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Research Article

Synthesis of [^{13}C]-isotopomers of indole and tryptophan for use in the analysis of indole-3-acetic acid biosynthesis

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Summary

The direct conversion of indole to indole-3-acetic acid without tryptophan as an intermediate has previously been shown to occur *in vivo*, as well as *in vitro*, with seedlings of plants. In order to facilitate the purification of the enzymes that carry out the enzymatic synthesis of indole-3-acetic acid from labeled indole, it was necessary to develop an assay that had both high sensitivity and analytical precision. To obtain the required analytical resolution and to allow definitive product identification, [$^{13}C_6$]indole was synthesized for use in GC-MS assays of the enzymatic conversion. Plants have been shown to be able to synthesize indole-3-acetic acid either directly from indole as well as by degradation of tryptophan. Thus, in order to allow the biochemical discrimination between these processes, the synthesized [$^{13}C_6$]indole was used as a starting material for a novel enzymatic synthesis of [^{13}C]isotopomers of L-tryptophan labeled at specific positions. Together, these isotope labeled indolic compounds offer powerful new approaches to understanding and differentiating routes of indole-3-acetic acid biosynthesis *in vitro* and *in vivo*.

Key Words: carbon-13 indole; enzymatic synthesis; indole-3-acetic acid metabolism; mass spectrometry standards; tryptophan isotopomers

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Introduction

The oxidative degradation of the amino acid tryptophan (trp) has long been considered to be the primary pathway for the production of the plant growth hormone auxin (indole-3-acetic acid, 1AA) in plants.[1] Isotope labeling experiments with *Lemna gibba*, carrot embryos and trp biosynthesis mutants in maize and *Arabidopsis thaliana*, however, have shown that IAA can be made by plants via a trp-independent biosynthetic pathway.[1–5] For example, the maize mutant *orange pericarp*, which lacks a functional tryptophan synthase β due to knockout mutations in both tryptophan synthase β genes, does not make trp but does synthesize IAA *de novo*. Additionally, neither normal nor mutant seedlings treated with ^{15}N- or deuterium-labeled trp showed significant conversion of trp into IAA. These results established the fact that IAA biosynthesis occurs by a pathway not using trp as an intermediate in both mutant and normal maize.[5]

In order to define the enzymes and intermediates involved in the trp-independent pathway to IAA, we conducted a series of experiments[6] that demonstrated the enzymatic synthesis of IAA from indole by an *in vitro* preparation from maize (*Zea mays* L.). These studies with light grown seedlings of normal and *orange pericarp* mutant maize showed that extracts contained the necessary enzymes and cofactors necessary to convert [^{14}C]indole to [^{14}C]IAA and that tryptophan was not an intermediate in this process.[6]

Our initial approaches, which involved the fractionation of the radio-labeled intermediates and their identification, were made difficult due to the low percentage of indole conversion to IAA relative to the formation of other indolic byproducts.[7] These results suggested that further progress in enzyme and intermediate isolation would depend on the availability of methods to clearly identify and distinguish components of the process from the competing reactions and resulting products in plant extracts. Thus, we have designed a robust stable isotope method for measuring indole to IAA conversion *in vitro* that provides a highly definitive quantitative measurement of IAA production in such extracts. The method we describe here involves the chemical synthesis of [$^{13}C_6$]indole for use as a substrate, then the quantitative analysis of the [$^{13}C_6$]IAA formed with *in vitro* reactions using [$^{2}H_4$]IAA as the internal standard for GC-MS measurements.

Chemically synthesized [$^{13}C_6$]indole was also used together with L-[$^{13}C_3$]serine in a novel enzymatic synthesis of specifically labeled L-tryptophans. These heavy labeled L-tryptophan isotopomers are thus suitable and easily produced for studies of alternative IAA biosynthetic pathways as well as for tracing the metabolism of the amino acid, trp containing peptides and other indolic signal messengers.

Results and discussion

In order to develop a stable isotope method for analysis of IAA biosynthesis from indole, several advances were necessary. First, highly enriched, multiple [^{13}C]-labeled indole needed to be synthesized so that indole to IAA conversion could be measured *in vitro* even in crude preparations where unlabeled tryptophan and indole might be present in the extract. Second, in order to measure the quantity of isotopic labeled IAA produced, an internal standard of heavy IAA was necessary for use for isotope dilution based analysis.[8] Several stable isotope labeled IAAs are available and [$^{2}H_4$]IAA, as described by Magnus *et al*.,[9] proved particularly suitable for the assay procedure since it is four mass units heavier than unlabeled IAA and two mass units lighter than the expected product from [$^{13}C_6$]indole. Tryptophan-independent IAA biosynthesis had previously been studied with *in vitro* experiments on young maize seedlings by the analysis of the conversion of radiolabeled [^{14}C]indole and [^{14}C]tryptophan to IAA. It was shown that in extracts from light-grown maize seedlings IAA was synthesized from indole but not from tryptophan.[6] To allow similar comparisons between the efficacy of IAA labeling by isotopic indole and tryptophan, several specifically labeled isotopomers of L-trp were required.

The assay for trp-independent IAA biosynthesis we describe in this report is based on the analysis of the formation of [$^{13}C_6$]IAA from [$^{13}C_6$]indole using an isotope dilution method with [$^{2}H_4$]IAA as the internal standard for quantification by GC-MS. It can be expected that with the ability to easily distinguish IAA from other products formed in the reaction that it will be possible to follow the specific enrichment in IAA forming activity upon further fractionation of the proteins that catalyze these reactions.

In any study of non-traditional IAA biosynthetic routes it is also important to have detailed knowledge of the fate of tryptophan both *in vivo* and *in vitro*. One significant limitation to such studies has been the lack of stable isotope labeled tryptophan containing multiple labels in specific positions. To advance such studies, we developed a cell-free enzyme system for the production of specifically labeled L-trp from [$^{13}C_6$]indole and [^{13}C]serine. Combinations of unlabeled and specifically labeled indole and serine provides a highly facile route to L-trp labeled in any of several specific patterns. The labeled L-tryptophan isotopomers produced in this way are particularly useful for studies of the biosynthesis of IAA and should find applications in the analysis of the routes to indolic compounds derived from tryptophan in plants as well as other organisms.

Synthesis of [$^{13}C_6$]indole

Indole is an important heterocyclic molecule that forms the structural basis for many natural products of great physiological and economic importance.

Synthesis of the indole ring itself has been the subject of many studies and numerous methods for its synthesis have been reported. Most methods use, as a starting compound, substituted benzene ring compounds subjected to a variety of cyclization reactions to form the required indole ring. It is, however, much more common to find methods for production of substituted indoles rather than indole itself. Although a number of methods for synthesis of indole are available, the synthesis of indole containing labels in specific ring positions is not straight-forward. As with many syntheses of labeled compounds there are several limiting factors, including the availability of appropriately labeled starting compounds and the suitability of the selected method for small scale synthesis, that determine the approach that can be used. The commercial availability at a modest price of [$^{13}C_6$]aniline was an important factor for selecting the synthetic approach. [$^{13}C_6$]Indole was synthesized in three steps using a modification of the general method of Nordlander *et al.*[10] with [$^{13}C_6$]aniline as a starting compound (Figure 1). The reaction of [$^{13}C_6$]aniline

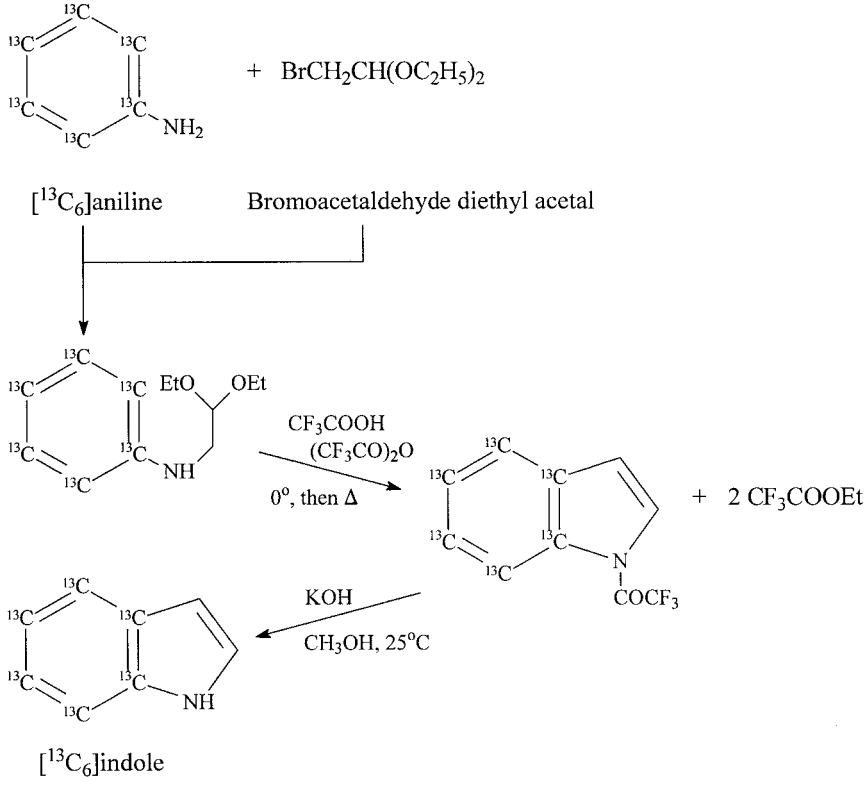

Figure 1. Synthetic steps leading from [$^{13}C_6$]aniline to [$^{13}C_6$]indole, as described in text. The reaction of [$^{13}C_6$]aniline and bromoacetaldehyde diethyl acetal gave 2-anilino acetal that in an acid-catalyzed cyclization-elimination reaction formed the indole ring

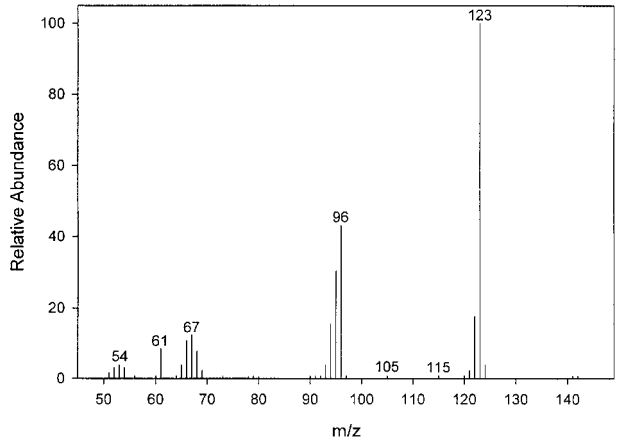

Figure 2. Mass spectrum of [$^{13}C_6$]indole synthesized from [$^{13}C_6$]aniline, from GC-MS analysis. Diagnostic ions for unlabeled indole,[11] *m/z* 117 and 90 (M^+ - HCN), are both fully labeled to yield the corresponding ions at *m/z* 223 and 96. Note the presence of the *m/z* 95 ion is due, in part, to the rearrangement to a cyclic structure with the loss of a hydrogen atom[11]

and the α-halo aldehyde acetal (bromoacetaldehyde diethyl acetal) gave 2-anilino acetal that in an acid-catalyzed cyclization-elimination reaction formed the indole ring. Essential for the reaction was the trifluoroacetylation of the amino function of 2-anilino acetal before the acid-catalyzed cyclization. Trifluoroacetylation of 2-anilino acetal was done *in situ* with excess of trifluoroacetic anhydride in trifluoroacetic acid before the cyclization reflux. Saponification of the product in methanolic KOH gave [$^{13}C_6$]indole. We were not able to achieve the higher yields reported in the original method, possibly because of the reduced scale and differences in purity between the unlabeled compound used for optimization and the [$^{13}C_6$]aniline provided by the isotope supplier. The reaction product was characterized by GC-MS without derivatization (Figure 2) and noting the ions at *m/z* 123 and 96 for [$^{13}C_6$]-labeled indole as compared to molecular ion *m/z* 117 and ion *m/z* 90 for the unlabeled compound.

Use of [$^{13}C_6$]indole as a substrate in maize seedlings assays for IAA biosynthesis

The supernatant from extracts of 9–10 day old maize seedlings was incubated with [$^{13}C_6$]indole for 5 h at 35°C, as previously described using [^{14}C]indole.[6] IAA was purified following the enzyme reaction by ethyl acetate partitioning, C_{18} HPLC, followed by methylation with diazomethane. The conversion of the substrate to [$^{13}C_6$]IAA was quantified by GG-MS monitoring four ions in the selected-ion mode. Ions at *m/z* 136 (quinolinium

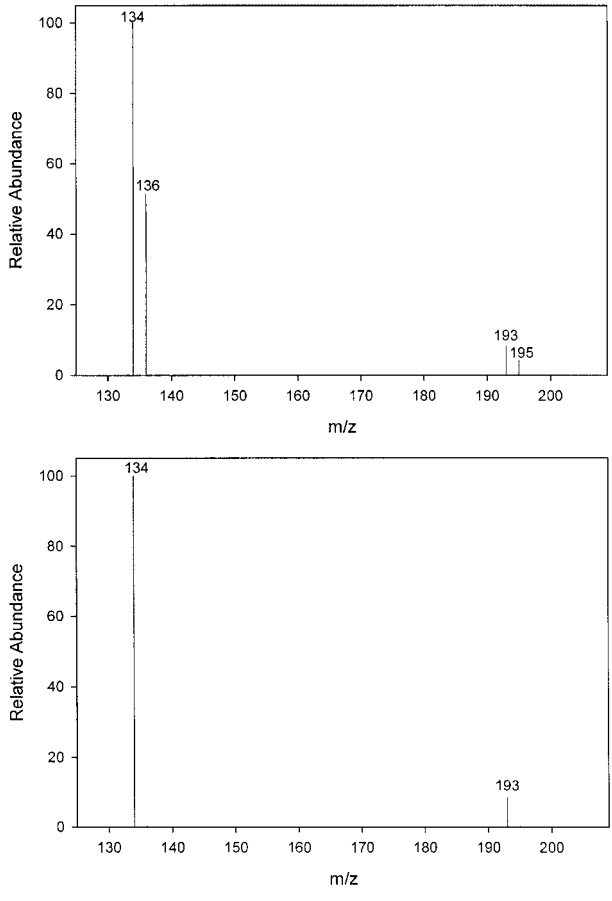

Figure 3. GC-MS selected ion analysis (monitoring *m/z* 134, 136, 193 and 195) of the [$^{13}C_6$]indole-3-acetic acid (analyzed as its methyl ester) formed from [$^{13}C_6$]indole by maize seedling extracts (upper spectrum). Selected ion spectrum of the internal standard [$^{2}H_4$]indole-3-acetic acid methyl ester showing the lack of ions at *m/z* 136 and 195 is also shown (lower spectrum)

ion) and *m/z* 195 were monitored for [$^{13}C_6$]IAA produced by the enzyme activity from [$^{13}C_6$]indole. The internal standard [$^{2}H_4$]IAA was analyzed simultaneously by monitoring the corresponding ions at *m/z* 134 and 193 (Figure 3). Using [$^{13}C_6$]indole and [$^{2}H_4$]IAA in this way for a selected ion monitoring GC-MS-based assay, the measurement of conversion of very small amounts of indole to IAA using partially purified extracts could be accomplished with an exact quantitative isotope dilution analysis of the product formed (Figure 3). For example, in the reaction shown in Figure 3, only 9 ng of IAA was produced from 1 μg of [$^{13}C_6$]indole added to the enzyme reaction.

Synthesis of [^{13}C]tryptophan isotopomers

Previously published methods for enzymatic tryptophan synthesis often rely upon tryptophan synthase β, the enzyme capable of catalyzing the formation of trp from indole and L-serine with pyridoxal phosphate as the coenzyme.[12] The enzyme frequently used is that obtained from *E. coli* mutants that overexpress tryptophan synthasel.[13–16] Based on the now classic work by Widholm[12] on tryptophan synthase activity in higher plants and the demonstration of rapid conversion of indole to trp by maize liquid endosperm preparations,[7,17] a facile enzymatic synthesis was designed. The tryptophan synthase from maize liquid endosperm was partially purified by dialysis, thus removing contaminating, naturally occurring, unlabeled indole, serine and tryptophan as well as cofactors for potentially competing reactions.

Chemically synthesized [$^{13}C_6$]indole was incubated with the dialyzed enzyme preparation, pyridoxal phosphate and [$^{13}C_3$]-L-serine. The resulting isotopomer with nine [^{13}C] carbons was isolated by a procedure modified from that of Michalczuk *et al.*,[18] derivatized to its *N*-acetyl methyl ester and characterized by the full scan GC-MS (Figure 4; characteristic ions at *m*/*z* 269 and 137). Other isotopomers were produced, derivatized and analyzed by GC-MS similarly. In the reaction of [$^{13}C_6$]indole and unlabeled L-serine, the product was [$^{13}C_6$]tryptophan with characteristic ions at *m*/*z* 266 and 136. The combination of unlabeled indole and [$^{13}C_3$]-L-serine produced [$^{13}C_3$]tryptophan with characteristic ions at *m*/*z* 263 and 131. The characteristic ions for unlabeled derivatized tryptophan were at *m*/*z* 260 and 130, identical to that previously described.[18]

Experimental

Synthesis of [$^{13}C_6$]indole

In a modification of Nordlander's procedure,[10] a solution of 0.250 g (2.68 mmol) [$^{13}C_6$]aniline (Cambridge Isotopes Laboratories, Inc., Andover, MA), 0.352 g (1.78 mmol) bromoacetaldehyde diethyl acetal (Aldrich Chemical Co. Inc., Milwaukee, WI) and 0.226 g (2.68 mmol) $NaHCO_3$ (Sigma Chemical Co., St. Louis, MO) in 5 ml 95% EtOH was brought to boiling under reflux for 96 h (Figure 1). After reflux, the reaction mixture was extracted with ether (3×10 ml) and the ether layer was washed with water (3×10 ml) and the ether was dried over anhydrous Na_2SO_4. Rotary evaporation of the solvent and separation of the residue on normal phase HPLC (Phenomenex Luna 5 µ Silica 250×10 mm column; elution solvent of isopropanol 25: heptane 75) gave 0.250 g of [$^{13}C_6$]2-anilinoacetaldehyde diethyl acetal as a major fraction. This acetal was added with a syringe to 3 ml of a 50/50 (v/v) mixture of trifluoroacetic anhydride/acid at 0°C under nitrogen. After 0.5 h, 2 ml of trifluoroacetic acid was added to this

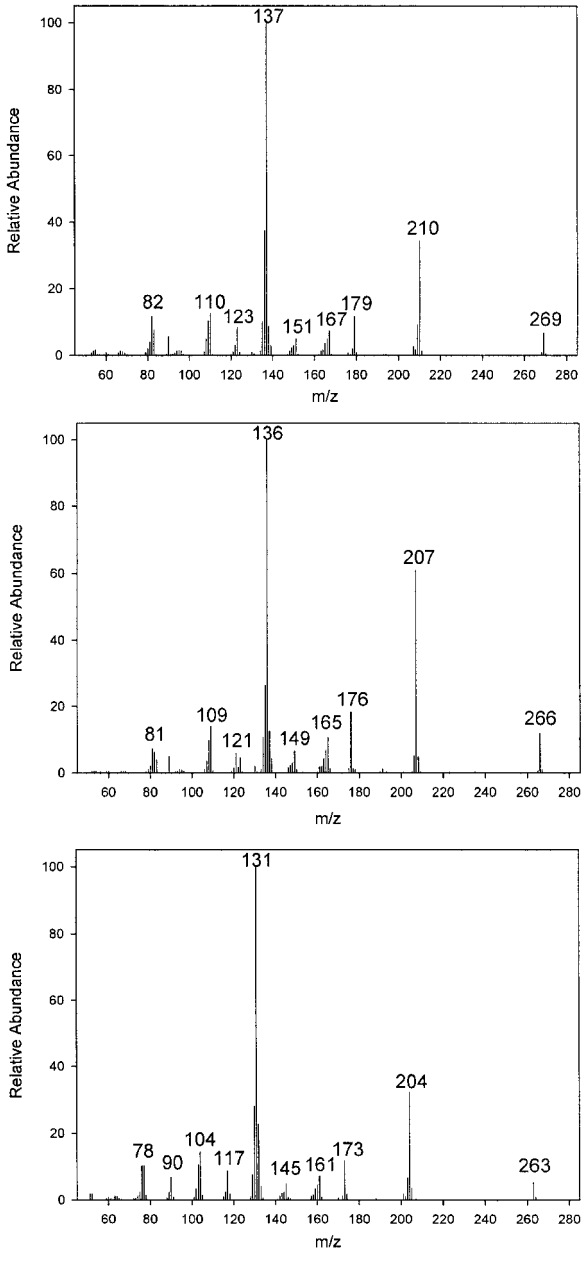

Figure 4. GC-MS characterization of the [^{13}C]tryptophan isotopomers. Note the characteristic ions for the isotopomer formed from [$^{13}C_6$]indole and [$^{13}C_3$]serine with 9 [^{13}C] carbons (*m/z* 269 and 137); with 6 [^{13}C] carbons from [$^{13}C_6$]indole and unlabeled indole (*m/z* 266 and 136; and with 3 [^{13}C] carbons from unlabeled indole and [$^{13}C_3$]serine (*m/z* 263 and 131). Note that only the methylene carbon (in the side chain derived from serine) is present in the quinolinium ion fragment (*m/z* 130 in the unlabeled compound)[11]

mixture and then refluxed for 72 h. After reflux, the reaction mixture was evaporated to dryness and stirred overnight at room temperature in 3 ml of 5% methanolic KOH. The methanol was evaporated and the product was dissolved in 20 ml of water and extracted with ether (3×20 ml). The ether phase was washed with water (2×15 ml) and dried over Na_2SO_4. The ether was evaporated and the product was run on a short (4×2.5 cm) silica column (Kieselgel 60, 70–230 mesh, Merck, Darmstadt, Germany) with toluene as the elution solvent. The first 50 ml of the eluent was collected, the solvent was evaporated and the sample purified on normal phase HPLC (as above). The fractions with [$^{13}C_6$]indole were collected and the solvent was evaporated, thus yielding 42 mg of product. The product was characterized by GC-MS and then dissolved in pentane for ease of use as a substrate in biochemical studies.

Maize seedling extract assays for IAA biosynthesis using [$^{13}C_6$]indole as a substrate

The kernels of sweet hybrid corn (Silver Queen, Meyer Seed Co., Baltimore, MD) were imbibed in the tap water overnight, planted in vermiculite and grown in a chamber at 25°C with constant cool white flourescent light at 25 μmol/m^2/s. The homogenization buffer used with the seedlings consisted of 75 mM Tris, 50 mM $(NH_2)_2SO_4$, 1.5% PVPP, 10–20% glycerol, 1% Zwittergent 3:10, 0.25 M ascorbic acid, 1 mM benzamide, 1 mM PMSF, 1 mM benzamidine, 5 mM ε-amino-*n*-caproic acid, 1 mM PHMB, 10 mM EGTA, 0.1 mg/ml pepstatin, 0.02 mg/ml antipain and 0.02 mg/ml leupeptin. The final pH was adjusted to 8.5 using KOH. Ten grams of 9–10 days old seedlings were homogenized in 50 ml of prechilled buffer at 4°C. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 12 000 g for 20 min at 4°C. Following centrifugation, 1 ml of supernatant was incubated with 1 μg of [$^{13}C_6$]indole for 5 h at 35°C. The enzymatic reaction was stopped by adding 40 μl of concentrated H_3PO_4. The reaction mixture was then extracted with 1 ml of EtOAc. The sample was evaporated under a stream of nitrogen gas and then dissolved in 100 μl of 50% MeOH together with 18.5 ng of [$^2H_4$]IAA and the radiotracer [^{3}H]IAA (20 000 dpm). The sample was then injected onto the reverse phase HPLC column (Ultracarb (30), 4.6×50 mm; Phenomenex, Torrance, CA) using 27% methanol/water plus 1% acetic acid as the mobile phase. The major radioactive fraction was methylated using diazomethane and the methylated sample was then analyzed by GC-SIM-MS, monitoring four ions: *m*/*z* 136 and 195 for enzymatically synthesized IAA and *m*/*z* at 134 and 193 for the methyl ester of the internal standard [$^2H_4$]IAA.

Synthesis of [^{13}C]-labeled tryptophan

The enzymatic conversion of indole to tryptophan by maize endosperm enzymes was done essentially as we previously reported using [^{14}C]indole.[7] Fresh liquid endosperm of sweet corn (*Zea mays* L. cv Silver Queen obtained from a local grocery) was collected by cutting the rows of kernels still on the cob with a razor blade and the kernel contents pressed out against the rim of a beaker and further squeezed through a layer of cheese cloth. The resulting liquid (80 ml) and 8 ml of grinding buffer (0.4 M potassium phosphate, pH 8.5, 10 mM mercaptoethanol and 40 μg/ml pyridoxal phosphates)[12] were then homogenized with a glass homogenizer and centrifuged at 10 000 g at 4°C for 20 min. After centrifugation and removal of a fatty layer, 20 ml of the supernatant was dialyzed against 3.5 l of half-strength grinding buffer, at 4°C for 16 h using dialysis membranes with a MWCO of 12–14 000 Da. Following the dialysis, 0.8 ml of dialyzate was incubated for 1 h at 30°C with 0.2 ml of a solution of 200 μmol potassium phosphate, pH 8.5, 4.32 mg (40 μmol) L-serine (alternatively, the same amount of [$^{13}C_3$]-L-serine (Cambridge Isotopes Labs) was used), 40 μg pyridoxal phosphate and 23.4 μg (0.2 μmol) of indole (unlabeled or [$^{13}C_6$]). The reaction was stopped by boiling for 5 min and centrifuged at 10 000 g for 5 min. Following the centrifugation step, approximately 50 000 dpm of L-[5-^{3}H] tryptophan (33.0 Ci/mmol; Amersham Life Science, Arlington Heights, IL) was added to the supernatant. The sample was then diluted 3 × with double-distilled water and applied to a 4 ml bed volume column of Dowex 50W-X2, 200-400 mesh (Sigma), preconditioned with 1 M HC1. The sample on the column was then washed with three bed volumes of double-distilled water and eluted with two bed volumes of 2 M NH_4OH. The eluate was evaporated to dryness, resuspended in dichloromethane and evaporated. After the evaporation of the dichloromethane, 1 ml of acetic anhydride (Supelco, Bellefonte, PA) and 2 ml of anhydrous methanol were added, the evaporation flask was closed with a glass stopper and clip, mixed for 1 min on a vortex mixer and placed in a sandbath at 65°C for 1 h in order to form the *N*-acetyl methyl ester of tryptophan.[18] Following the methanol-acetic anhydride derivatization procedure the sample was again evaporated to dryness, resuspended in 100 μl of 50% methanol and purified using an HPLC equipped with a C_{18} Nova-Pak 4 μm column (3.9 × 150 mm; Waters, Milford, MA). The mobile phase was 30% methanol/water (v/v) at a flow rate 1 ml/min. Labeled fractions were collected, evaporated to dryness and resuspended in 20 μl of ethyl acetate. The sample was then injected onto the GC-MS and data was collected in the full scan mode. The variously labeled tryptophan isotopomers from the enzymatic syntheses were characterized by monitoring the ions at m/z 130 (quinolinium ion) and 260 (molecular ion) for unlabeled tryptophan, and at $M^+ + 3$, $M^+ + 6$ and $M^+ + 9$ in heavy labeled

experiments (for $M^+ + 3$: 131 and 263 ions; for $M^+ + 6$: 136 and 266 ions and for $M^+ + 9$: 137 and 269 ions).

Conclusion

A facile synthesis of [$^{13}C_6$]indole from readily available [$^{13}C_6$]aniline provides a useful new approach to measuring the metabolic pathways involved in the synthesis of tryptophan and indole. [$^{13}C_6$]indole was synthesized in particular for use in GC-MS based analysis of IAA biosynthesis in order obtain the required analytical resolution and to facilitate definitive product identification. Also, in order to allow the biochemical discrimination between pathways using indole directly or via a tryptophan intermediate, the synthesized [$^{13}C_6$]indole was used as the starting material for a novel enzymatic synthesis of [^{13}C]isotopomers of L-tryptophan labeled at specific positions. Together, this suite of labeled indolic compounds provides critical tools for understanding and differentiating routes of indole-3-acetic acid biosynthesis *in vitro* and *in vivo*.

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