SYNTHESIS OF ^{14}C-LABELED HALOGEN SUBSTITUTED INDOLE-3-ACETIC ACIDS

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SUMMARY

A general method for microscale synthesis of ^{14}C-labeled indole-3-acetic acids with halogen substitutions in the benzene ring is described. The method utilizes halogen substituted phenylhydrazines reacted with [^{14}C]-2-oxoglutarate to generate the halogenated indole-3-acetic acid. 3-Chlorophenylhydrazine yielded a mixture of the 4 and 6 chloro compounds that was resolved by C_{18}-reverse phase high performance liquid chromatography.

Key Words: Chloroindole-3-acetic acid, Fischer indole synthesis, indole-3-acetic acid analogues, radiolabeled indoles.

INTRODUCTION

4-Chloroindole-3-acetic acid (4-Cl-IAA) is a naturally occurring compound in Pisum sativum, Vicia faba, and Lathyrus sp. (6-9,11,12). Some monochloro substituted indole-3-acetic acids show plant growth promoting activity greater than or equal to that of the phytohormone, indole-3-acetic acid (IAA), when applied in a variety of test systems (1,15). The greater

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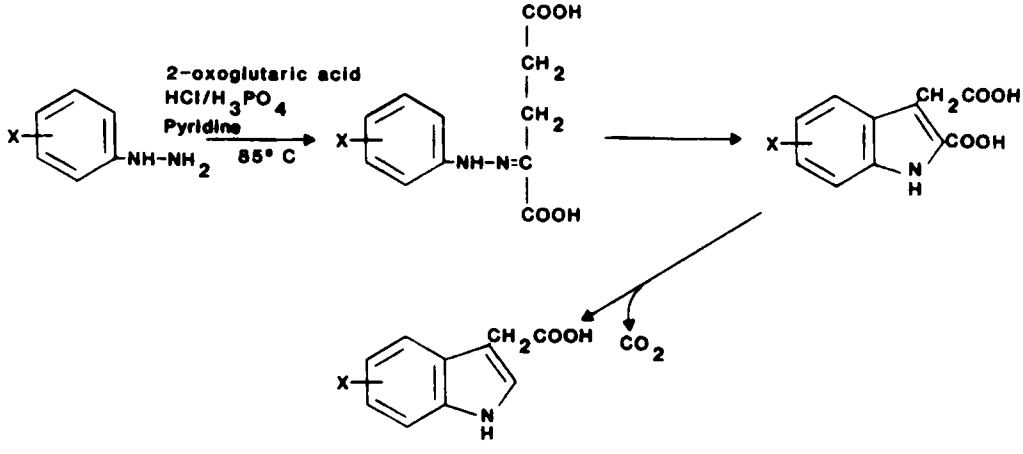
biological activity of 4-Cl-IAA relative to IAA has been attributed to its resistance to attack by oxidative enzymes (13); however, this hypothesis has never been tested directly in vivo. Several of the dichloroindole-3-acetic acids have also been studied and found to be toxic, or to inhibit IAA induced growth (1,5,15).

Studies of the metabolism of the halogenated IAAs have been limited by the unavailability of the radiolabeled compounds. The only reported production of a radioactive halogenated IAA was the biological labeling of 4-Cl-IAA by irrigation of pea plants with a solution containing radioactive chloride (3). However, the product obtained was of low specific activity and was obtained in amounts insufficient for further study. The availability of a variety of radioactive halogen substituted IAAs at relatively high specific activity would greatly facilitate isolation of these materials from plant sources, would aid in studies utilizing these substances to determine the molecular requirements for phytohormone action (see Ref. 10), and would be useful in studies of the metabolism of these compounds. We have developed a general method for the production of radiolabeled halogen substituted IAAs. The products obtained are suitable for a variety of studies or, alternately, can be further elaborated to ester or amide conjugates by methods that have been previously described (2,14).

DISCUSSION

Several possible routes exist for the production of radioactive halogen substituted IAAs. The major routes which appeared to show promise for our work were as follows: 1) The elaboration of a halogen substituted indole. A variety of monohalogen substituted indoles are available from commercial sources[3] (i.e. Aldrich, Sigma, K and K/ICN, Pfaltz and Bauer) and these can be converted to the substituted gramine. The conversion of the gramine to IAA using [^{14}C]cyanide is a well established procedure (17). The major disadvantage of this approach is that the di-substituted compounds are not readily available commercially. 2) A variety of halogen substituted

[^{14}C]anilines are available (Pathfinder Laboratories) and these could be converted to the corresponding IAA (16). However, the process is a two step synthesis and we have found that the yields are usually low (<5%). In addition, the compounds are available at only moderate specific activity and a large number of different labeled compounds would be required in order to synthesize a variety of different positional isomers. 3) Substituted IAA can be generated from the appropriate phenylhydrazine by a two step Fischer indole synthesis using 3-formylpropionate (from glutamic acid) as described for preparative scale synthesis by Engvild (4). Alternatively, we have used the single step synthesis from a phenylhydrazine and 2-oxoglutarate described by Robinson (16) for synthesis of unsubstituted [^{14}C]IAA (see reaction scheme) and have found this to be more consistent for micro-scale preparations than the method described by Engvild (4). In our hands this method proved to be generally applicable although, as with the method of Engvild, the yields varied with the different substituted phenylhydrazines. Radioactive 2-oxoglutarate is available in a variety of different forms which allows for the synthesis of either carboxyl labeled compounds or compounds with label in four positions (ring 2, 3 and both side chain carbons). Two typical syntheses that yield multiple labeled compounds are



described in detail in this report. In addition, we have produced labeled 4,6-di-Cl-IAA and have run non-radioactive microscale syntheses of four other halogenated IAAs.

The identities of the reaction products were established by a number of criteria. First, a parallel synthesis was run on a larger scale starting with 1-10 g of the appropriate phenylhydrazine and the product of this synthesis was confirmed as follows. The synthetic products had the same R_f values on Silica Gel thin layer chromatograms and R_t on C_{18}-HPLC as authentic compounds (a gift from Dr. K. C. Engvild (4)). The samples were analyzed by direct probe mass spectrometry on a Finnigan 4000 series instrument. The bis-chloro compounds had predominant ions at m/z 243 (m^+) and at 198 (base peak) and the monochloro derivatives had major ions at m/z 209 and 164, thus confirming the expected structures. The ^{14}C-labeled compounds were then compared with the unlabeled compounds. For each compound synthesized, the authentic standard, unlabeled compound and the ^{14}C-labeled product all had the same UV spectra, R_t on C_{18}-HPLC and R_f on Silica Gel TLC. Finally, an injection on HPLC of a mixture of the ^{14}C compound and the authentic standard gave a single peak which, when collected, contained >95% of the radioactivity and the specific activity was constant over the time of elution of the peak.

The specific activity of the final product, when starting with 50 µCi of [^{14}C-UL]-2-oxoglutaric acid diluted to 1 µmole with unlabeled 2-oxoglutaric acid, should have been approximately 40 µCi/µmole after loss of one ^{14}C by decarboxylation. We usually obtained specific activities in the range of 22-27 µCi/µmole. The reason for the lower than expected specific activity was probably due to a combination of transfer loss, degradation of the radiolabeled compound prior its dilution by unlabeled 2-oxoglutaric acid and a possible inaccurate determination of concentration by the manufacturer.

EXPERIMENTAL

Based on the manufacturer's stated concentration, 50 µCi (0.21 µmole) of [^{14}C-UL]-2-oxoglutaric acid (240 mCi/mmole, ICN Isotopes) in ethanol:water (20:80) was mixed with 0.8 µmole of a freshly prepared solution of unlabeled 2-oxoglutaric acid (Sigma). This solution was dried in vacuo in a 10 x 70 mm ignition tube and the residue resuspended in 35 µl pyridine. 20 µmole of the appropriate phenylhydrazine (Aldrich) was added in three 50 µl aliquots from a chilled, 0.13 M suspension of the halogen substituted phenylhydrazine in HCl (conc):85% phosphoric acid (2:1), with swirling and cooling between aliquots. The reaction mixture was sealed under vacuum in the ignition tube after flushing with N_2 gas and freezing with liquid nitrogen. The sealed ampule was heated at 85°C for 17-20 hr. in a dry block heater.

After chilling on ice, the vial was opened and the solution acidified with 1 ml 0.1 M HCl. This solution was extracted 3X with 1 ml ether using a Mixxor extraction system (Lidex). Ether fractions were pooled and dried over sodium sulfate. After filtering, 0.5 ml water was added and the ether was removed in vacuo. 2-Propanol (0.3 ml) was added and the solution fractionated on a 1 x 15 cm column of Sephadex LH-20 (Pharmacia) using 2-propanol:water (1:1).

100 µl of the solution was used to determine specific activity and radiochemical purity using C_{18} reverse phase high performance liquid chromatography on a 4.6 mm x 25 cm Whatman Partisil ODS-3 column with elution by methanol:water:acetic acid (60:39:1) and detection at 225 nm. [^{14}C]5,7-di-Cl-IAA eluted as a single peak between 18 and 22 ml. The final yield was 22% from 2-oxoglutarate (specific activity 27.3 µCi/µMole) and radiochemical purity (by HPLC) was determined to be 97%.

Micromole Synthesis of Halogen Substituted Indole-3-Acetic Acids

Compound	% Yield	Specific Activity
[^{14}C]5,7 di-Cl-IAA	22%	27.3 µCi/µmole
[^{14}C]4-Cl-IAA + [^{14}C]6-Cl-IAA	13.4% (mixture) 6.0% 4-Cl 7.4% 6-Cl	 21.8 µCi/µmole 21.1 µCi/µmole
[^{14}C]4,6 di-Cl-IAA[1]	1.0%	90.1 µCi/µmole
4,6 di-Cl-IAA	15%[2]	Unlabeled
4,7 di-Cl-IAA	25%[2]	Unlabeled
4,5 di-Cl-IAA + 5,6 di-Cl-IAA	10%[2] (mixture)	Unlabeled
7-Cl-IAA	15%[2]	Unlabeled

[1]This reaction was at the 200 nmole scale

[2]Yields of unlabeled compounds are estimates based on spot color from thin layer chromatograms.

Starting with 3-chlorophenylhydrazine yielded a mixture of [^{14}C]-4-Cl-IAA and [^{14}C]-6-Cl-IAA. The [^{14}C]-4-Cl-IAA and [^{14}C]-6-Cl-IAA eluted together from the LH-20 column between 18 and 22 ml. This solution was reduced in volume to 100 µl and then the compounds separated using the same HPLC system as before but with elution by water:methanol:acetic acid (59:40:1). [^{14}C]-4-Cl-IAA eluted between 48 and 53 ml and was determined to be a 6% yield from 2-oxoglutarate (specific activity 21.8 µCi/µMole) with radiochemical purity of 95%. [^{14}C]-6-Cl-IAA eluted between 67 and 73 ml. Yield was 7.4% from 2-oxoglutarate (specific activity 21.1 µCi/µMole) and radiochemical purity was 96%.

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