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Radiosynthesis of C-11 labeled auxin (3-indolyl[1-^{11}C]acetic acid) and its derivatives from gramine

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3-Indolylacetic acid (IAA) is the major auxin in higher plants and plays a key role in plant growth and development. We report the rapid radiolabeling of the important plant hormone using carbon-11 (half life: 20.4 min) enabling *in vivo* imaging of its distribution and movement in whole plants. 3-Indolyl[1-^{11}C]acetic acid was synthesized in 2-steps: (1) reaction of gramine with [^{11}C]cyanide to give 3-indolyl[1-^{11}C]acetonitrile in >99% radiochemical purity; (2) hydrolysis of the intermediate in aqueous sodium hydroxide solution to give 3-indolyl[1-^{11}C]acetic acid in >98% radiochemical purity after HPLC purification. The overall nondecay corrected radiochemical yield was 28%, synthesis time was 68 min and specific activity was (0.7 mCi/nmol). Hydrolysis proceeded through the formation of 3-indolyl[1-^{11}C]acetamide and by varying the temperature of this step, either C-11 labeled acid or amide were obtained. This procedure provides unexpectedly high C-11 incorporation in a short time and using a simple and selective hydrolysis without the need of an indole-nitrogen protecting group or a typical leaving group. Since 3-indolylacetonitrile and 3-indolylacetamide are also intermediates in the biosynthesis of IAA, and also function as auxins, this versatile reaction makes all three of these labeled compounds available for imaging studies in whole plants *in vivo*.

Keywords: auxins; plant imaging; cyanide; gramine

Introduction

Auxins (Figure 1) are critical plant hormones that play key roles in almost every growth and regulatory function in plants including response to light, stem elongation, lateral root initiation and organ formation.[1] Auxin activity is context dependent, for example, stimulating elongation in stems, and stimulating formation of adventitious and lateral roots, but inhibiting both lateral branch formation and elongation in primary roots.[1] 3-Indolylacetic acid (**1**, IAA), the most abundant and most potent naturally occurring auxin, was first structurally characterized in the 1930s[2] and it has since been shown to occur in both above- and belowground plant structures. Endogenous concentrations of auxin in tissues and organs of the plant are highly variable and depend on the plant species and organ, but are typically very low, for example, 5–100 ng gfw^{-1} in leaves, and perhaps 10–100 times lower in roots.[3] Further, the biological activity of auxins is often observed at very low concentrations, for example, 10 μM IAA inhibits root elongation. The auxin regulatory system is extremely complex, and can be modulated by *de novo* auxin biosynthesis, degradation, conjugation, de-conjugation, cell-to-cell transport and vascular transport. However, only recently was the first auxin receptor, transport inhibitor response 1 (TIR1), identified and characterized. TIR1 binds auxin directly and mediates transcriptional responses.[4,5] To date, the biosynthesis of IAA is only partially understood with two precursors being proposed, either tryptophan or a tryptophan precursor (indole or indole-glycerophosphate).[6] Several probable intermediates have been proposed for the tryptophan-dependent pathway including 3-indolylpyruvate, 3-indolylaldoxime, 3-indolylacetaldehyde, 3-indolylacetonitrile (**5**), 3-indolylacetamide (**6**) or tryptamine.[7,8]

Recent interest in the use of *in vivo* imaging in whole plants to monitor carbon and nitrogen resource allocation patterns and the translocation of key plant signaling molecules in real time[9] has stimulated the development of rapid methods to label various substrates and signaling molecules with carbon-11 (half life; 20.4 min) and nitrogen-13 (half life: 10 min), which decay by positron emission.[10–12] These isotopes are produced from a cyclotron in very high specific radioactivity and can substitute for stable carbon and nitrogen in a molecule thus preserving the biological properties of the parent compound and allowing the study of biochemical transformations at true radiotracer levels.[13]

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The resulting energetic 511 keV annihilation photons from positron decay can be imaged externally using a PET scanner. Thus, the concentration and movement of a labeled compound can be imaged in real time in the whole plant[11] and quantified taking into account positron escape from thin plant tissues such as leaves.[14]

Because of the importance of auxin in plant growth and development we devised a rapid radiolabeling scheme for the major auxin, 3-indolylacetic acid with carbon-11. We modeled the synthesis on an early report that the methiodide salt of gramine (**4**, 3-(dimethylaminomethyl)-indole) reacts with cyanide to yield 3-indolylacetonitrile (**5**), which could then be hydrolyzed to IAA (**1**).[15] To date, there have been many examples of the use of this general strategy to synthesize 3-indolylacetonitrile derivatives.[15–17] This general approach has also been used for the preparation of [^{14}C]IAA from [^{14}C]gramine.[18–20] [^{14}C]Gramine, prepared *via* the Mannich reaction using indole, [^{14}C]formaldehyde and dimethylamine, reacts efficiently with cyanide to give 3-indolyl[2-^{14}C]acetonitrile via a presumed quaternary ammonium intermediate. 3-Indolyl[2-^{14}C]acetonitrile was then hydrolyzed to give 3-indolyl[2-^{14}C]acetic acid.

The long synthesis times reported for these reactions were not practical for synthesis with carbon-11, due to its short half-life. However, using [^{11}C]HCN, a synthetic precursor that can be produced routinely and in large amounts starting with [^{11}C]CO_2,[21] we developed conditions to rapidly convert gramine (**4**) to 3-indolyl[1-^{11}C]acetic acid (**[^{11}C]1**). The synthetic scheme is shown in Figure 2. This process required optimizing the displacement step with [^{11}C]cyanide as well as the hydrolysis of the resulting labeled nitrile (**[^{11}C]5**) to carboxylic acid (**[^{11}C]1**). We also considered that manipulation of the hydrolysis conditions could potentially give the labeled amide (**[^{11}C]6**), which would also be useful for *in vivo* studies. We note that endogenous auxin concentrations in plants are very low. Thus for tracer studies in whole plants with **[^{11}C]1** we required that [^{11}C]HCN be used in no-carrier-added form (i.e. high specific radioactivity). This constraint results in a large excess of the gramine precursor at the end of the displacement step, and thus required rapid removal of the excess gramine as well as removal of other unwanted side-products during final formulation.

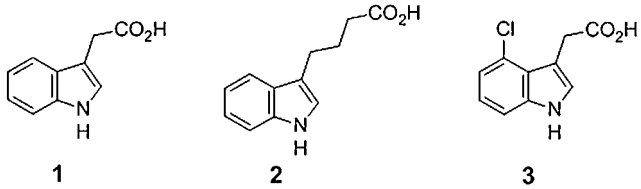

Figure 1. Naturally occurring auxins, 3-indolylacetic acid (**1**, IAA), 3-indolylbutyric acid (**2**), 4-chloro-3-indolylacetic acid (**3**).

Experimental

General

All reagents and solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) except for gramine (Winthrop Chemical Co.). ^{11}C was generated as [^{11}C]CO_2 using 17.4 MeV proton irradiation of a N_2 gas target containing 100 ppm O_2 to induce the ^{14}N(p,α)^{11}C nuclear reaction. Irradiations were carried out on the BNL EBCO TR-19 cyclotron. [^{11}C]CO_2 produced from this process was collected, catalytically reduced to $^{11}CH_4$ over reduced Ni at 365°C, which was converted to [^{11}C]HCN over Pt at 1000°C using gaseous NH_3. Radioactivity measurements were made in a Capintec CRC-712MV radioisotope dose calibrator (Capintec Inc., Ramsey, NJ). Semi-preparative HPLC was performed using a Knauer HPLC system equipped with a model K-1001 pump, a model 87 variable wavelength monitor, a NaI detector and a SRI Peak Simple Integration System. Analytical HPLC was performed using a Knauer model K-1001 pump, a Knauer model K2501 UV detector (254 nm), a Geiger Muller ionization detector and a SRI Peak Simple Integration System. For thin layer chromatography (TLC) analyses of radiotracers, Macherey-Nagel (MN) polygram sil G/UV254 plastic-back TLC plates and a Bioscan System 200 imaging scanner were used. Radiochemical yields (decay-corrected back to EOB) were calculated based on the total radioactivity trapped in the reaction vessel at the start of the reaction. Specific activities, decay corrected back to end of beam (EOB) and recorded in mCi/nmol, were determined from the carbon-11 activity in the product peak from the HPLC (obtained from Capintec readings) and the mass of compound (determined by the UV absorbance of the radioligand and the use of calibration curves of unlabeled reference compounds). Total synthesis times were calculated from EOB to end of radiotracer formulation.

3-Indolyl [1-^{11}C]acetonitrile ([^{11}C]5)

To a solution of gramine (**4**, 1 mg) in DMSO (0.3 ml) was bubbled H^{11}CN with stream of nitrogen at room temperature until maximum radioactivity was trapped as indicated by a NaI pin diode detector. The vessel was sealed and heated in an oil bath at 140°C for 5 min. The reaction mixture was diluted with water (20 ml) and passed through a C-18 light sep-pak (Waters, Milford, MA) cartridge and rinsed with HCl (1 M, 5 ml) followed by water (10 ml). 3-Indolyl[1-^{11}C]acetonitrile (**[^{11}C]5**) was eluted into a V-shaped vial with 1 ml of ether, which was removed with a stream of argon. The labeled nitrile was hydrolyzed without further purification. It could also be purified by semi-preparative HPLC on a PFP column with an eluent mixture acetonitrile/0.01 M formic acid (40/60), at a flow rate of 5.0 ml/min. Elution time for **[^{11}C]5** was 14–17 min. The fraction containing the labeled tracer was collected, and the solvent concentrated to dryness on a rotary evaporator. Using this procedure **[^{11}C]5** was

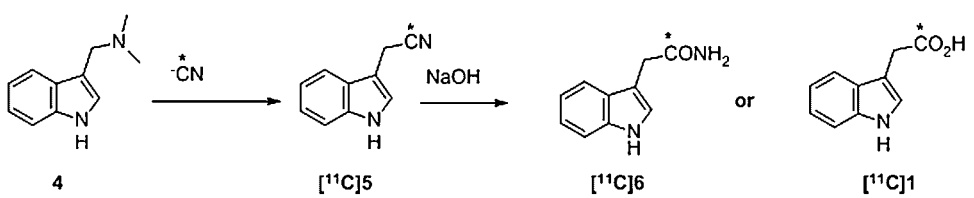

Figure 2. Synthesis of C-11 labeled 3-indolylacetonitrile (**[^{11}C]5**), 3-indolylacetic acid (**[^{11}C]1**) and 3-indolylacetamide (**[^{11}C]6**) from gramine (**4**) and [^{11}C]cyanide. *Carbon-11.

produced in 54% radiochemical yield with a specific activity up to 1.4 mCi/nmol at EOB and a radiochemical purity of >99%.

3-Indolyl[1-^{11}C]acetic acid ([^{11}C]1)

Aqueous sodium hydroxide solution (1 M, 0.3 ml) was added to **[^{11}C]5** and the mixture was heated for 5 min at 140°C. The reaction mixture was neutralized by adding hydrochloric acid (1 M, 0.3 ml) and diluted with HPLC solvent (1 ml) and purified with HPLC using formic acid (0.1%) 75%/acetonitrile (25%) at a flow rate of 5 ml/min on a PFP column (Phenomenex, 250 × 10 mm, 5 μ). Under these conditions, **[^{11}C]1** eluted at 19–20 min. The fraction containing **1** was collected, solvent concentrated to almost dryness on a rotary evaporator and diluted with water for plant tracer studies. The overall radiochemical yield of **[^{11}C]1** based on total [^{11}C]cyanide at the start of synthesis was 28%. Radiochemical purity as determined by TLC (solvent: hexane 40%/EtOAc 60%) and analytical HPLC was >98%. Total synthesis time was 68 min, while specific activity at EOB was 0.7 mCi/nmol.

3-Indolyl[1-^{11}C]acetamide ([^{11}C]6)

The conditions for hydrolysis of **[^{11}C]5** to acetamide (**[^{11}C]6**) were identical to those for **[^{11}C]1** except that the temperature was 100°C. The reaction mixture purified with HPLC using formic acid (0.1%) 85%/acetonitrile (15%) at a flow rate of 5 ml/min on a PFP column (Phenomenex, 250 × 10 mm, 5 μ). The labeled acetamide **6** was eluted at 17–18 min. HPLC solvent was removed as described above. The overall radiochemical yield of **6** based on total [^{11}C]cyanide at the start of synthesis was 10%. Radiochemical purity as determined by TLC (solvent: EtOAc 95%/MeOH 5%) and analytical HPLC was >98%. Total synthesis time was 66 min, while specific activity at EOB was 1.6 mCi/nmol.

Positron autoradiography of 3-indolyl[1-^{11}C]acetic acid ([^{11}C]1) movement in maize

To assess the extent of loading and short-distance movement of 3-indolyl[1-^{11}C]acetic acid (**[^{11}C]1**) and/or its radiolabeled metabolites, we administered a water solution to a leaf tip of a maize plant and imaged tracer distribution using positron autoradiography. Approximately 1 cm of a leaf tip of a maize plant was abraided prior to submerging the abraided section in 0.25 ml of de-ionized water containing approximately 1 mCi/ml of the **[^{11}C]1**. Plant tissue was exposed to tracer for 1 h prior to autoradiography, exposing the leaf section by direct contact to a phosphor-imaging plate for 10 min and using a Fuji BAS2500 imager to register the radioactivity distribution.

Results and discussion

Reaction of gramine with cyanide and [^{11}C]cyanide

Our initial investigations of the reaction between cyanide and the *N*-Boc protected 3-bromomethyl- or 3-acetoxymethyl-indole derivatives did not give the desired displacement product, leading to the current investigation of the use of gramine as a substrate for labeling (data not shown). In the previously reported syntheses of 3-indolylacetic acid, gramine was converted to the more reactive quaternary methiodide salt either *in situ* or prior to the displacement with cyanide.[15–17] However, we found that gramine itself reacts very rapidly and efficiently with cyanide to give 3-indolylacetonitrile (**5**) *without* prior or *in situ* formation of the quaternary ammonium salt as a leaving group (data not shown). This has precedent with the synthesis of 4-halo or nitro-substituted 3-indolylacetic acid derivatives[22] as well as 5-substituted 3-indolylacetonitrile derivatives[23] and 7-azaindoles.[24] Based on our results with unlabeled cyanide, we investigated the reaction of gramine (**4**) using [^{11}C]HCN trapped in different solvents (Table I). While EtOH (95%) as a solvent gave high yield with unlabeled cyanide, it gave a low yield (25%) with [^{11}C]HCN. Changing the reaction solvent to DMF/DMSO (3:1) gave even poorer yields of **[^{11}C]5** as compared to reaction in EtOH (95%). In contrast, carrying out the displacement reaction in DMSO gave **[^{11}C]5** almost exclusively as the reaction product at 140°C for 5 min. Even at room temperature after [^{11}C]HCN trapping, the radiochemical yield reached up to 60% reproducibly.

Table I. Reaction conditions for the reaction of gramine with [^{11}C]cyanide[a] to form **[^{11}C]5**

Condition[b]	Solvent (0.3 ml)	Radiochemical yield[c]
a	EtOH (95%)	25 ± 9% (n = 6)
b	DMF/DMSO (3:1)	20 ± 1% (n = 2)
c	DMSO	90 ± 10% (n = 16)

[a][^{11}C]CN trapped at room temperature for condition c and at −42°C for a and b.
[b]140°C, 5 min.
[c]Radiochemical yield was determined by TLC and HPLC.

Hydrolysis of 3-indolyl[1-^{11}C]acetonitrile ([^{11}C]5)

Alkaline hydrolysis of **[^{11}C]5** without removal of DMSO led to very poor yields of **[^{11}C]1** and **[^{11}C]6**. Hydrolysis of the total reaction mixture in the absence of the DMSO gave only slightly higher yields. For optimum reaction yields, it was necessary to isolate **[^{11}C]5** from the reaction mixture prior to hydrolysis. Rapid isolation was achieved by dilution of the crude reaction mixture with water and trapping of the organics on a C-18 light sep-pak. Rinsing the sep-pak with dilute HCl removed the unreacted gramine and **[^{11}C]5** was then eluted from the column with ether (1 ml). Evaporation of the ether at room temperature under a stream of argon gave the radiolabeled nitrile for subsequent hydrolysis.

Both acid and base hydrolysis conditions were investigated, but hydrolysis of nitrile (**[^{11}C]5**) using aqueous hydrochloric acid generally gave very poor yields of **[^{11}C]6** and **[^{11}C]1**. The yields of **[^{11}C]6** and **[^{11}C]1** changed significantly by varying base concentration and reaction temperature. Higher concentrations of NaOH (10 and 5 M) gave more of an unidentified labeled nonpolar side product. Of the hydrolysis conditions investigated, optimum hydrolysis of the labeled nitrile to **[^{11}C]1** required aqueous 1 M NaOH at 140°C for 5 min. For **[^{11}C]6** aqueous 1 M NaOH at 100°C for 5 min is required.

Purification and formulation of [^{11}C]1 and [^{11}C]6

Both **[^{11}C]1** and **[^{11}C]6** could be purified by HPLC on a semi-preparative PFP column. Removal of the HPLC solvent under vacuum followed by formulation in pure water provided either **[^{11}C]1** or **[^{11}C]6** in greater than 95% radiochemical purity. Specific activities varied depending on the production of H^{11}CN but were typically 0.6–0.7 mCi/nmol for **[^{11}C]1** and 0.6–1.6 mCi/nmol for **[^{11}C]6**. The total synthesis time for **[^{11}C]1** and **[^{11}C]6** was 68 and 66 min respectively (Figure 3).

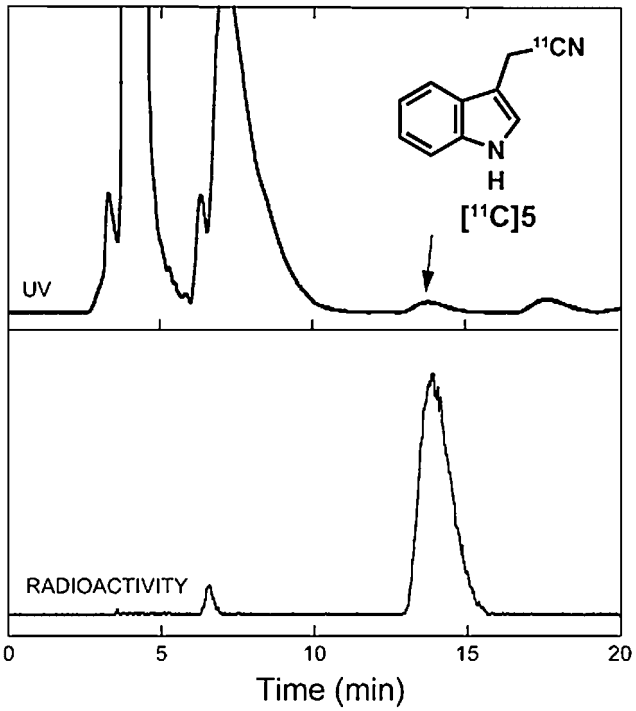

Figure 3. Preparative HPLC profile of the crude reaction mixture from the cyanation of gramine (**4**) with [^{11}C]cyanide. Reaction solvent, DMSO; Temperature, 140°C.

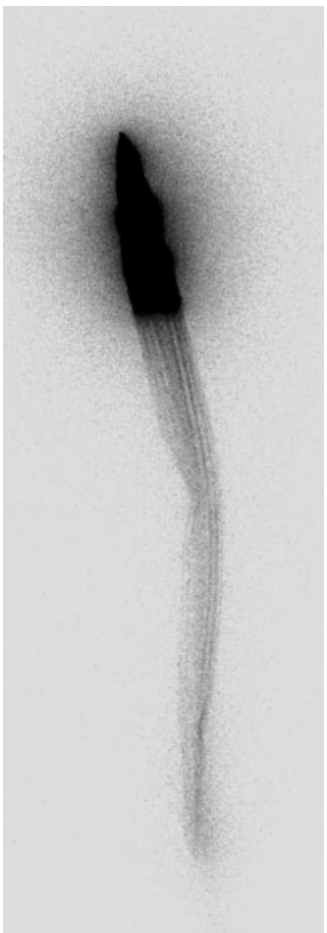

Figure 4. Positron autoradiograph of maize leaf after 1 hour topical exposure to 0.25 mCi of **[^{11}C]1**. The image shows signs of vascular loading and movement through the length of the leaf tissue.

Positron autoradiography of [^{11}C]1

Figure 4 shows the distribution of radioactivity in an intact maize leaf after 1 hr of topical exposure to **[^{11}C]1**. The image shows C-11 distribution outside the region of administration. Studies are ongoing to characterize the species which moves (**[^{11}C]1** or a labeled metabolite or conjugate) as well as the nature of the movement.

Summary

In the prior syntheses of 3-indolylacetic acid (**1**), gramine was converted into the quaternary ammonium salt using the potent electrophiles, methyl iodide or dimethyl sulfate, either *in situ* or prior to the reaction with cyanide so that cyanide displaces trimethylamine. However, there is precedent for cyanide substitution directly from 3-dimethylaminomethyl indole derivatives using excess alkali cyanide in either acidic or neutral medium at high temperature *without* prior formation of the quaternary ammonium salt.[22–24] We found that gramine (**4**) itself reacts rapidly with [^{11}C]cyanide to give **[^{11}C]1** *without* formation of the quaternary methiodide salt. In addition, by adjusting the temperature of the hydrolysis reaction, we could form either the labeled acid or the amide. Since 3-indolylacetonitrile and 3-indolylacetamide are also intermediates in the biosynthesis of 3-indolylacetic acid (**1**), and also function as auxins, this versatile reaction produces all three of these labeled compounds available for metabolic studies and imaging studies in whole plants *in vivo*.

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