JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Potent Enantioselective Auxin: Indole-3-Succinic Acid

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Hoya/NM Schools Chemobile, 2017 Calle Lejano, Santa Fe, New Mexico 87501-8745

Low levels of synthetic auxins are used as root growth promoters for a wide variety of botanicals. When properly used, they can produce earlier and more prolific root growth. Indole-3-succinic acid (ISA) is a chiral compound that can be synthesized as a racemate. Its enantiomers were resolved by both chromatography and diastereomeric crystallization. The absolute configuration of each enantiomer was determined by X-ray crystallography and then tested for auxin activity. The relative effectiveness of the ISA enantiomers was determined and compared to the achiral synthetic auxin indole-3-butyric acid and to the natural auxin indole-3-acetic acid.

KEYWORDS: Rooting hormone; enantiomeric separation; plant propagation

INTRODUCTION

Auxins are well-known plant growth or development hormones that were first extensively studied in the mid 1930s (*1*−*5*). Auxin is actually involved in a variety of plant activities although its ability to promote cell elongation is perhaps best known (*5*, *6*). The most widely occurring, natural auxin is indole-3-acetic acid (IAA). It occurs in both free and conjugated states in plants and seeds (*3*, *5*, *6*). Early on, the use of IAA was shown to be advantageous in stimulating root formation in plant cuttings (*4*). Subsequently, synthetic materials such as indole-3-butyric acid (IBA) and naphthleneacetic acid (NAA) were found to be even more useful at least in part to their greater stability (*5*, *7*). Most recently, it has been found that IBA also occurs naturally in some plants albeit at very low levels (*8*). Today, IBA and NAA are widely used as synthetic rooting hormones. They are most often applied to the base of plant (stem and leaf) cuttings, to transplantings since it is known that auxin is required for initiation of adventitious roots on stems, and to stimulate root growth in general (*7*−*11*). Rooting hormones are widely used for plant propagation because they hasten root initiation, improve rooting percentages, produce more uniform rooting, and increase the number and quality of roots (*6*, *7*).

Recently, we noticed that another synthetic compound, indole-3-succinic acid (ISA), seemed to be much more effective in promoting the growth of some seedlings than either the natural auxin, IAA, or the widely used synthetic hormones, IBA and NAA. However, unlike the other rooting hormones, ISA contains one stereogenic center and can exist in two enantiomeric forms (**Figure 1**). Most chiral biologically active compounds are known to be stereoselective. Indeed, enantiomers can have different biological actions and potencies (*12*−*14*). However, there have been few reports on enantioselective growth-promoting auxins, to our knowledge (*15*). ISA has been synthesized only as the racemate (*16*−*18*). It has never been resolved nor have any reports appeared as to its activity as an auxin. In this work, we synthesize ISA, resolve its enantiomers, determine their absolute configuration, demonstrate that its auxin activity can be stereoselective, and show that both ISA enantiomers and the racemate can have a greater effect on root growth than either the natural auxin, IAA, or the most widely used synthetic analogue, IBA (*19*).

MATERIALS AND METHODS

Apparatus. An assembled high-performance liquid chromatography (HPLC) system was used to separate racemic ISA. It consisted of a LC-6A pump, a SPD-2AM UV detector, and a CR 601 Chromatopac recorder from Shimadzu (Kyoto, Japan). The analytical column used in this study was a Cyclobond I 2000 RSP column, 250 mm × 4.6 mm (id) obtained from Advanced Separation Technologies, Inc. (Whippany, NJ) as was the analogous semipreparative Cyclobond I 2000 RSP 500 mm × 10 mm (id). The mobile phase was methanol/water/acetic acid (30:70:0.1, volume ratio), and the flow rate was 1 mL/min. The detection wavelength was 254 nm. Chiral liquid chro-

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10.1021/jf010991f CCC: $22.00
Published on Web 12/29/2001

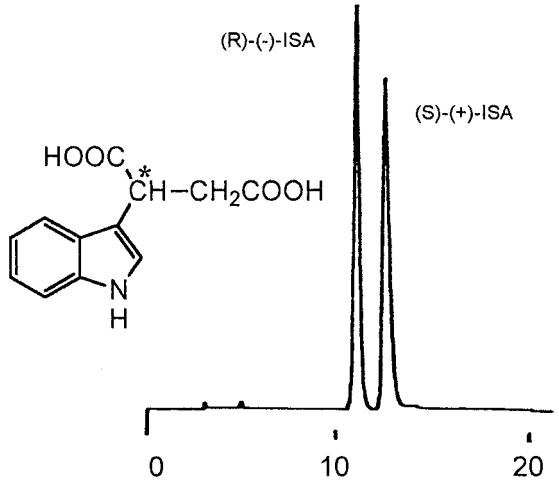

Figure 1. Structure of ISA and a LC chromatogram showing the separation of its enantiomers on a Cyclobond I-RSP column (25 cm × 0.46 cm (id)) using a mobile phase of 40:60:02 (v:v:v) methanol:water:glacial acetic acid (flow rate = 1.0 mL/min). The optical rotation of the compound represented by each peak was determined with an on-line laser polarimeter (see Materials and Methods). The absolute configuration was determined as indicated in **Figures 2** and **3**.

matography (LC) was used to confirm the enantiomeric purity of the products. The optical rotation (at 675 nm) of the eluted enantiomers was determined with an in-line chiroptical LC detector (i.e., the PDR Chiral Advanced Laser Polarimeter, Palm Beach Gardens, FL). In most cases, this rotation correlates with that found for the sodium D-line (*20*). Elemental analysis was performed by Galbraith Laboratories, Inc. (Knoxville, TN). Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were uncorrected. The absolute configuration was confirmed by X-ray crystallography as outlined below.

Reagents and Solvents. ISA was prepared according to the method introduced by Noland and Hammer (*16*, *17*). Indole and maleic anhydride were purchased from Aldrich Chemical Co. Cinchonidine was purchased from Fluka Chemika-Biochemica Analytika. Solvents and organic modifiers (methanol, ethanol, acetic acid, and methyl tertbutyl ether) were of Ommisolve or HPLC grade and were supplied by Fisher Scientific (St. Louis, MO).

Preparation of ISA. A total of 14.57 g (0.1486 mole) of Maleic anhydride was dissolved in 11.0 mL of acetyl acetate, and then, 34.81 g (0.297 mole) of indole was added with magnetic stirring. The deep orange-red solution was stirred until all of the indole went into the solution. Yellow-orange crystals formed after standing at 4°C for 2 days. Filtration and washing with ethanol (10 mL) gave 28.48 g (yield, 58%) of yellow-orange crystals of maleyldiindole. The 28.48 g (0.0857 mole) of maleyl diindole was refluxed for 3 h with 30% aqueous potassium hydroxide solution (150 mL). The reaction mixture was cooled and extracted with ether, and the ether was evaporated, yielding crude indole (10.050 g, 0.088 58 mole). The alkaline solution was acidified to pH 4.4 with concentrated sulfuric acid. After water was removed under reduced pressure, the residue was subject to soxlet extraction with 250 mL of methyl-*tert*-butyl ether for 36 h, which gave racemic ISA, 11.96 g (yield 60%). Recrystallization from ethanol−water yielded pale pinkish white platelets, mp 196−198°C (with gas evolution); (literature, mp 197−198°C).

Optical Resolution of Racemic ISA by Preferential Crystallization as Cinchonidine Salts. A mixture of 1.26 g (4.29 mmol) of cinchonidine and 1.00 g (4.29 mmol) of racemic ISA in 10 mL of 96% ethanol was heated on a steam bath with magnetic stirring until all of the solid dissolved. The solution was cooled slowly to room temperature, and the white precipitate formed was collected and washed with ether. After the mixture was recrystallized 11 times from 96% ethanol and one solution of 96% ethanol:methanol, 80:20 by volume, and dried, colorless fine needles of (*S*)-ISA-cinchonidine salt was obtained; 0.296 g; yield, 13.1%; enantiomeric excess (ee), 99.3%; mp, 196−198 °C (with gas evolution). Elemental analysis calcd for $C_{31}H_{33}N_3O_{55}$: C, 70.57%; H, 6.30%; N, 7.96%. Found: C, 70.00%; H, 6.42%; N, 7.84%. IR: 3500−2900, 1592, 1460 cm^{-1}. By fractional removal of (*S*)-ISA salt, followed by rotary evaporation of the solvent and recrystallization (supra vide), the (*R*)-ISA-cinchonidine salt was obtained.

The ammonium salts of both ISA enantiomers were obtained by suspending the respective ISA-cinchonidine salts in concentrated aqueous NH_4OH. As the suspension was heated, the solid dissolved. When the solution was cooled to room temperature, cinchonidine precipitated from the solution. After the precipitate was removed, the volume of the supernatant liquid was reduced by rotary evaporation. Additional cinchonidine precipitated when this solution was cooled to room temperature. This process was repeated if any cinchonidine was left in solution (or determined by reversed phase LC (using a 250 mm × 46 mm, id, Astec C_{18} column). The ee values of (*S*)-ISA and (*R*)-ISA were found to be 98 and 94%, respectively, as determined by enantioselective HPLC (**Figure 1**). The ee is calculated as follows: ee = (A − B)/(A + B) × 100 where "A" is the predominant enantiomer and "B" is its antipode.

Crystallographic Data for Cinchonidine Indole Succinate Salt. X-ray diffraction analysis was carried out on a Siemens SMART CCD system at 173 K. The structure of the salt ($C_{19}H_{23}N_2O^+C_{12}H_{10}NO_4^-$, MW 527.60 amu) was determined from an orthorhombic crystal of dimensions 0.355 × 0.1 × 0.1 mm^3 (space group C_2), with unit cell a = 20.0944(2) Å, b = 6.6716(6) Å, c = 22.294(2) Å, β = 113.646(2)°, V = 2737.8(4) $Å^3$, Z = 4, D_x = 1.28 g cm^{-3}, and m = 0.087 mm^{-1}. The absolute configuration was determined by the known stereochemistry of the cinchonidine; Mo Kα (λ, −0.71070 Å), 3696 reflections, 2253 with $I > 3\sigma(I)$, R = 0.058. The following tables can be obtained as supporting information to this paper: Table 1, crystal data and structure refinement; Table 2, atomic coordinates and equivalent isotropic displacement parameters; Table 3, band lengths and angles; Table 4, isotropy displacement parameters; Table 5, hydrogen coordinates and isotropic displacement parameters; Table 6, torsian angles; and Table 7, observed and calculated structure factors.

Root Growth Studies of ISA Enantiomers and Traditional Auxins. Fresh solutions were used in each experiment. Weights of hormone salts were measured to ±0.01 mg on a Mettler H-16 single-pan mechanical balance (reproducibility = ±0.03 mg). Using ISA *R* and *S* enantiomers [(*R*)-ISA or (*S*)-ISA] as the 2× NH_4^+ salt, 1.40 mg was dissolved in 95% ethanol (20 mL) and slowly diluted to 500 mL with deionized water to make a stock solution of 1.047 × 10^{-5} molar concentration. Dilutions were then made in the following manner: 10^{-5} molar, 50 mL stock solution used directly; 10^{-6} molar, 5.0 mL stock solution + 45.0 mL R. O. water; 10^{-7} molar, 0.50 mL stock solution + 49.5 mL R. O. water; 10^{-8} molar, 0.50 mL of 10^{-6} molar solution + 49.5 mL R. O. water; 10^{-9} molar, 0.50 mL of 10^{-7} molar solution + 49.5 mL R. O. water. For the concentration study, (*S*)-ISA $2NH_4^+$ (98% ee, 1.41 mg) and IBA Na (1.13 mg) were used to make stock solution of 1.055 × 10^{-5} and 1.005 × 10^{-5} molar, respectively. Deionized water was also used as a control.

Five new growth cuttings of two different varieties of *Fuchsia hybrida* (i.e., varigated fuchsia (VF) and swingtime fuchsia (SF)) were placed into the 50 mL solutions contained in orange plastic pill containers that were blackened with black electrician's tape. The solutions were placed in a fiberglass solar prism greenhouse (90% of UV light is filtered out of sunlight) in which the temperatures varied from ∼55 °F (night) to 100 °F (day). The solutions were topped with deionized water daily to the 50 mL mark to compensate for water expiration and evaporation. Readings of the root growth were measured in total millimeters for the cuttings in a particular solution on days 7, 10, 14, 18, 22, 26, 30, and 34. These were recorded as the number of rootlets/total length (mm). Occasionally, one cutting from the groups of five did not survive. Therefore, all data reflect the average millimeter root growth per survived cutting.

RESULTS AND DISCUSSION

The synthesis of ISA produces a racemic mixture. This compound, like the natural plant auxin IAA, can be photodeactivated (this causes plants to grow toward light—one form

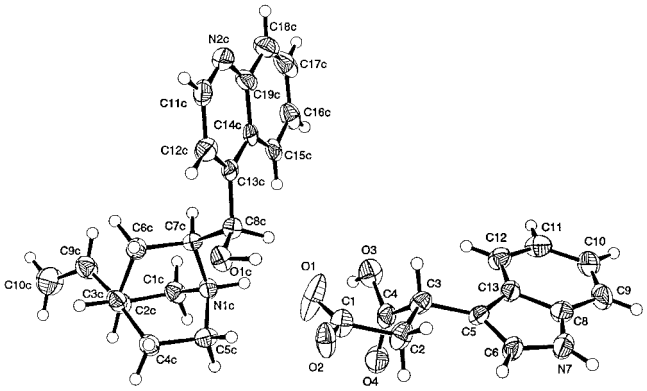

Figure 2. X-ray crystal structure of the (*S*)-ISA-cinchonidine salt.

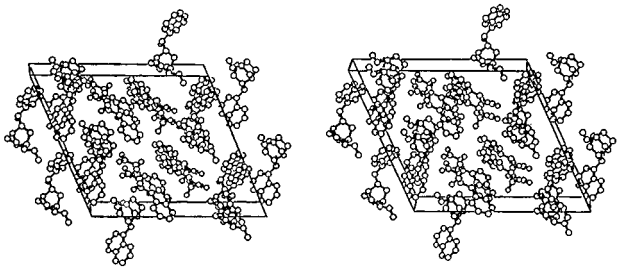

Figure 3. Crystal packing for the unit cell of the (*S*)-ISA-cinchonidine salt. All crystallographic data, atomic coordinates, torsion angles, etc. are available as supplemental data.

of phototropism). Also, ISA can undergo racemization and decomposition at extremes of pH and at higher temperatures. A simple, highly efficient analytical resolution of ISA can be achieved by HPLC using an appropriate chiral stationary phase (see **Figure 1**). With this method, enantiomeric purities can be determined to >99.9% for both enantiomers.

Although the chromatographic separation was simple, effective, and used to determine enantiomeric purities, it did not allow determination of the absolute configuration of the enantiomers of this synthetic auxin. Also, the free acid of ISA was difficult to crystallize. ISA can be resolved in large quantities by recrystallization as the (−) cinchonidine salt (see Materials and Methods). This produced a crystalline product that was suitable for X-ray diffraction studies. The enantiomeric purity of the crystallized product was determined by the aforementioned chromatographic method (see Materials and Methods). **Figure 2** shows the structure (and absolute configuration) of one enantiomer of ISA (as the cinchonidine salt). This enantiomer has the *S*-configuration and corresponds to the second peak in **Figure 1**. **Figure 3** shows the packing of the unit cell for the diastereomeric salt. Because the stereochemistry of the cinchonidine alkaloid was known, the determination of the absolute configuration of ISA was greatly simplified (**Figures 2** and **3**). The resolved, purified enantiomers of ISA were tested for their plant growth properties, along with other natural and synthetic auxins. Two strains of *F. hybrida* (i.e., SF and VF) were tested in a hydrophonic system (see Materials and Methods). A hydrophonic system is beneficial in such a study where total root growth is the dependent variable being evaluated.

Figure 4 shows typical results for the total root growth of SF. These data illustrate several trends that were found throughout the study. First, the ISA activity is very concentration dependent, as are all natural and synthetic auxins. The maximum effect for both ISA enantiomers is in the range of 10^{-7}−10^{-9} M. Concentrations of ISA either above or below this range have much less effect. Indeed, it is well-known that higher concentrations (above optimal levels) of both natural and synthetic auxins can have an inhibiting effect on root growth. The (*R*)- and (*S*)-enantiomers of ISA can have different effects (**Figure 4a**,**b**). Finally, root growth tends to occur earlier and proceeds more prolifically for plants exposed to the optimal amounts of ISA than either untreated plants or plants treated with the recommended concentration of the leading commercial synthetic auxin, IBA. It should be noted that the effects of any single plant growth hormone can vary between plant species, and even strains. Therefore, even though 10^{-8} M of the *R*-enantiomer of ISA is more potent for SF, the opposite enantiomer is observed to be more potent for VF at all concentrations of ISA (**Figure 5**).

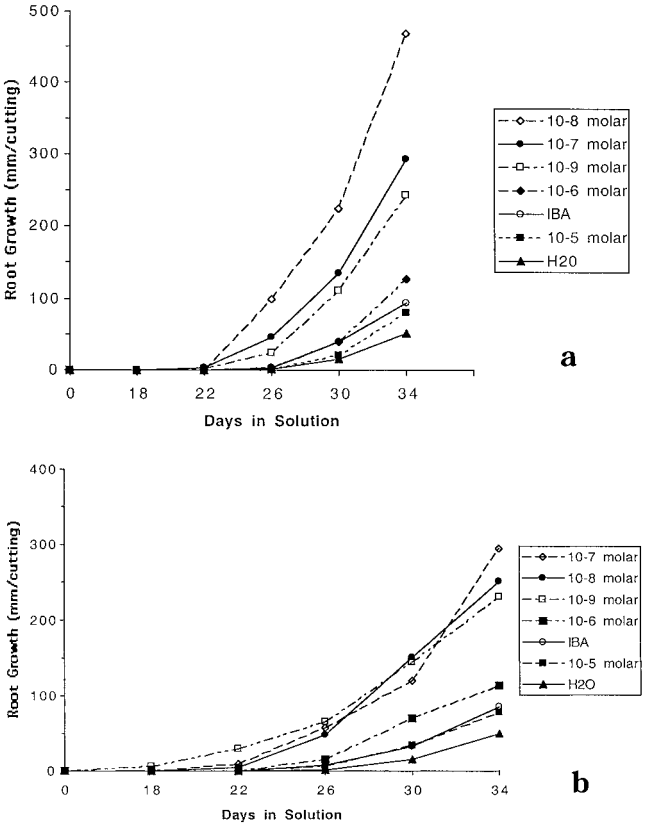

Figure 4. (**a**) Plots showing the effects of different concentrations of (*R*)-ISA, as well as IBA, on the root growth of SF. (**b**) Plots showing the effect of different concentration of (*S*)-ISA, as well as IBA, on the root growth of SF. Note that there is an optimum concentration range that produces the greatest effect for each synthetic auxin. The growth curve for deionized water (H_2O) is given as well.

Table 1 is a comparison of the relative potencies of various forms of ISA (i.e., the (*R*)-enantiomer, (*S*)-enantiomer, and racemate) and the commercial synthetic auxin IBA. In every case but one, both enantiomers of ISA and racemic ISA had significantly higher activity than the IBA. In one case, a higher than optimum level of 10^{-7} M (*R*)-ISA had nearly the same activity as the optimum level of IBA. Also included in **Table 1** is the relative potency of racemic ISA (a 10^{-7} M solution). Its potency is significantly higher than that of IBA as well. However, a direct comparison of the racemate to the single enantiomer is difficult to make since the level of each enantiomer in the racemate is half that of the corresponding single enantiomeric solution. The natural auxin, IAA, always had substantially lower levels of activity than either IBA or ISA (**Table 1**). These data show once again that the enantiomers of ISA have different activities.

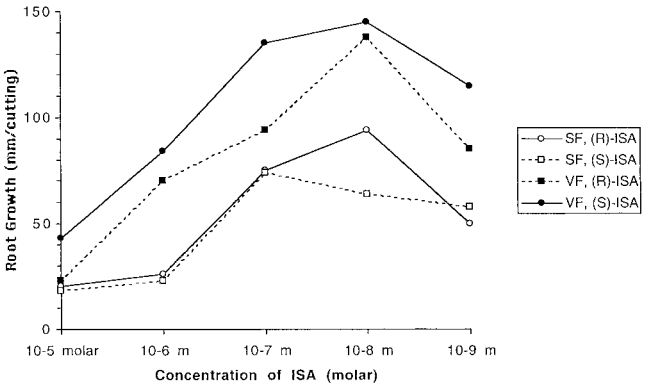

Figure 5. Plots showing the effect of different concentrations of (*S*)-ISA and (*R*)-ISA on root growth of SF (bottom two curves with the open symbols) and VF (top two curves with the solid symbols). Note that the optimum concentration for these synthetic auxins is in the 10^{-7}–10^{-8} molar range. Also, these two varieties of Fuchsia appear to have the opposite ISA enantioselectivity.

Table 1. Relative Effectiveness (in Promoting Root Growth) of Different ISA Solutions as Compared to 10^{-7} M IBA[a]

compd	concn (M)	plant tested	enhancement ratio[a]
(*S*)-ISA	10^{-7}	VF	3.8
(*S*)-ISA	10^{-7}	SF	5.7
(*S*)-ISA	10^{-8}	VF	4.6
(*S*)-ISA	10^{-8}	SF	4.8
(*R*)-ISA	10^{-7}	VF	0.7
(*R*)-ISA	10^{-7}	SF	5.6
(*R*)-ISA	10^{-8}	VF	4.4
(*R*)-ISA	10^{-8}	SF	7.1
racemic ISA	10^{-7}	SF	7.0
IAA	10^{-7}	VF	0
IAA	10^{-7}	SF	0

[a] The relative effectiveness or enhancement ratio (*R*) was calculated using the following formula: $R = (ISA_x - H_2O)/(IBA - H_2O)$ where ISA_x is the average root growth obtained per cutting with ISA_x solution, IBA is the average root growth per cutting with IBA, and H_2O is the average root growth per cutting with only deionized water.

CONCLUSIONS

Racemic ISA is easily synthesized and can be resolved by both chromatography and crystallization as diastereomeric cinchonidine salt. The absolute configuration of its enantiomers can be determined by X-ray diffraction. Both enantiomers of ISA and its racemate have significantly greater "root growth promoting activity" than the popular IBA and the naturally occurring auxin (IAA) on the plants tested. The optimum concentration range for all ISA isomers was between 10^{-7} and 10^{-9} M. The (*R*)- and (*S*)-enantiomers of ISA showed different activities in these studies. Because different plants do not respond in the same way to all auxins, it may be beneficial to use racemic ISA in many cases. This is because a mixture of these two stereoselective growth promoters could elicit a broader range of responses than other simple synthetic auxins that currently are in use.

Supporting Information Available: Tables of crystallographic data for ISA salts. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review July 27, 2001. Revised manuscript received November 6, 2001. Accepted November 6, 2001.

JF010991F