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Rapid radiosynthesis of [^{11}C] and [^{14}C]azelaic, suberic, and sebacic acids for *in vivo* mechanistic studies of systemic acquired resistance in plants

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A recent report that the aliphatic dicarboxylic acid, azelaic acid (1,9-nonanedioic acid) but not related acids, suberic acid (1,8-octanedioic acid) or sebacic (1,10-decanedioic acid) acid induces systemic acquired resistance to invading pathogens in plants stimulated the development of a rapid method for labeling these dicarboxylic acids with ^{11}C and ^{14}C for *in vivo* mechanistic studies in whole plants. ^{11}C-labeling was performed by reaction of ammonium [^{11}C]cyanide with the corresponding bromonitrile precursor followed by hydrolysis with aqueous sodium hydroxide solution. Total synthesis time was 60 min. Median decay-corrected radiochemical yield for [^{11}C]azelaic acid was 40% relative to trapped [^{11}C]cyanide, and specific activity was 15 GBq/μmol. Yields for [^{11}C]suberic and sebacic acids were similar. The ^{14}C-labeled version of azelaic acid was prepared from potassium [^{14}C]cyanide in 45% overall radiochemical yield. Radiolabeling procedures were verified using ^{13}C-labeling coupled with ^{13}C-NMR and liquid chromatography–mass spectrometry analysis. The ^{11}C and ^{14}C-labeled azelaic acid and related dicarboxylic acids are expected to be of value in understanding the mode-of-action, transport, and fate of this putative signaling molecule in plants.

Keywords: azelaic acid; 1,9-nonanedioic acid; systemic acquired resistance; plant signaling; plant hormone

Introduction

Localized foliar infections in plants subsequently result in a higher resistance of the entire plant to secondary infections, a phenomenon termed systemic acquired resistance (SAR).[1,2] SAR seems to require salicylic acid and possibly methyl salicylate.[3,4] A recent study reported that azelaic acid (1,9-nonanedioic acid) accumulates in the leaves after primary infections and also that exogenous application of azelaic acid primes the plant to generate salicylic acid upon secondary infections in other parts of the plant, a process involving activation of the AZI1 (AZELAIC ACID INDUCED 1, At4g12470) gene.[5] Furthermore, the same study also reported that whereas application of azelaic acid can induce SAR, the related dicarboxylic, suberic (1,8-octanedioic acid), and sebacic acids (1,10-decanedioic acid) were inactive, indicating that the induction of SAR is specific to azelaic acid over chemically similar acids differing by only one methylene group in chain length.

In order to investigate the transport and fate of azelaic acid in living plants *in vivo*, we investigated approaches to label azelaic acid, as well as the chemically related but inactive suberic and sebacic acids, with either ^{11}C or ^{14}C. ^{11}C-labeled version of the acids were synthesized to enable short-term tracking of movement of labeled acids away from the site of application via external detection of gamma or β^+ emissions in the living plant, whereas the corresponding ^{14}C-labeled versions of the acids were synthesized to enable longer duration studies via detection of β^- decay using autoradiography or liquid scintillation counting in dried plant material.

Labeling with ^{11}C (half-life, 20.4 min) requires chemistry that can be conducted rapidly and with a minimal number of postlabeling steps. In prior studies involving ^{11}C-labeling of aliphatic dicarboxylic acids, Thorell *et al.* [6] employed a scheme in which the two-carbon dicarboxylic acid, oxalic acid, was labeled in a three-step procedure from [^{11}C]cyanide via methyl [^{11}C]cyanoformate. De Spielgeer *et al.* [7] synthesized the three-carbon dicarboxylic acid, [^{11}C]malonic acid, via a shorter two-step procedure from [^{11}C]cyanide using either chloro-acetate, bromo-acetate, or iodo-acetate as the precursor. To our knowledge, longer chain dicarboxylic acids have not yet been labeled with ^{11}C.

In the current study, we developed a two-step ^{11}C-labeling of suberic, azelaic, and sebacic acids via reaction of ammonium [^{11}C]cyanide with the corresponding bromonitrile precursors

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followed by hydrolysis with aqueous sodium hydroxide solution (scheme 1). ^{14}C-labeled versions of these acids were subsequently prepared using a similar procedure using potassium [^{14}C]cyanide.

Experimental

General

Commercial reagents and solvents were purchased from Sigma–Aldrich. Organic solvents were distilled before use. Anhydrous DMSO was stored over molecular sieves. Flash chromatography was performed using silica gel 60 (Acros Organics). Analytical thin layer chromatography (TLC) measurements were conducted with 0.25 mm silica Gel 60-F254 and radioTLC with Macherey–Nagel polygramsil G/UV254 plastic-back TLC plates. Radioactivity distribution of TLC plates was determined using a Bioscan system 200 imaging scanner (Bioscan Inc., Washington DC). RadioHPLC was performed using a HPLC system (Knauer, Berlin, Germany). ^{1}H and ^{13}C-NMR spectra were measured with a Bruker spectrometer (Bruker Biospin Corp., Billerica, MA) at 400 and 100 MHz, respectively. Mass spectrometry data was obtained using an Agilent 6300 ion trap LC-MS (Agilent Technologies Inc., Santa Clara, CA).

Typical procedure for the preparation of 7-bromoheptanenitrile (2a)

To a solution of sodium cyanide (6 g, 120 mmol) in water (13.5 mL) was slowly added 1,6-dibromohexane (25 g, 100 mmol) in isopropyl alcohol (66 mL). The reaction mixture was refluxed for 15 h and then extracted with toluene. The toluene layer was washed with aqueous NaOH (1 M) followed by distilled water. The toluene was evaporated under vacuum to obtain a yellow liquid, which was purified by chromatography (silica gel, ethyl acetate/n-hexane, 1/4). Following evaporation of the solvent, 6.4 g (33%) of 7-bromoheptanenitrile was obtained as colorless oil. NMR (400 MHz, $CDCl_3$), δ (ppm) = 1.3 (m, 4H), 1.7 (m, 2H), 1.79 (m, 2H), 2.4 (t, 2H), 3.2 (t, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): 119.6, 33.6, 32.3, 27.8, 27.3, 25.5, 17.06.

8-bromooctanenitrile (2b)

Synthesis of 8-bromooctanenitrile (2b) was performed using 1,7-dibromoheptane (10 g, 39 mmol) and an equimolar amount of NaCN. After addition of 1 M NaOH (8 mL), the product was extracted with ethyl ether and purified by chromatography (silica gel, 1:5 ethyl acetate:hexane) to give 8-bromooctanenitrile (2.8 g, 35%) as a pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$), δ (ppm): 1.30–1.40 (m, 6H), 1.63 (m, 2H), 1.80 (m, 2H), 2.28 (t, J = 7.0 Hz), 3.45 (t, J = 6.6 Hz). ^{3}C-NMR (100 MHz, $CDCl_3$): 119.5, 37.8, 34.1, 33.7, 32.5, 28.4, 27.9, 17.9.

9-bromononanenitrile (2c)

Synthesis of 9-bromononanenitrile (2c) was performed using 1,8-dibromooctane (25 g, 90 mmol) and an equimolar amount of NaCN. After addition of 1 M NaOH (25 mL), the product was extracted with ethyl ether and purified by chromatography (silica gel, 1:5 ethyl acetate:hexane) to give 9-bromononanenitrile (6.9 g, 34%) as a pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$), δ (ppm): 1.30–1.44 (m, 8H), 1.65 (m, 2H), 1.81 (m, 2H), 2.29 (t, 2H, J = 7.0 Hz), 3.4 (t, 2H, J = 6.6 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$): δ119.8, 33.8, 32.5, 29.1, 28.5, 27.9, 26.1, 25.3, 17.8).

Labeling of azelaic and related acids with ^{11}C

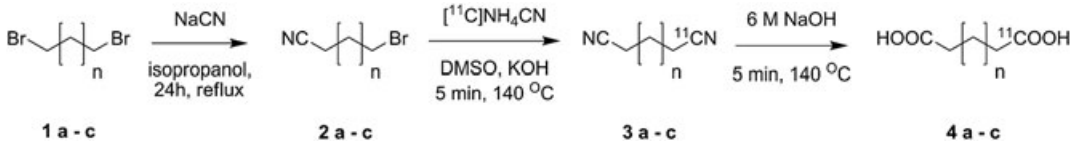
^{11}C was generated by proton bombardment of nitrogen gas (^{14}N(p,α)^{11}C) with a trace of oxygen to produce [^{11}C]CO_2. For [^{11}C]cyanide production, the [^{11}C]CO_2 was first reduced to [^{11}C]CH_4 via a nickel catalyzed hydrogenation at 390 °C for 3 min. [^{11}C]CH_4 was then mixed with ammonia gas and passed through a platinum furnace at 950 °C to produce [^{11}C]NH_4CN. The latter was trapped in DMSO (200 µL) to which aqueous potassium hydroxide solution (6 N 1 µL) had been added. To this solution, the bromonitrile precursor (1 mg, 4.7 µmol) in DMSO (100 µL) was then added, and the mixture was heated to 140 °C for 5 min. After cooling, a sample of the reaction mixture was in some cases analyzed by radioTLC to determine the extent of incorporation of [^{11}C]cyanide into the dintrile. Mean incorporation was 90% for both the C-8 and C-9 dintrile and 83% for the C-10 dintrile. Water (1 mL) was then added to the remaining reaction mixture, and the mixture passed through a solid-phase extraction cartridge (Waters, Sep-Pak®, C18 Plus). The cartridge was washed with water and then with diethyl ether (2 mL) to obtain the [^{11}C]dinitrile intermediate. Following evaporation under the stream of argon, NaOH solution (6 M, 300 µL) was added to the [^{11}C]dinitrile, and the reaction mixture was heated at 140 °C for 5 min. The mixture was neutralized with 6 M HCl and purified HPLC using a Gemini seimipreparative C-18 column (250 mm × 10 mm) at a flow rate of 5 mL/min. Retention times and mobile phases were as follows: [^{11}C]suberic acid RT 13 min (0.1% formic acid/acetonitrile, 80/20), [^{11}C]azelaic acid RT 12 min (0.1% formic acid/acetonitrile, 75/25), [^{11}C]sebacic acid RT 13 min (0.1% formic acid/acetonitrile, 70/30). Total synthesis time was approximately 60 min relative to end-of-bombarbment (EOB). All three carboxylic acids were analyzed by Liquid chromatography–mass spectrometry; for suberic acid ($C_8H_{14}O_4$ [M–H]$^-$ calcd 173.1 found 173.1), for azelaic acid ($C_9H_{16}O_4$ [M–H]$^-$ calcd 187.1 found 187.1), and sebacic acid ($C_{10}H_{18}O_4$ [M–H]$^-$ calcd 201.1 found 201.1).

Identification of labeled carboxylic acids using [^{13}C]KCN

Radiosynthesis was performed as described earlier except that [^{13}C]KCN (0.5 mg, 7.6 µmol) was added as a carrier to the DMSO solvent prior to trapping [^{11}C]NH_4CN. The labeled dicarboxylic acids were analyzed by ^{13}C-NMR after decay of the ^{11}C radioactivity. Only one ^{13}C signal for each carboxylic acid was observed, with a chemical shift consistent with the expected acid.

Labeling of azelaic acid with ^{14}C

To a solution of [^{14}C]KCN (390 µCi, 14 MBq) and 6 M KOH (1 µL) in DMSO (400 µL), the bromonitrile precursor (2 mg, 9.4 µmol) was



Scheme 1. Synthesis of ^{11}C-labeled dicarboxylic acids; a: n = 4, b: n = 5, c: n = 6.

added, and the mixture was heated at 140° for 15 min. After cooling, subsequent hydrolysis of the dinitrile and purification of the dicarboxylic acid by semipreparative HPLC were performed as described for the [^{11}C] radiosynthesis. A slightly modified procedure involving extraction of the labeled dintrile into ether rather than sep-pack purification was employed for the other acids,but with lower yields.

Autoradiography

For ^{11}C autoradiography, a solution of the labeled acid (1–2 mCi; 37–74 MBq) in water (0.5 mL) was applied to the tip of a leaf. Following uptake, the radioactivity was imaged by placing a phosphor plate over the fresh leaf. The high activity of the ^{11}C coupled with the ease of detection of β^+ particles and gamma rays emanating from the thin leaf tissue allowed the image to be collected using just a brief 15 min exposure to the plate, after which it was scanned using a Fuji BAS2500 phosphoimager (Fujifilm Corporation, Tokyo, Japan). For ^{14}C autoradiography, a solution of the labeled acid (1 μCi; 37 KBq) in 10 mm phosphate buffer (50 μL, pH 7) containing 0.1% Triton X-100 was applied to the tip of a leaf. Following uptake, the whole plant was placed over a Saran®-wrapped phosphor screen. The plant and phosphor plate were then placed in a vacuum bag and covered with several layers of paper and a few grams of drying agent (Drierite®) to dry the plant *in situ*. The bag was evacuated and sealed, and the plate was exposed to the radioactive plant for 2–3 days. Following exposure, the phosphor plate was scanned using a Cyclone phosphoimager (Packard Instrument Co. Inc., Downers Grove, IL).

Results and Discussion

Bromonitriles were chosen for the cyanide displacement rather than the corresponding chloronitriles because of their higher reactivity. Gas chromatography–mass spectrometry analysis of the bromonitrile precursors showed only one peak; and thus, they could be used directly after column chromatography without additional purification.

A series of optimization experiments with unlabeled KCN determined that the maximum yields of the dinitrile from the bromonitrile were obtained using DMSO at 140 °C for 5 min with added 6 M NaOH or KOH (Table 1). Yields were significantly reduced by using lower temperatures or by eliminating the base.

Nitriles can be hydrolyzed to carboxylic acids using either strong acids or strong bases. Initial optimization of the hydrolysis conditions for conversion of aliphatic nitriles to carboxylic acids were conducted using 4-phenylbutyronitrile as a model compound due to its ease of detection in the HPLC UV chromatogram. In a direct comparison of hydrolysis of 4-phenylbutyronitrile using either aqueous HCl, H_2SO_4, KOH, or NaOH (3, 6, and 9 M; 5 min at 140 °C), NaOH gave the highest yields of the carboxylic acid. In our hands, acidic hydrolysis conditions resulted in the formation of a side product in addition to the free carboxylic acid. This side product was not further characterized but likely represented the amide. On the basis of these results, 6 M NaOH was selected as the preferred conditions for the labeled dinitrile hydrolysis. In subsequent optimization experiments using the [^{11}C]dinitriles, direct one-pot hydrolysis without removal of DMSO from the previous labeling step was not successful. An intermediate extraction of the [^{11}C]dinitriles using a Sep-Pak cartridge prior to hydrolysis with NaOH was thus implemented.

Table 1. Effect of solvent conditions and temperature on reaction of 7-bromooctanenitrile with KCN

Entry[a]	Solvent	Temperature (°C)	Yield[b]
1	DMSO only	90	10%
2	DMSO only	120	15%
3	DMSO only	140	20%
4	DMSO, NaOH (6 M, 1 μL)	140	70%
5	DMSO, KOH (6 M, 1 μL)	140	90%
6	DMSO, KOAc (6 M, 1 μL)	140	50%
7	DMSO, Triethylamine (1 mg)	140	40%

[a]Reaction conditions: 7-bromooctanenitrile (1 mg), KCN (1 mg), DMSO (300 μL), 5 min at indicated temperature.
[b]Measured by GC-MS (area integration of total ion counts)

Because of the poor UV absorbance of the aliphatic dicarboxylic acids and their trace concentration due to the use of no-carrier-added [^{11}C]cyanide, neither ultraviolet absorption nor refractive index detection could be used to detect the acids eluting from the HPLC column. To address this problem during the development of the labeling procedure, we spiked the reaction solvent with [^{13}C]KCN prior to trapping the [^{11}C]NH_4CN. When the ^{11}C had decayed, the formation of the ^{13}C-dicarboxylic acids in the reaction mixture could be confirmed by ^{13}C-NMR. A chemical shift at 177 ppm, specific for the carboxylic group, was clearly observed. This procedure was also used to confirm the formation of labeled suberic and sebacic acids. A similar strategy of using [^{13}C]cyanide to monitor the [^{11}C]cyanide reaction was used in the development of a novel synthesis of [^{11}C]formaldehyde from [^{11}C]methyl iodide.[8]

ESI-MS analysis was used to further confirm the labeling chemistry for the dicarboxylic acids. By generating a standard curve from the total ion chromatogram (negative mode) and comparing it to that from the labeled product fraction, the specific activity of the acids could be determined. Using this approach, the specific activity for [^{11}C]azelaic acid following HPLC purification was calculated as 0.4 Ci/μmol (15 GBq/μmol). Median decay-corrected radiochemical yield for [^{11}C]azelaic acid relative to the amount of trapped [^{11}C]cyanide was 40% (range 35%–50%; $n = 6$). For suberic and sebacic acids, the median radiochemical yields relative to trapped [^{11}C]cyanide were 30% ($n = 4$) and 35% ($n = 1$), respectively.

[^{14}C]Azelaic acid, as well as related acids, was prepared using a similar procedure as with the ^{11}C compounds. Because of the lower specific activity of ^{14}C over ^{11}C and thus higher mass of compound eluting from the HPLC, a small UV absorbance peak (wavelength set at 200 nm) from the radiolabeled compound could be observed above the background signal (Figure 1). The specific activity of [^{14}C]azelaic acid, calculated from the UV absorbance, was 16 mCi/mmol (0.6 GBq/mmol). [^{14}C]-labeled suberic and sebacic acids gave similar specific activities. Final radiochemical yield for [^{14}C]azelaic acid following purification was 45% ($n = 1$).

Autoradiographic studies were performed with the ^{11}C and ^{14}C-labeled azelaic acids. An example of an autoradiogram taken on a tobacco leaf after application of [^{11}C]azelaic acid to the tip is shown in Figure 2. The site of application is clearly visible and

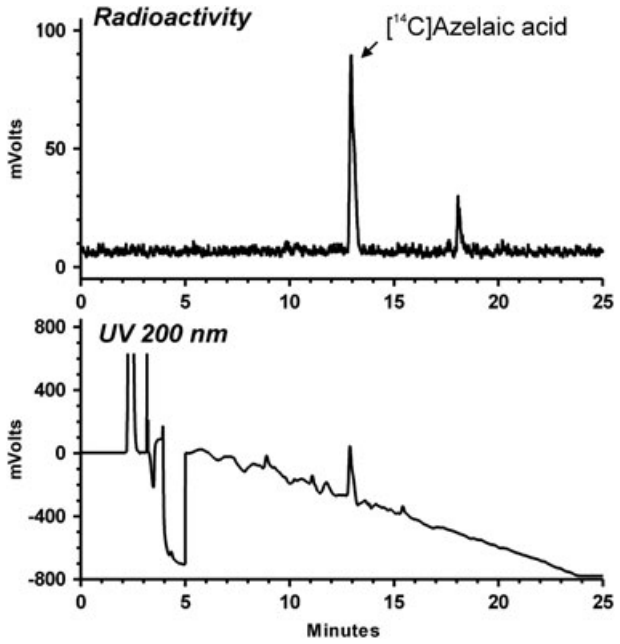


Figure 1. HPLC purification of [^{14}C]azelaic acid. HPLC conditions: C-18 column 250 × 10 mm, flow rate 5 mL/min, gradient from 10% acetonitrile, 0.1% formic acid to 80% acetonitrile (no formic acid) over 25 min.

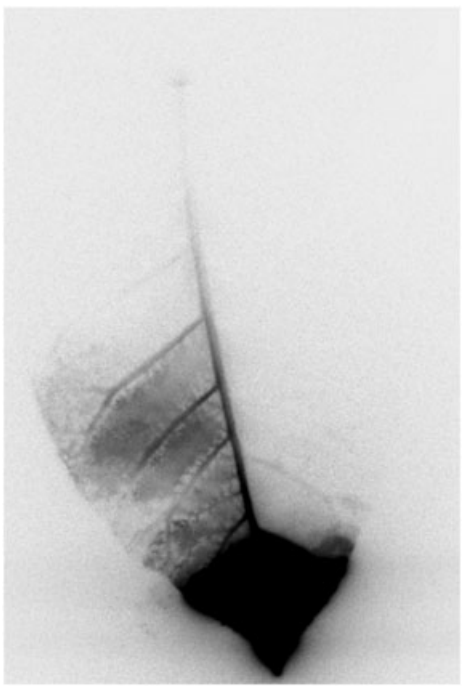


Figure 2. Phosphor-screen autoradiogram of a fresh tobacco leaf at 50 min after topical application of [^{11}C]azelaic acid to the tip of the leaf.

some early movement of the label up the vascular tissue of the leaf is apparent. Figure 3 shows an example of an autoradiogram of a whole *Arabidopsis* plant after application of ^{14}C-labeled azelaic acid to one of the leaves. Movement of ^{14}C-label throughout the plant is apparent.

Labeled compounds should preferably be at tracer levels to avoid interfering with the physiology of the organism to which

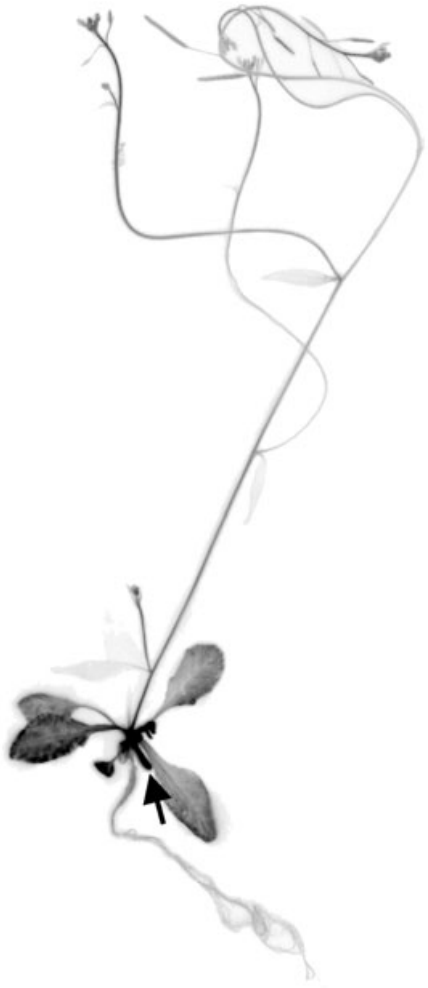


Figure 3. Phosphor-screen autoradiogram of a dried *Arabidopsis* plant at 24 h after topical application of [^{14}C]azelaic acid to a leaf. Arrow indicates the location of the application leaf. The application site was removed immediately prior to imaging to reduce 'fogging' of the phosphor screen around the site.

they have been administered. In the case of azelaic acid, endogenous levels in the plant sap range from 5 μm under basal conditions to 30 μm following pathogen attack.[5] For the [^{11}C]azelaic acid, specific activity was high and unlikely to perturb the physiology of the plant. However, for the ^{14}C-experiments, specific activity is several orders of magnitude lower. For the ^{14}C autoradiography experiments, approximately 0.06 μmol of labeled compound was applied to the surface of the leaf and 6%–10% of this was found to be exported from the application site after 24 h. Assuming the latter distributes evenly throughout the plant, it can be calculated that the final average concentration of the ^{14}C-labeled azelaic acid in a 1 g plant will be approximately 4–6 μm.

Conclusion

In summary, we have developed a rapid two-step radiolabeling scheme for labeling the putative plant signaling molecule, azelaic acid, as well as related inactive dicarboxylic, suberic, and sebacic acids, with ^{11}C or ^{14}C. These radiolabeled molecules should facilitate further investigations into the movement and metabolism of these compounds in plants and the role of azelaic acid in priming plants for systemic acquired resistance.

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Conflict of Interest

The authors did not report any conflict of interest.

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