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METHODS TO OBTAIN RADIOLABELLED MONOCROTALINE

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Summary

Crotalaria spectabilis, a plant found in many areas of the world is associated with the pyrrolizidine alkaloid monocrotaline. Monocrotaline when injected subcutaneously in Sprague Dawley rats has been utilized for years to create a condition known to mimic pulmonary hypertension in humans. We attempted to determine the optimum conditions for the biosynthesis of radiolabelled monocrotaline. Our work describes the plant growth conditions and the time periods associated with the production of radiolabelled monocrotaline. In addition, the incorporation of $^{14}CO_2$ or [2,3-^{3}H]-putrescine dihydrochloride and the specific activity plus the amount (s) of recovered radiolabelled monocrotaline are discussed. We conclude that the most efficient and cost effective method for the biosynthesis of radiolabelled monocrotaline is still the utilization of $^{14}CO_2$.

Introduction

Plants containing pyrrolizidine alkaloids (PAs) are distributed worldwide and involve diverse botanical families such as the Asteraceae, Boraginaceae, Fabaceae and the Orchidaceae (1, 2). Many of the PAs are esters of hydroxylated 1-methylpyrrolizidines; their acid moieties are known as "necic acids" and the amino alcohols "necines". (3). The

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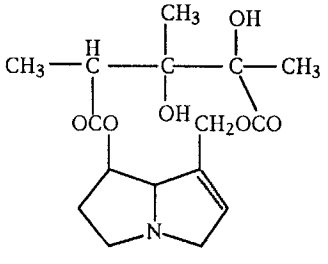
PAs exhibiting hepatotoxicity characteristically are esters of 1,2-dehydro-1-hydroxymethyl pyrrolizidine or "unsaturated PAs" (4). Following ingestion and metabolic activation by hepatic enzymes, the PAs with an unsaturated necine base form reactive and highly toxic dehydroalkaloids (5,6).

Understanding the metabolism and detoxification of PAs has been an interest of numerous investigators including our own laboratory. The limiting factor in many metabolism and pathology studies involving the PAs has been the lack of sufficient specific radioactivity associated with the PA of choice.

The biosynthesis of radiolabelled PAs has been challenging for many investigators. Numerous investigators have used small amounts of radioactivity (either ^{14}C or ^{3}H labelled precursors) to determine the biosynthesis of specific components of the macrocyclic PAs. In particular, PAs derived from the *Senecio* spp. have drawn the majority of interest (7-10). Studies performed in various laboratories have focused primarily on the initial synthesis of the retronecine base by examining the incorporation of substrates such as putrescine, spermidine, etc., and enzymes such as homospermidine synthase (7, 11-13). However, their objectives and experimental designs have not proven feasible in obtaining either high enough specific activity or enough labelled PAs required for use in future metabolic or toxicological studies.

A number of years ago, we published a manuscript detailing the results of radiolabelled biosynthesis studies performed in our laboratory in an effort to obtain labelled PAs for future hepatic metabolism studies (14). We as well as other investigators, obtained somewhat low specific activities in PAs associated with the incorporation of numerous radiolabelled substrates (14-19).

Few investigators have attempted to radiolabel the PAs associated with the *Crotalaria spp*., in particular the alkaloid monocrotaline (20,21).



Monocrotaline

Monocrotaline when injected subcutaneously in Sprague Dawley rats has been utilized for many years to create a condition known to mimic pulmonary hypertension in humans (22). Following a single injection of 60 mg/kg of monocrotaline, rats develop a proliferative pulmonary vasculitis, pulmonary hypertension and cor pulmonale within 3 weeks (23). Having uniformly radiolabelled monocrotaline would greatly facilitate our understanding of the initiating mechanism of pneumotoxicity associated with the monocrotaline model. We describe in the following sections labelling experiments using *Crotalaria spectabilis* performed with the specific radiolabelled precursor putrescine (24) or the utilization of $^{14}CO_2$. Our objective was to determine the "optimum conditions" to facilitate the radiolabelling of monocrotaline for future toxicological studies.

Methods

Plant Growth Chamber and Accessories:

The plant growth chamber utilized for radiolabelling experiments and appropriate accessories were identical in all respects with that described in our prior publication (14). The exception was the actual construction of a second plant growth chamber which was manufactured from stainless steel with a top window consisting of glass encased in a steel frame lined with screws to facilitate access. The dimensions of the new chamber are 61 cm (height) x 66 cm (length) x 38 cm (width).

Radiation Safety

All protocols were checked and approved by the University of California Radiation Safety committee. Spot monitoring of all radiolabelled experiments were completed by the Office of Environmental Safety.

Biosynthesis and Isolation of ^{14}C-Monocrotaline from *Crotalaria spectabilis*

All chemicals and supplies unless otherwise noted were obtained from Fisher Scientific (Santa Clara, CA). *Crotalaria spectabilis* were grown from seed in clay pots (21.6 cm in diameter and 19.3 cm in depth) in modified University of California (UC) mix (25% each of horticultural sand, peat moss, redwood compost and ground pumice rock), following inoculation with *Rhizobium leguminosarum.* Up to a maximum of 8 plants per pot were grown on a twelve hour light cycle under metal halide lights supplemented with tungsten illumination in a plant growth chamber. Plants received only distilled H_2O and received a 0-10-10 granular fertilizer administered at 2 week intervals. When plants were approximately one month of age, exhibiting an average height of 16 cm, they were placed in the atmospherically isolated radiolabelling plant growth chamber. Plants were allowed to equilibrate within this growth chamber for one day prior to the administration of $^{14}CO_2$. Total CO_2 (CO_2 augmented with $^{14}CO_2$) within the box was maintained within the limits of 260 to 300 ppm; the light cycle and the elements providing illumination were maintained as described above.

Four separate labelling experiments were conducted in which 50, 100, or 250 mCi of $^{14}CO_2$ was provided over a five day period in equal allotments. For instance, in the two experiments conducted with 100 mCi of $^{14}CO_2$, the first day of exposure began at 9:00 am in which 20 mCi was added as a solution of 50 mCi/mmol $NaHCO_3$ (American Radiolabeled Chemicals Inc., St. Louis, MO) administered to a solution of 18N H_2SO_4 over a 4 hr period releasing $^{14}CO_2$. Following the final exposure at 9:00 am on the fifth day, an additional 72 hr elapsed before the plants were harvested. To ensure safety, the CO_2 level was allowed to decrease to 75 ppm (maximal plant uptake) and the plants harvested.

All aerial parts of the plant were immediately homogenized in methanol and the material soxhlet extracted for 48 hr. Methanol was removed under reduced pressure and

the residue was dispersed in 0.25 N HCl and extracted with 3 vol of hexane followed by 3 vol of ethyl ether. The acid layer was stirred overnight with an excess of zinc dust to reduce the monocrotaline-N-oxides. Zinc dust was removed by filtration through Whatman #4 paper. The filtrate was immediately made basic (pH 9 to 10) with NH_4OH and extracted with 4 vol of $CHCl_3$. Each extraction was filtered through a layer of anhydrous Na_2SO_4 into a common reservoir with additional Na_2SO_4. The drying agent was not removed from the $CHCl_3$ layer until the following day; the agent was removed by filtration through Whatman #4 paper and the $CHCl_3$ removed under reduced pressure. The radiolabelled monocrotaline residue was recrystalized from ethanol.

For studies using small amounts of plant material, the crude alkaloid mixture was resuspended in a minimal volume of methanol and applied to a 5 mL column of Dowex-50W resin (12% cross linked, mesh 200-400, hydrogen form, Sigma, St. Louis, MO). The column was washed with several volumes of methanol followed by water. ^{14}C-monocrotaline was eluted from the matrix with 1N NH_4OH. The NH_4OH layer was extracted with 3 vol of $CHCl_3$ which was dried as previously described before removal under reduced pressure. The purity of the radiolabelled monocrotaline was checked by reverse phase HPLC (fig 1) using non-radiolabelled monocrotaline as the standard. Data for the four separate labelling experiments is recorded in Table 1.

[2,3-^{3}H]-Putrescine Incorporation into Monocrotaline.

[2,3-^{3}H]-Putrescine dihydrochloride (30.0 Ci/mmol) or [1,4-^{14}C]-putrescine dihydrochloride (90.4 mCi/mmol) both from DuPont , Wilmington, DE, were administered to *C. spectabilis* (age $1^1/_2$ months, 50 to 60 cm in height, one plant per pot) by the wick method (10). Briefly, the procedure entailed the insertion of a cotton thread through the stock of the plant with both ends being inserted into a vial containing the

Table 1

Specific activity of ^{14}C-Monocrotaline (MCT) obtained from Crotalaria spectabilis exposed to different levels of $^{14}CO_2$

[a] Total mCi of $^{14}CO_2$	[b] Grams of Plant Material	Quantity of ^{14}C-MCT in mg	Specific Activity of ^{14}C-MCT (mCi/mmol)
50	42.39	114.1	1.95
100	73.87	147.2	1.88
100	22.90	34.0	4.36
250	89.06	136.9	2.48

a) Total amount of $^{14}CO_2$ administered to plants over a 5 day period.
b) Wet weight plant material at the time of harvest; 72 hr after the last administration of $^{14}CO_2$

radiolabelled putrescine. The length of wick between the mouth of the feeding vessel and the insertion point in the plant was kept as short as possible. The labelled material was usually administered in a volume no greater than 5 mL of water and at the time of administration the soil was dry, but the plant retained it's normal hydration. Volumes of 5 mL were completely consumed by the plant within 6 hr or less. The vial containing the radiolabelled putrescine was washed with an additional 5 mL of water to remove residual material in the vessel and the wick.

A number of 5 mCi experiments were performed which varied the times for the administration of the [2,3-^{3}H]-putrescine dihydrochloride and the lapse of time between the harvest of the plant and the last feeding of radiolabelled putrescine; data and conditions are recorded in Table 2. Monocrotaline was extracted and purified as described previously. In addition, to determine if the [2,3-^{3}H]-putrescine dihydrochloride had incorporated into the necine base of monocrotaline as expected, radiolabelled monocrotaline was mixed with one gram of non-labelled monocrotaline. The mixture was hydrolyzed with a solution of $Ba(OH)_2$ and extracted according to the method of Adams and Rogers 1939 (25). Retronecine obtained from this extraction was further purified by the methods of Hoskins et al (26). Retronecine recrystalized from acetone had a melting point of 117-118°C (uncorrected). For both the ^{14}C and the ^{3}H putrescine precursors, only the necine base and not the necic acid was found to contain radiolabel.

Table 2

Specific activity of ^{3}H or ^{14}C-MCT obtained from Crotalaria spectabilis exposed to radiolabelled putrescine

Total mCi ^{3}H or μCi ^{14}C	[a] Grams of Plant Material	Quantity of ^{3}H or ^{14}C-MCT (mg)	Specific activity mCi/mmol
[b] 250	38.81	62.0	0.006
[c] 5	33.35	106.9	0.039
[d] 5	52.20	185.8	0.063
[e] 5	43.74	109.8	0.068
[f] 5	31.14	106.4	0.041
[e] 50	48.31	220.0	0.357

a) Total wet weight of plant material at the time of harvest.
b) Experiment was conducted with [1,4-^{14}C]-putrescine, 250 μCi. Material administered in approximately equal aliquots (125 μCi) over a 2 day period. Plant harvested at 8:30 am of the third day.
c) Radiolabel administered in a single bolus at 8:30 am and harvested 24 hr later.
d) Same as (c) but harvested 48 hr after administration.
e) Same as (c) but harvested 72 hr after administration.
f) Radiolabel administered at 8:30 am for 3 days at 1 mCi/day on day 4, 2 mCi were given; plant harvested 24 hr after the last dose. On the fourth day , the site of wick insertion was becoming necrotic.

Discussion

This article mainly presents two types of labelling experiments, the incorporation of $^{14}CO_2$ (Table 1) and the uptake of [2,3-^{3}H]-putrescine dihydrochloride (Table 2). A single labelling experiment involving [1,4-^{14}C]-putrescine, 250 μCi was also performed (Table 2). Initially, we were hoping to obtain tritium labelled monocrotaline exhibiting high enough specific activity for future use with autoradiography studies. We had suspected based on previous experimental data, that the amount of radiolabel incorporation would be greater when using [2,3-^{3}H]-putrescine dihydrochloride as the appropriate precursor (7). Other reports (20,21), have also shown that the majority of radiolabel associated with ornithine, a precursor of putrescine, would be found in the necine portion, which is where we would prefer the label to be located for future autoradiography studies. Unfortunately, as can be observed in Table 2, the specific activity obtained when 50 mCi of [2,3-^{3}H]-putrescine dihydrochloride was utilized was somewhat low (0.357 mCi/mmol). The data presented in the accompanying tables indicates that the amount of radiolabel incorporation into monocrotaline was far greater with the $^{14}CO_2$ than the [2,3-^{3}H]-putrescine dihydrochloride.

Rao et al (21) using *C. retusa* , examined the utilization of a variety of precursors including radiolabelled acetate, threonine, isoleucine, valine and ornithine. The majority of the radioactivity associated with acetate, threonine, isoleucine and valine was incorporated in the necic acid fraction of monocrotaline and thus we did not include them in our studies. The only precursor Rao et al used which was significantly incorporated into the retronecine portion was radiolabelled ornithine. Nowacki and Byerrum (20) used ^{14}C labelled acetate, propionate and ornithine in their experiments with *C. spectabilis*. Their results showed that the majority of the acetate and propionate were incorporated in the necic acid fraction and that only ornithine was incorporated into the necine portion of monocrotaline. We utilized [2,3-^{3}H]-putrescine dihydrochloride since it follows ornithine in the biosynthetic pathway in the formation of other *Senecio* derived PAs.

A most important factor when considering the biosynthesis of radiolabelled monocrotaline using radiolabelled putrescine or CO_2 has been the plants and the conditions used to raise them. Preliminary experiments indicated a major growth spurt began to occur when *C. spectabilis* plants were approximately one month of age (or exhibiting an average height of 16 cm). Plants of this size yielded reasonable levels of monocrotaline for future toxicology experiments. We have noted variability associated with the specific activity associated with either the $^{14}CO_2$ or the [2,3-^{3}H]-putrescine dihydrochloride data. The presence of varying amounts of direct PA precursors such as arginine, ornithine and isoleucine as noted by prior investigators using *Senecio* spp. could play a major role in the final specific activity of monocrotaline obtained in these experiments. In addition, the cost associated with these radiolabelling experiments prevented us as it has prior investigators from performing as many experiments as we would have liked.

As the reader can observe with our 5 mCi experiments, we attempted to determine the optimum time of harvest following the up-take of the [2,3-^{3}H]-putrescine dihydrochloride. In addition, we also attempted to determine the optimum uptake method using 5 mCi of [2,3-^{3}H]-putrescine dihydrochloride: a single bolus or numerous aliquots delivered over a number of days. The results indicate that using a single bolus was most effective when using [2,3-^{3}H]-putrescine dihydrochloride.

The data presented in the accompanying tables show that the amount of radiolabel incorporation was far greater with the $^{14}CO_2$ than the [2,3-^{3}H]-putrescine. The obvious advantage with $^{14}CO_2$ labelled monocrotalinefor *in vivo* metabolism is the uniform incorporation throughout the PAs. In spite of all our efforts, we must conclude that obtaining a tritium labelled monocrotaline was just not practical by utilizing presently available substrates and their associated financial costs. We are however, encouraged by the increased specific activity of ^{14}C-monocrotaline obtained utilizing far less $^{14}CO_2$ than in our prior studies (14).

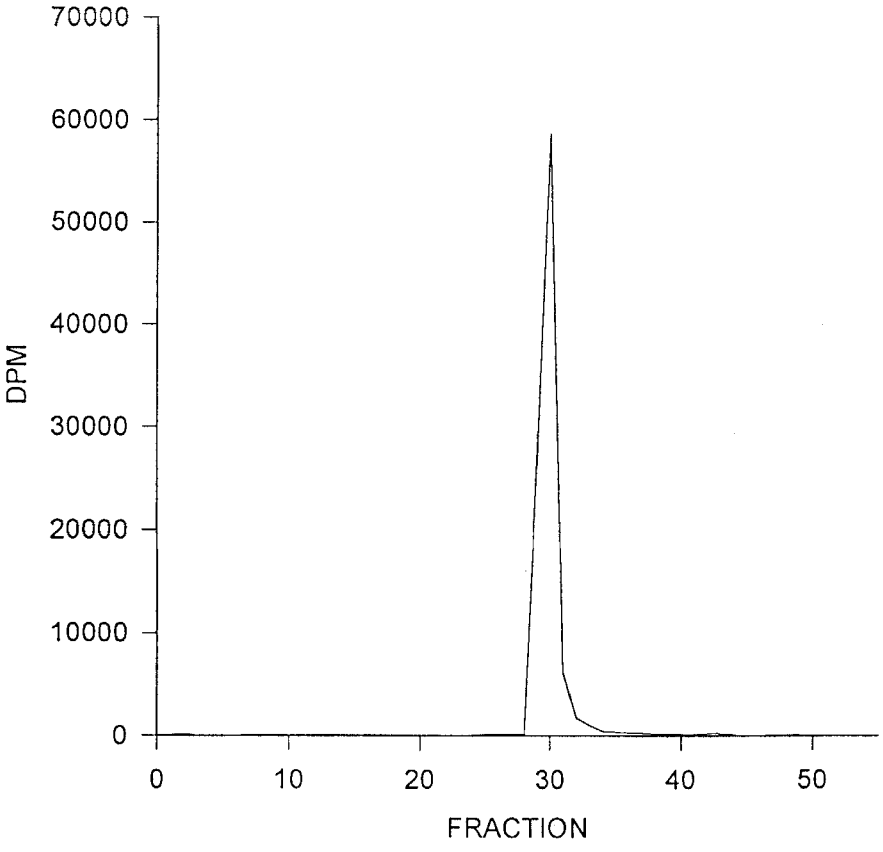


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Figure Legend

Figure 1: Radioprofile of ^{3}H-MCT produced from [2,3-^{3}H]-putrescine (50 mCi). Purity was checked with a 5μ PRP-1 column (150 x 4.1mm, Hamilton, Reno, NV) . Conditions were CH_3CN: NH_4OH (10 mM) (5:95) , isocratic for 15 min followed by a 15 min linear gradient to CH_3CN: NH_4OH (10 mM) (25:75); flow rate was 1ml/min. Fractions (1ml) were collected for scintillation counting.