more sensitive even for the diols.

EXPERIMENTAL

Plant material. Seven-week-old plants, propagated from roots of a single greenhouse grown plant cultivated under good light, temp., water, and nutrient conditions, were used. Pots with intact plants were covered with aluminum foil leaving a small opening for the protruding stem. Fresh cut stems were immediately immersed in slightly acidified water.

The plants were exposed to labelled CO_2 in a 60 l. Plexiglass chamber having a small electric fan for air circulation, an injection septum on the upper surface and inlet and outlet valves on opposite ends. The injection septum was connected via Tygon tubing to the innermost well of a central glass container having three concentric wells graduated in height with the innermost well the shortest. The inlet valve was connected to a polyethylene tube filled with Ascarite to absorb CO_2 from the entering air during flushing; the outlet valve was connected to three gas washing bottles in series, the first two containing 10% NaOH and the third containing 5% $Ba(OH)_2$. The chamber could be water cooled if necessary.

At time zero, 12 intact and 8 de-rooted plants were placed in the chamber; 3.5 mCi [^{14}C]$BaCO_3$ and 48 mg Na_2CO_3 were transferred to the innermost well; the middle well and the outer contained 106 and 424 mg Na_2CO_3, respectively. The chamber was sealed and the labelled CO_2 in the innermost well released by lactic acid. The unlabelled CO_2 in the middle and outer wells was released 2 and 4 hr later by lactate in excess. The released amount of $^{14}CO_2$, combined with CO_2 present in the air (amounting to *ca* 13 mg carbon) allowed a photosynthesis rate *ca* 2 mg CO_2/dm · hr for the first 2 hr.

After 12 hr exposure in light the chamber was flushed with air and four intact and four de-rooted plants were sampled. The chamber was resealed, placed in darkness for 12 hr, reflushed in the morning, and four intact plants were sampled. The remaining eight were exposed for an additional 12 hr light/12 hr dark period to unlabelled CO_2 and then sampled. The leaves were separated from the stem, the roots, including rhizomes, cut off, cleaned by floating the Vermiculite away in tap H_2O and blotted dry. The organs were immediately weighed and frozen. They were analysed in two replicates, two plants each. At time zero, four unexposed plants were sampled as controls. The H_2O from de-rooted plants as well as NaOH and $Ba(OH)_2$ solns were collected and analysed.

Analyses. The samples were treated (×3–4) with boiling EtOH, homogenized with sonication, centrifuged, and the supernatant collected. The supernatants were combined, the sediments resuspended, and their radioactivities measured.

After EtOH removal *in vacuo*, the residue was extracted with 2 N H_2SO_4 and *N*-oxides reduced by shaking overnight in the presence of excess Zn. The acidic soln was purified twice with $CHCl_3$, the organic solvent washed with 0.1 N H_2SO_4, and the washings combined with the original. (Washing with Et_2O at this stage did not result in any significant radioactive purification.)

The PA's were extracted with $CHCl_3$ at pH 10.5–11.0 and the purification and extraction repeated. The PA's were hydrolysed with 10% aq. NaOH (2 ml/sample) at 100° for 2 hr. The necines were extracted with $CHCl_3$ in two steps: (a) directly from the aq. soln; this fraction contained trachelanthamidine, supinidine, trace of lindelofidine, and small amounts of retronecine not exceeding 10% of its total; and (b) after reducing the vol. of the basic soln to 0.5 ml; this fraction yielded retronecine only. A complete extraction

of retronecine could be achieved either when a two-fold treatment with $CHCl_3$ was followed by binding the remaining H_2O with dry Na_2SO_4 and an additional $CHCl_3$ extraction or when the aq. soln was extracted with $CHCl_3$–EtOH (2:1). The $CHCl_3$ extracts were dried (Na_2SO_4) and aliquots taken for analyses, including GC/MS (the combined amounts of bases, determined by titration, were similar to those found before hydrolysis). After the solvent was removed, the residue was acidified, purified with Et_2O, basified, and re-extracted with $CHCl_3$. Each necine fraction, pooled together from two replicates, was separated by TLC plates coated (150–600 μm) with 0.1 N NaOH-pre-treated Si gel G (Merck). MeOH was used as the solvent. The necines were detected with I_2 or Dragendorff reagent. The localized bands and bordering areas were eluted with ammoniacal MeOH, the solvent removed, and the necines extracted with $CHCl_3$ from aq. solns at pH 10.5–11.0 as described above. Before elution the necine bands were halved longitudinally and each half was analysed separately. The differences between specific activities of the necines obtained from two halves of their bands ranged from 7 to 13%. The retronecine band from fraction (a) was also assayed. The R_f values of the marker lindelofidine, isoretronecanol, (−)-trachelanthamidine, (−)-supinidine, retronecine and heliotridine were 0.06, 0.08, 0.09, 0.13, 0.26 and 0.35, respectively.

GC/MS did not reveal any impurities, detectable under the experimental conditions, in the TLC-separated necines. Labelled trachelanthamidine and retronecine, obtained from leaves of intact and de-rooted plants, after 24 and 48 hr, were diluted with cold necines and twice recrystallized; trachelanthamidine picrate from EtOH and retronecine from Me_2CO. Their specific radioactivities were similar to those found prior to crystallization. The specific activities of necines in Table 2 represent the lowest values obtained after TLC separation.

To determine the ratios between individual necines, GC/MS was quantitatively calibrated using (−)-trachelanthamidine (R_T 2.8 min), (−)-supinidine (3.1 min), lindelofidine (3.1 min), retronecine (9.3 min), and heliotridine (10.1 min), at 0.25–4.0 μg/injection. The sensitivity of the instrument to individual amino alcohols was continuously monitored: the R_T of isoretronecanol was 3.1 min. (−)-Trachelanthamidine was isolated from *Heliotropium spathulatum* [37] or *H. gregii* [6]; lindelofidine from *H. ternatum* [6]; (−)-supinidine from *Heliotropium spathulatum* or *H. karwinskyi* [6]; retronecine from *H. indicum* [37] or monocrotaline (S. B. Pennick, Lyndhurst, NJ); heliotridine and isoretronecanol were gifts from C.C.J. Culvenor.

PA's and necines were analysed by titration [7] and/or by the methyl orange method [48]. All chemicals were reagent grade; spectrophotometric grade MeOH was used for GC/MS. The *p*-dioxane-based scintillation fluid contained naphthalene, POP and POPOP at 60, 4 and 0.2 g/l., respectively; absolute MeOH and ethylene glycol at 100 and 20 ml/l. respectively. The non-radioactive plant organs, sampled at time zero, were used to evaluate quenching effects. These organs were also exposed to similar procedures as the radioactive ones; no differences in PA recovery were found when either fresh frozen or dried material was used.

The Hewlett–Packard 5992 GC/MS system was equipped with a 180 cm × 2 mm glass column packed with 3% OV-17 on 100–120 mesh GasChrom Q. The injection port temp. was 200°. A jet separator was used for the GC/MS interface. Samples in MeOH were injected directly onto the glass column. The column temp. was programmed at 140° for 7 min, then to 180° at 16°/min: carrier gas He at 24 ml/min. The run times of necine fractions (a) and (b) as well as of the TLC-separated necines were often prolonged to 60–70 min in order to monitor for possible contaminations. Radioactivity was determined using a Unilux Nuclear Chicago scintillation counter.

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