Plant Physiol. (1988) 86, 1236–1239
0032-0889/88/86/1236/04/$01.00/0

Changes in the Level of [^{14}C]Indole-3-Acetic Acid and [^{14}C]Indoleacetylaspartic Acid during Root Formation in Mung Bean Cuttings[1]

Received for publication May 26, 1987 and in revised form January 4, 1988

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ABSTRACT

Changes in the levels of [^{14}C]indole-3-acetic acid (IAA) and [^{14}C]indoleacetylaspartic acid (IAAsp) were examined during adventitious root formation in mung bean (*Vigna radiata* [L.] R. Wilcz. 'Berken') stem cuttings. IAAsp was identified by GC-MS as the primary conjugate in IAA-treated cuttings. During root formation in IAA-treated cuttings, the level of [^{14}C]IAAsp increased rapidly the first day and then declined; [^{14}C]IAA was rapidly metabolized and not detected after 12 hours.

One of the most frequently reported conjugates of IAA found in IAA-treated plants is IAAsp[3] (7), although the glycine (12), valine (12), alanine (12), and glutamic acid (12, 22, 27) conjugates have also been found. IAAsp is a major amino acid conjugate in IAA-treated mung bean (18), bean (28), pea (1, 19), and soybean (6) but has not been detected in mung bean as a natural conjugate (RH Hamilton, personal communication).

Recently it was hypothesized that amide-conjugated IAA may have a role in adventitious root formation in those species in which it is the predominant form of IAA (21). It was suggested that if auxin is added during the early stages of primordium development, then the level of amide-conjugated IAA would increase until the endogenous IAA could no longer support cell division in all root initial cells. At that point, amide-conjugated IAA would then be utilized as a source of free IAA and meristematic activity would be sustained resulting in maximum root formation. These hypotheses concerning the relationship between free and amide-conjugated auxin during rooting are consistent with the research which shows that exogenous auxin has its greatest promotive effect during early primordium development (13, 24, 25). Furthermore, in a recent study it was suggested that endogenous IAAsp accumulates during periods of low IAA demand and is utilized during periods of higher IAA demands (9).

In this study, IAAsp was unequivocally identified as the primary amino acid conjugate of IAA in IAA-treated mung bean cuttings. Changes in the levels of [^{14}C]IAAsp in the hypocotyl of cuttings treated with [^{14}C]IAA were then examined during adventitious root formation using labeled IAA.

MATERIALS AND METHODS

Plant Material. Mung beans from the same seed lot were surface-sterilized in 0.5% sodium hypochlorite (v/v) for 10 min, and then rinsed in tap water. The mung beans were germinated and grown as previously described (21). The seedlings were watered each day with tap water except on the fifth day after planting, when the beans were watered with a full strength Hoagland solution (14).

Metabolism of IAA. One hundred and thirty uniform cuttings with 3-cm hypocotyls were prepared from 10-d seedlings (5). Groups of 10 cuttings were placed at random into 13 sterilized shell vials containing 1 ml of 1 mM IAA in sterile distilled water (21). All cuttings then were placed back in the growth chamber and allowed to take up the solution under the photoperiod and temperature regime described above.

Extraction and Preliminary Purification of IAAsp. The plants were harvested 21 h after placement in the treatment solution. One hundred stems (14.1 g fresh weight; 270 mg dry weight), excluding the leaves and apical buds, were quartered and then immediately boiled in 150 ml of 100% methanol for 4 min. The methanol was decanted and the stems reextracted for 40 min in 150 ml methanol.

The extract was concentrated *in vacuo* at 40°C to the aqueous phase (6–7 ml). The aqueous extract was acidified (pH 2–3) with 3 N HCl and then partitioned four times against equal volumes of peroxide-free diethyl ether and then water-saturated *n*-butanol. The ether was concentrated to dryness under a stream of air and the residue resuspended in 250 μl 95% ethanol containing 50 μl diethyl ether. Both the butanol and aqueous phases were concentrated *in vacuo* at 50°C to less than 1 ml; 250 μl methanol was added to each concentrate to dissolve precipitates.

Aliquots from all three fractions and authentic standards were streaked on 20 × 20 cm Silica Gel G (0.50 mm) thin layers and developed for 15 cm in chloroform:methanol:28% ammonia (5:4:1). Metabolites of IAA were located by short wavelength UV light and the Salkowski reagent (26). Substances that cochromatographed with authentic IAAsp were eluted from the gel with 4 ml 90% methanol:water (v/v). The eluate was centrifuged (10,000*g*, 3 min), the supernatant concentrated *in vacuo* at 40°C to dryness, and the residue redissolved in 30 μl methanol.

Presence of IAA and IAAsp. Putative IAAsp was hydrolyzed to IAA and aspartate using methods adapted from Bandurski and Schulze (3, 4).

The presence of IAA and IAAsp then was determined by TLC.

[1] Contribution No. 112, Department of Horticulture, The Pennsylvania State University. Authorized for publication as paper No. 7662 in the journal series of the Pennsylvania Agriculture Experiment Station.

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[3] Abbreviations: IAAsp, indoleacetylaspartic acid; BHT, butylated hydroxytoluene; mlz, mass to charge ratio.

Aliquots of hydrolyzed and unhydrolyzed acidic ether-soluble substances along with authentic IAA and IAAsp were spotted on a 20 × 20 Silica Gel G thin layer (0.50 mm) and developed for 15 cm in chloroform:ethyl acetate:formic acid (5:4:1). Both IAA and IAAsp were localized by the Salkowski reagent. Aliquots of hydrolyzed and unhydrolyzed water-soluble substances along with authentic aspartate and glutamate were spotted on a similar Silica Gel G thin layer and developed to 15 cm in chloroform:methanol:formic acid (5:4:1); ninhydrin was used to detect the amino acids.

High Performance Liquid Chromatography. Putative IAAsp isolated from TLC was purified additionally on a Waters HPLC in preparation for GC-MS. The system consisted of a 30 cm × 7.8 mm μ-Bondapak C_{18} column (Waters Assoc.), pump (model 6000A), and injector (model U6K); it was equipped with a glassy carbon electrochemical detector (LC-4B; Bioanalytical Systems, West Lafayette, IN) operated at +0.850 V with a detection range of 50 nA/V. The mobile phase consisted of methanol:buffer (1:5, v/v) and the buffer was composed of 50 mM sodium perchlorate, 5 mM sodium acetate, and 1 mM sodium EDTA adjusted to pH 3.5 with NaOH. The flow rate was 1.3 ml/min. The fraction corresponding to authentic IAAsp was collected and concentrated *in vacuo* at 40°C to dryness. After the residue was redissolved in 40 μl HPLC-grade methanol, the putative IAAsp was methylated with ethereal diazomethane (11).

GC-MS. The identity of the putative IAAsp was confirmed using a type 9000B LKB Gas Chromatograph-Mass Spectrometer. Samples of putative and authentic bis-methyl IAAsp were injected into a 6 ft × 2 mm column containing 3% SP2250 on 80/100 Supelcoport (Supelco, Inc., Bellefonte, PA). The He flow rate was 23 ml/min and the injector temperature was 325°C. A temperature program of 250 to 300°C at 10°C/min was started upon injection. Mass spectra were recorded at an ionizing voltage of 70 eV with a source temperature of 250°C.

Changes in the Level of [^{14}C]IAAsp during Root Formation. Two hundred and fifty cuttings with 3-cm hypocotyls were prepared as before. Lots of 10 cuttings were selected at random and placed into each of 21 sterile shell vials containing 1 ml of treatment solution. The treatment solution was composed of 1 mM IAA containing 57.4 nCi [1-^{14}C]IAA (50 mCi/mmol; Amersham) and 1 mg/L boric acid. Prior to the experiment the [^{14}C]IAA was purified by descending paper chromatography developed with isopropanol:28% ammonia:water (8:1:1, v/v/v). The cuttings were placed in a growth chamber and allowed to take up the solution under the temperature and light regime described previously. The time at which the cuttings had completely taken up the solution was defined as the 0 h (all test solutions were taken up within 20 min of each other).

In order to estimate the actual amount of IAA taken up by the cuttings, the degree of [^{14}C]IAA decarboxylation was determined over the course of the 96-h experiment. Ten defoliated cuttings with 2-cm epicotyls were placed into each of four vials containing 1 ml of the treatment solution. Each vial was capped with foil to prevent evaporation. At 0 and 96 h, three 250-μl aliquots were removed from two vials for liquid scintillation counting.

Cuttings from three vials chosen at random were harvested at 0, 6, 12, 24, 36, 48, and 96 h. The epicotyls were excised from the hypocotyls at a point immediately below the cotyledonary node. The hypocotyls were placed in 20 ml boiling methanol (containing 2 mg BHT) for 4 min and then filtered. The tissue was ground in a mortar and reextracted in an additional 20 ml of methanol for 40 min in the dark. The extract was filtered and pooled with the original extract; three 250-μl aliquots were removed for counting.

After the extracts were concentrated *in vacuo* at 42°C (0.5–1.0 ml), the [^{14}C]IAA metabolites were analyzed by TLC. Aliquots (20–25 μl) from each sample were streaked on 5 × 20 cm Silica Gel G thin layers (0.50 mm) and developed in either chloroform:ethylacetate:formic acid (35:55:10, v/v/v) for [^{14}C]IAAsp analysis or chloroform:methanol:28% ammonia:water (5:4:1, v/v/v) for [^{14}C]IAA analysis. A basic solvent was required for IAA isolation because IAA was unstable after TLC in the acidic solvent. To determine the R_F values of IAA and IAAsp, authentic standards were run with a sample and located by short wavelength UV light and the Salkowski reagent. The regions containing ^{14}C corresponding to IAA and IAAsp were located by a Packard model 7201 Radiochromatogram Scanner (1.3 kV; FS = 300 or 1000 cpm; time constant = 10; 1% isobutane in helium with a flow of 280 ml/min) and scraped from the plates into scintillation vials containing 1 ml 95% ethanol. The amounts of IAA and IAAsp were calculated after correcting for background and quenching.

RESULTS

The major IAA metabolite detected was an acidic ether-soluble substance that chromatographed similarly to authentic IAAsp in TLC developed in basic (Table I) and acidic (Table II) solvents. Upon strong alkaline hydrolysis, the putative IAAsp yielded substances that had R_F values identical to IAA and aspartic acid released from authentic IAAsp after strong alkaline hydrolysis (Tables II and III). Furthermore, putative IAAsp purified by TLC co-eluted with authentic IAAsp in HPLC (Fig. 1, R_t = 26.5 min). The identify of the putative IAAsp was confirmed by combined GC-MS (Fig. 2).

Two other less abundant Salkowski-positive metabolites were

Table I. *Acidic Ether-Soluble IAA Metabolites in Mung Bean Stems*

Acidic-ether soluble IAA metabolites were isolated from the stems of mung bean cuttings harvested 21 h after treatment with 1 mM IAA. The substances present in this crude extract were separated on a Silica Gel G thin layer (0.50 mm) developed in chloroform:methanol:28% ammonia (5:4:1, v/v/v); detection was by short wavelength UV light and the Salkowski reagant.

Substance	R_F Region
Unknown No. 1	0.11–0.15
Unknown No. 2	0.19–0.23
IAA (authentic)	0.43–0.45
IAAsp (authentic)	0.11–0.16

Table II. *Analysis of Acidic-Ether Soluble IAA Metabolites Before and After Alkaline Hydrolysis*

The acidic ether-soluble substances isolated from the stems of mung bean cuttings 21 h after treatment with 1 mM IAA were analyzed by TLC (Silica Gel G, 0.50 mm; chloroform:methanol:formic acid, 5:4:1, v/v/v) before and after strong alkaline hydrolysis (7 N NaOH at 100°C for 3 h); detection was by short wavelength UV light and the Salkowski reagent.

	R_F Region
Substances—before hydrolysis	
Acidic ether-soluble fraction of crude extract	0.15–0.18
	0.23–0.29
	0.68–0.74
Putative IAA-aspartate	0.24–0.29
IAA-aspartate	0.22–0.31
Substances—after hydrolysis	
Acidic ether fraction of crude extract	0.56–0.58
IAA	0.54–0.59
Acidic ether-soluble substances of IAA-aspartate	0.56–0.59

Table III. *Amino Acids Present in Hydrolysates of Putative and Authentic IAAsp*

Putative IAAsp was isolated from the acidic ether-soluble fraction described in Table I. After authentic IAAsp and the putative IAAsp were subject to strong alkaline hydrolysis, the amino acids present in the H_2O fraction (after acid partitioning) were analyzed by TLC (Silica Gel G, 0.50 mm; chloroform:methanol:acetic acid, 5:4:1, v/v/v); amino acids were detected with ninhydrin.

Sample	R_F Region
Acidic ether-soluble fraction of crude extract, unhydrolyzed	0.82–0.89
Same sample as above plus 0.75 μg aspartate and glutamate	0.61–0.66 0.68–0.75 0.82–0.89
H_2O fraction from hydrolyzed putative IAA-aspartate	0.51–0.58
H_2O fraction from hydrolyzed authentic IAA-aspartate	0.51–0.58

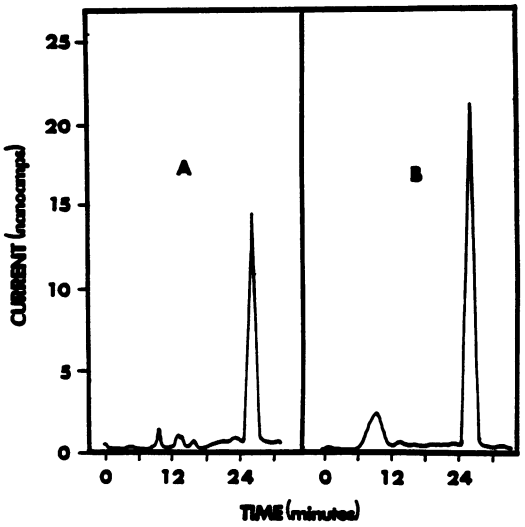

FIG. 1. HPLC elution profiles of (A) putative IAAsp isolated from mung bean stem cuttings 21 h after treatment with 10^{-3} M IAA and (B) the same extract plus authentic IAAsp. Column: Waters 30 cm × 7.8 mm μ-Bondpak C_{18}; mobile phase: 20% methanol in 50 mM sodium perchlorate, 5 mM sodium acetate, 1 mM sodium EDTA, at pH 3.5; flow rate: 1.3 ml/min; electrochemical detection at +0.850 V with 50 nA/V full scale.

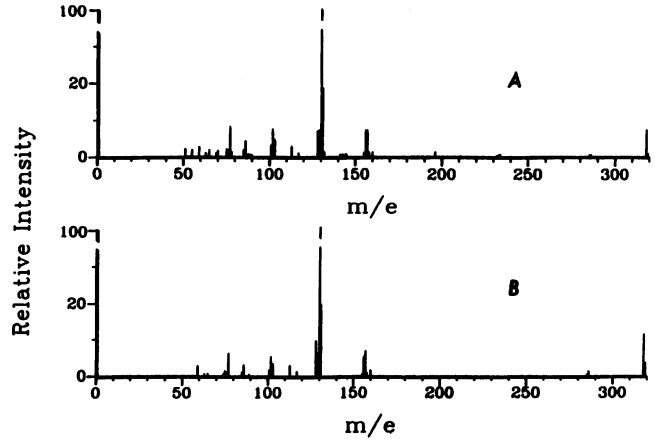

FIG. 2. The 70 eV electron mass spectra of (A) a methylated sample of putative IAAsp isolated from mung bean cuttings treated with 10^{-3} M IAA, and (B) authentic *bis*-methyl-IAAsp. Ions characteristic of *bis*-methyl-IAAsp are m/z[4] 318 (m^+), 286, 157, and 130 (base peak).

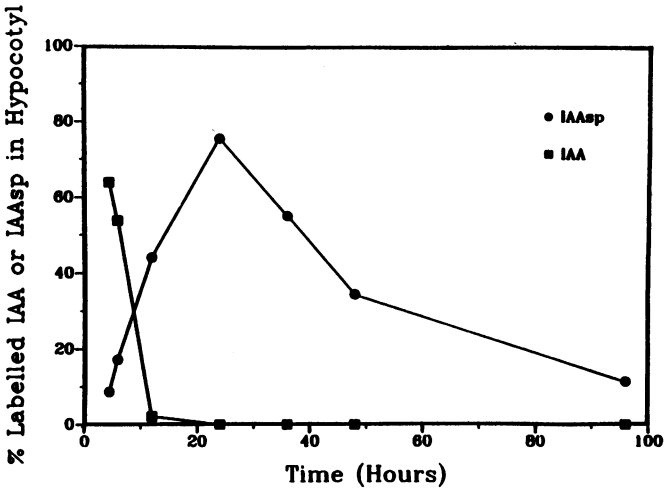

FIG. 3. Percentages of [^{14}C]IAAsp and [^{14}C]IAA in the hypocotyls of mung bean stem cuttings during adventitious root formation. For each time examined, 3 sets of 10 cuttings received a 1-ml pulse of 10^{-3} M IAA containing 57.4 nCi [1-^{14}C]IAA (50 mCi/mmol). Values represent the percentage of ^{14}C as [^{14}C]IAAsp and [^{14}C]IAA.

detected in the acidic ether-soluble fraction also. One was detected by TLC in the metabolism study (R_F 0.19–0.23, Table I) and the other by TLC in the time course study (R_F 0.04 in chloroform:ethylacetate:formic acid, 35:55:10, v/v/v). These may have been the same substance. In the time course study a substantial amount of a second labeled substance (R_F 0.27, basic solvent) was detected, however it gave a negative Salkowski test.

Time Course Study. Nearly 78% of the total IAA in the treatment solution was taken up by the cuttings. The IAA was rapidly metabolized and reduced to undetectable levels between 12 and 24 h (Fig. 3). As the percentage of ^{14}C in IAA decreased to zero by 24 h, the percentage of extractable ^{14}C in the hypocotyl as [^{14}C]IAAsp increased sharply to a peak during the first day and then steadily declined (Fig. 3).

DISCUSSION

This study is one of the few in which IAAsp has been unequivocally identified as a metabolite. IAAsp was the primary conjugate in mung bean cuttings treated with IAA. It was reported to be a major metabolite in mung bean (18) and other plants (8, 20, 22, 30) but was only positively identified in *Parthenocissus* crown gall (12), pine (23), and pea root nodules (2).

The other two Salkowski-positive low R_F TLC components may have been the same substance. Neither was an ester conjugate of IAA as [^{14}C]indoleacetamide was not detected in any of the extracts when samples were analyzed by paper chromatography developed in isopropanol:28% ammonia:water (10:1:1, v/v/v) (results not shown) (29).

During adventitious root formation, the percentage of IAAsp in the hypocotyl increased during the first day, the time of the first cell division, and then decreased steadily as the primordium developed and elongated. This observation provides additional evidence for the hypothesis that amide-conjugated IAA functions as a source of IAA during the later stages of rooting when IAA supply is low and IAA demand is high (21). Research on the effect of timing of auxin application on root formation further supports this hypothesis. Auxin enhanced rooting the most when applied early in root primordium development in mung bean

(16), *Vigna catjang* (24, 25), and brittle willow (13).

If a build-up of amide-conjugated auxin during the early stages of root development is essential for optimum rooting, as suggested above, then any factor which inhibits this build-up should inhibit rooting. This is the case with kinetin, which inhibits IAAsp formation in etiolated mung bean hypocotyl segments (18) and rooting in mung bean cuttings (5). Exogenous cytokinin inhibits root formation in pea (17) and bean (15) as well. In all three cases amide-conjugated IAA is the predominant form of endogenous IAA (4, 21). Furthermore, Eriksen (10) found that in decapitated pea cuttings the inhibitory influence of applied cytokinins gradually disappeared during the later stages of root initiation, a period during which amide conjugate formation apparently is not important (13, 21, 24, 25).

In conclusion, the results of this research are consistent with the idea that amide-conjugated auxins may regulate adventitious root formation, especially in those species in which it is the major form of auxin.

LITERATURE CITED

1. Andrea W, NE Good 1955 The formation of indole-acetylaspartic acid in pea seedlings. Plant Physiol 30: 380–382
2. Badenoch-Jones J, RE Summons, BG Rolfe, DS Letham 1984 Phytohormones, *Rhizobium* mutants, and nodulation in legumes. IV. Auxin metabolites in pea root nodules. J Plant Growth Regul 3: 23–29
3. Bandurski RS, A Schulze 1974 Concentrations of indole-3-acetic acid and its esters in *Avena* and *Zea*. Plant Physiol 54: 257–262
4. Bandurski RS, A Schulze 1977 Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiol 60: 211–213
5. Blazich FA 1977 Adventitious root initiation in cuttings of *Phaseolus aureus* Roxb. PhD thesis. The Pennsylvania State University, University Park
6. Cohen J 1982 Identification and quantitative analysis of indole-3-acetyl-L-aspartate from seeds of *Glycine max* L. Plant Physiol 70: 749–753
7. Cohen JD, RS Bandurski 1982 Chemistry and physiology of the bound auxins. Annu Rev Plant Physiol 33: 403–430
8. Davies PJ 1972 The fate of exogenously applied indole-acetic acid in light grown stems. Physiol Plant 27: 262–270
9. Elkinawy M 1985 Metabolic significance of indoleacetyl-aspartic acid in developing cotton leaves. Physiol Plant 63: 370–374
10. Eriksen EN 1974 Root formation in pea cuttings. III. The influence of cytokinin at different developmental stages. Physiol Plant 30: 163–167
11. Fales, HM, TM Jaouni 1973 Simple device for preparing ethereal diazomethane without resorting to codistillation. Anal Chem 45: 2302–2303
12. Feung C-S, RH Hamilton, RO Mumma 1976 Metabolism of indole-3-acetic acid. III. Identification of metabolites isolated from crown gall tissue. Plant Physiol 58: 666–669
13. Haissig BE 1970 Influence of indole-3-acetic acid on adventitious root primordia of brittle willow. Planta 95: 27–35
14. Hoagland DR, DI Arnon 1950 The water-culture method for growing plants without soil. California Agriculture Experiment Station Circular 347, Berkeley
15. Humphries EC 1960 Inhibition of root development on petioles and hypocotyls of dwarf bean (*Phaseolus vulgaris*) by kinetin. Physiol Plant 13: 659–663
16. Jarvis BC, AHN Ali, AI Shaheed 1983 Auxin and boron in relation to the rooting response and ageing of mung bean cuttings. New Phytol 95: 509–518
17. Kaminek M 1967 Root formation in pea stem sections and its inhibition kinetin, ethionine, and chloramphenicol. Biol Plant 9: 86–91
18. Lau O-L, SF Yang 1973 Mechanism of a synergistic effect of kinetin on auxin-induced ethylene production. Suppression of auxin conjugation. Plant Physiol 51: 1011–1014
19. Law DM, RH Hamilton 1982 A rapid isotope dilution method for analysis of indole-3-acetic acid and indoleacetyl aspartic acid from small amounts of plant tissue. Biochem Biophys Res Commun 106: 1035–1041
20. Morris DA, RE Briant, PG Thomson 1969 The transport and metabolism of ^{14}C-labelled indoleacetic acid in intact pea seedlings. Planta 89: 178–197
21. Norcini JG, CW Heuser, RH Hamilton 1985 Changes in free and conjugated indole-3-acetic acid during initiation and early development of adventitious roots in mung bean. J. Am Soc Hortic Sci 110: 528–533
22. Purves WK, SM Hollenberg 1982 Metabolism of exogenous indoleacetic acid to its amide conjugates in *Cucumis sativus* L. Plant Physiol 70: 283–286
23. Riov J, HE Gottlieb 1980 Metabolism of auxin in pine tissues: Indole-3-acetic acid conjugation. Physiol Plant 50: 347–352
24. Sircar PK, SK Chatterjee 1973 Physiological and biochemical control of meristemization and adventitious root formation in *Vigna* hypocotyl cuttings. The Plant Propagator 19(1): 17–26
25. Sircar PK, SK Chatterjee 1967 Physiological and biochemical changes associated with adventitious root formation in *Vigna* hypocotyl cuttings. III. Effects of indole acetic acid. The Plant Propagator 22(3): 3–8
26. Tang YW, J Bonner 1947 The enzymatic inactivation of indoleacetic acid. Arch Biochem 13: 11–25
27. Thurman DA, HE Street 1962 Metabolism of some indole auxins in excised tomato roots. J Exp Bot 13: 369–377
28. Tillberg E 1974 Occurrence of indol-3yl-aspartic acid in light and dark-grown bean seedlings, (*Phaseolus vulgaris*). Physiol Plant 31: 271–274
29. Zenk MH 1961 1-(Indole 3-acetyl)-D-glucose, a new compound in the metabolism of indole-3-acetic acid in plants. Nature 191: 493–494
30. Zenk MH 1964 Isolation, biosynthesis, and function of indoleacetic acid conjugates. *In* JP Nitsch, ed, Regulateurs Naturels de la Crossance Vegetale. CNRS, Paris, pp 241–249