J Plant Growth Regul (1992) 11:19–28

Journal of
Plant
Growth
Regulation

© 1992 Springer-Verlag New York Inc.

N-(Indol-3-ylacetyl)Amino Acids as Sources of Auxin in Plant Tissue Culture

Volker Magnus,[1] Biljana Nigović,[1] Roger P. Hangarter,[2] and Norman E. Good[3]

[1]Ruđer Bošković Institute, P.O. Box 1016, YU-41001 Zagreb, Yugoslavia; [2]Department of Plant Biology, Ohio State University, Columbus, Ohio 43210-1293, USA; and [3]Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824-1312, USA

Received April 26, 1991; accepted October 29, 1991

Abstract. N-(Indol-3-ylacetyl) derivatives (IAA conjugates) of aliphatic amino acids with a two- to six-carbon backbone including α-L-amino acids, ω-amino acids, and the α,ω-diamino acids ornithine and lysine were prepared, chemically characterized, and tested as sources of auxin in plant tissue culture. Stimulation of unorganized growth in *Solanum nigrum* L. callus and callus induction and developmental effects in tomato (*Lycopersicon esculentum* Mill. cv. Marglobe) hypocotyl explants were studied systematically. Relative auxin activities were estimated by comparing physiologically equivalent concentrations, in the optimal and suboptimal range, of the individual IAA conjugates. While the growth-promoting properties of some of the conjugates were species-dependent, those containing straight-chain two- to four-carbon α-L-amino acid moieties were generally up to 100 times more active than those of their five- to six-carbon homologues. Branching of the amino acid backbone at C-β (norvaline vs. valine and norleucine vs. isoleucine) and C-γ (norleucine vs. leucine) had a minor effect, but substitution of H-α by a methyl group (α-amino-L-butyric vs. α-aminoisobutyric acids) almost completely blocked growth-promoting activity. IAA conjugates of ω-amino acids were, in most cases, nearly as active as those of their α-amino-L-isomers. Among the conjugates of α,ω-diamino acids N_δ-(IAA) ornithine was less active than N_ϵ-(IAA)lysine. The activity of N_α-(IAA)lysine was less than for the ε-(IAA) isomer, and that of N_α,N_ϵ-(IAA)$_2$-lysine was different in tomato and *Solanum nigrum*. The L-alanine and ε-lysine conjugates were also found to be useful for induction and development of *Oenothera* leaf callus and in tomato cell-suspension culture, two systems which require highly active sources of auxin.

N-(Indol-3-ylacetyl)amino acids, in particular those containing aspartic and glutamic acid moieties, occur naturally in plants (Andersson and Sandberg 1982, Cohen 1982, Epstein et al. 1986, Percival 1986, Sembdner et al. 1980, Sonner and Purves 1985), and have been proposed to function as auxin storage pools which are converted to free IAA as required during plant growth and development. The plant parasite *Pseudomonas savastanoi* (Smith) Stevens produces εIAA-Lys[1] and its N_α-(acetyl) derivative which may play a role in pathogenesis (Evidente et al. 1986, Hutzinger and Kosuge 1968). A number of N-(IAA) amino acids have already been tested as sources of auxin in plant tissue culture (e.g., Feung et al. 1977, Hangarter et al. 1980). Their overall growth-sustaining activities varied within wide limits and specific developmental effects were noticed in some cases (Hangarter et al. 1980). Generally, interest has been focused on IAA conjugates of the protein amino acids, which cover a heterogeneous array of structural features. Series of systematically modified conjugates are, however, needed to establish structure–activity correlations which can help to design more active compounds, or more selective ones in terms of developmental effects. Here we describe the preparation of additional IAA conjugates with homologous mono- and diamino acids and compare their biological activities in several tissue culture systems. The molecular structures of the respective compounds have been studied independently (Duddeck et al. 1989, Kojić-Prodić et al. 1991). εIAA-Lys is introduced as one of the most active promoters of callus growth.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indol-3-ylacetic acid; the abbreviations for N-(indol-3-ylacetyl)amino acids are listed in Table 1.

Materials and Methods

General

Commercial chemicals of the highest available purity were employed unless indicated otherwise. The use of peroxide-free solvents in the syntheses of *N*-(IAA)amino acids is essential. The conjugates and intermediates in their syntheses were stored in the dark, at refrigerator temperature.

Optical rotations were measured with an automatic polarimeter (Anglia Instruments Ltd., model AA-10). Melting points (uncorrected) were determined in open capillaries (Culatti apparatus) or in thin layers inserted between microcover glasses (Fisher-Johns apparatus); the two methods gave identical results. NMR spectra were taken on JEOL FX 100 or FX 90 spectrometers operating at 100 or, respectively, 90 MHz for ^{1}H, and 25 or 22.5 MHz for ^{13}C.

Thin-layer chromatography was performed on silica gel GF (Merck) coated on glass plates using the following solvents: A, dichloromethane:methanol:acetic acid (90:10:1, vol/vol/vol); B, 2-propanol:ethyl acetate:aqueous ammonia (35:45:20, vol/vol/vol); C, 1-butanol:acetic acid:water (80:3:17, vol/vol/vol). Detection was by UV fluorescence, Ehmann's reagent (for indoles; Ehmann 1977), and Ninhydrin (0.2% solution in 95% ethanol; for free amino groups). IAA-D-ala and its L-isomer were separated on a column (5 × 0.46 cm i.d.) of Chiralpak WH (Daicel Chemical Industries, Ltd., Japan) operated at a temperature of 50°C and eluted with 1 ml/min of 0.5 mM $CuSO_4$. The effluent was monitored with a fluorescence detector (Shimadzu RF-535, Xe lamp) using excitation at 280 nm and measuring at 360 nm.

Preparation of N-*(IAA)Amino Acids: General Procedure*

IAA-Gly was prepared by dicyclohexylcarbodiimide-mediated condensation of IAA and glycine benzyl ester, followed by removal of the protective group by short alkaline hydrolysis (Good 1956; N. E. Good, unpublished). εIAA-Lys was synthesized according to Hutzinger and Kosuge (1968). The same method was used to prepare δIAA-Orn. Conjugates of the ω-amino acids were obtained by aminolysis of *N*-(indol-3-ylacetoxy)succinimide, a method originally devised for the synthesis of IAA-εAhx (Fuchs et al. 1971). The remaining amino acid conjugates, if not stated otherwise below, were prepared by the mixed-anhydride method as described in detail by Wieland and Hörlein (1955) and by Hangarter et al. (1980). Briefly, ethyl (indol-3-ylacetoxy)formate was prepared from equimolar quantities of IAA, ethyl chloroformate, and redistilled dry triethylamine in dry tetrahydrofurane, at −10°C. As soon as the reaction was complete, an equimolar amount of the amino acid, converted immediately before use to its potassium salt by treatment with the stoichiometric quantity of KOH, was added as an aqueous solution, at −5°C. The mixture was kept at this temperature for 10 min and then left to warm up to room temperature.

The neutral to slightly alkaline reaction mixtures obtained in the succinimide ester and mixed-anhydride methods were partitioned against ether to remove byproducts. In most cases, the amino acid conjugates were then crystallized on acidification of the aqueous phase, after removing organic solvent residues by a stream of nitrogen. Otherwise, the conjugates were extracted into ethyl acetate. Compounds which did not crystallize on evaporation of the dried (Na_2SO_4 sicc.) extracts were passed through a column (60 × 2.5 cm) of Sephadex LH-20 eluted with 2-propanol:water (1:1, vol/vol). The same column was used for purification of εIAA-Lys and δIAA-Orn in their hydrochloride forms. These conjugates precipitated on neutralization of the concentrated effluents. Final purification was, in all cases, by repeated crystallization (see Table 1 for solvents).

N_{α}-*(IAA)-L-Lysine*

N_{ϵ}-(Phthalimido)-L-lysine (Nefkens et al. 1960) was condensed with IAA by the mixed-anhydride method as above. The crude product was extracted with chloroform (removes contaminant IAA) and recrystallized from ethyl acetate to yield white crystals with a melting point (mp) of 173–174°C and homogeneous by TLC (R_F = 0.17; solvent A). The ε-phthaloyl group was removed by boiling (4 h) with hydrazine acetate in methanol solution (Schwyzer et al. 1963) and the deprotected product was passed through a column of Bio-Rex 70 (H^{+}-form) eluting with water adjusted to pH 6.5 with ammonia. The fractions containing αIAA-Lys were evaporated to dryness, redissolved in a minimal amount of warm (50°C) water, and cooled to 0°C. The precipitate (impurities) was removed. Concentration of the aqueous solution and addition of a 10-fold amount of acetone afforded the IAA conjugate, mp 173–175°C, R_F = 0.13 (solvent C). The product did not contain indolic contaminants detectable by TLC. Unfavorable solubility properties prevented rigorous purification and characterization.

N_{α},N_{ϵ}-*Bis-(IAA)-L-Lysine*

L-Lysine monohydrochloride (1.83 g, 10 mmol) suspended in a buffer containing K_2CO_3 (1 M) and $KHCO_3$ to pH 9.9 (overall volume: 30 ml) was condensed with a threefold excess of IAA by the mixed-anhydride method. Additional buffer (together approximately 30 ml) was added as the reaction proceeded to keep the pH constant. The crude product mixture (5.9 g) was extracted with ether at pH 2.5 and aliquots (together 2.5 g) were purified by preparative TLC in two steps using solvent B and dichloromethane:methanol:acetic acid (75:20:5, vol/vol/vol) for development. The zone containing the conjugate was eluted with methanol which was treated with charcoal and filtered. The filtrate was evaporated and recrystallized from water to yield the pure title compound (282 mg, 14%).

N-*(IAA) α-Aminoisobutyric Acid*

While the title compound may be prepared by the mixed-anhydride method in about 2% yield, attempts to proceed via *N*-(indol-3-ylacetoxy)succinimide were unsuccessful. The following procedure was finally adopted (Wang et al. 1981): to a mixture of α-aminoisobutyric acid methyl ester hydrochloride (1.84 g, 12 mmol) prepared according to Klieger and Gibian (1961), redistilled dry triethylamine (1.64 ml, 11.8 mmol), IAA (2.10 g, 12 mmol), and 4-dimethylaminopyridine (0.24 g, 2 mmol) in dry tetrahydrofurane (200 ml), and dicyclohexylcarbodiimide (2.47 g, 12 mmol) were added at −10°C. After stirring at −10°C for 1 h and at room temperature for 24 h, dicyclohexylurea was removed by filtration. The filtrate was diluted with ethyl acetate

Table 1. Physical constants of the *N*-(indol-3-ylacetyl)amino acids used in this study.

Compound		Solvent for crystalization[a]	Melting point (°C)	$[\alpha]_D^{25}$ (c = 2 g/100 ml)
Full name	Abbreviation			
N-(Indol-3-ylacetyl)glycine[b]	IAA-Gly	I	86–87	—
N-(Indol-3-ylacetyl)-L-alanine[b]	IAA-Ala	IV	134–135	−14.4[od]
N-(Indol-3-ylacetyl)-D-alanine[b]	IAA-D-Ala	I	135–136	+13.8[od]
N-(Indol-3-ylacetyl)-α-amino-L-butyric acid	IAA-αAbu	II	189	−1.4[od]
N-(Indol-3-ylacetyl)-α-aminoisobutyric acid	IAA-αAib	I	188	—
N-(Indol-3-ylacetyl)-L-norvaline	IAA-Nva	III	181–183	−6.6[od]
N-(Indol-3-ylacetyl)-L-valine[b]	IAA-Val	III	185–187	+10.3[od]
N-(Indol-3-ylacetyl)-L-norleucine	IAA-Nle	III	138–139	±0.0[od,i]
N-(Indol-3-ylacetyl)-L-leucine[b]	IAA-Leu	I	183–184	−19.2[od]
N-(Indol-3-ylacetyl)-L-isoleucine	IAA-Ile	III	180–182	+15.9[od]
N-(Indol-3-ylacetyl)-β-alanine	IAA-βAla	II	204–205	—
N-(Indol-3-ylacetyl)-γ-aminobutyric acid	IAA-γAbu	VI	89–90	—
N-(Indol-3-ylacetyl)-δ-aminovaleric acid	IAA-δAva	V	120–122	—
N-(Indol-3-ylacetyl)-ε-aminohexanoic acid	IAA-εAhx	VI	103–105	—
N_ϵ-(Indol-3-ylacetyl)-L-lysine	εIAA-Lys	I	240–245[c]	+18.4[oe]
N_ϵ-(Indol-3-ylacetyl)-D-lysine	εIAA-D-Lys	VII	234–235	ND[g]
N_α-(Indol-3-ylacetyl)-L-lysine	αIAA-Lys	VII	173–175	ND[g]
N_α,N_ϵ-Bis(Indol-3-ylacetyl)-L-lysine	$(IAA)_2$-Lys	I	155–160	−3.8[of]
N_δ-(Indol-3-ylacetyl)-L-ornithine	δIAA-Orn	I	234–235	+21.2[oe]

R_F values: solvent A: 0.00 (conjugates containing free amino groups), 0.10 [IAA-Gly, $(IAA)_2$-Lys], 0.35 (IAA-βAla, IAA-γAbu), 0.40 (IAA-δAva), 0.47 (IAA-εAhx), 0.25 ± 0.05 (other conjugates), 0.60 (free IAA); solvent B: 0.34 (δIAA-Orn, εIAA-Lys), 0.50 [$(IAA)_2$-Lys], 0.40 ± 0.05 (other conjugates and free IAA; αIAA-Lys not examined); solvent C: 0.13 (αIAA-Lys), 0.25 (δIAA-Orn, εIAA-Lys), 0.50 (IAA-Gly), 0.53 [$(IAA)_2$-Lys], 0.61 (IAA-jAla), 0.77 (IAA-εAhx), 0.70 ± 0.05 (other conjugates), 0.82 (free IAA).

[a] Solvents for crystallization: I, water; II, 40% (vol/vol) aqueous 2-propanol; III, 30% (vol/vol) aqueous 2-propanol; IV, 10% (vol/vol) aqueous 2-propanol; V, ethyl acetate:benzene (3:1, vol/vol); VI, ethyl acetate:benzene (1:1, vol/vol); VII, precipitated from aqueous solution with acetone. In the final recrystallization 2-propanol and benzene should be replaced by ethanol and cyclohexane which are more readily released from the crystals by drying.

[b] Melting points reported by Wieland and Hörlein (1955): IAA-Gly, 86–87°C; IAA-DL-Ala, 162–163°C; IAA-D-Ala, 136–137°C; IAA-DL-Val, 176–177°C; IAA-DL-Leu, 173–174°C.

[c] Refers to sample used in tissue culture experiments. Extensive drying (7×10^{-4} Pa, 100°C, 5 days) increased the melting point to 256–257°C which is close to the value given by Hutzinger and Kosuge (1968) (i.e., 259–261°C).

[d] In 95% ethanol.

[e] In 2 N HCl.

[f] In dimethylformamide.

[g] ND, not determined.

[i] Refer to text for comments.

(200 ml) and partitioned against 4% H_3PO_4 saturated with NaCl (4 × 50 ml), 5% $NaHCO_3$ (4 × 50 ml), and a saturated NaCl solution (2 × 10 ml), in this order. The residue obtained on evaporation of the dried (Na_2SO_4 sicc.) organic phase was dissolved in boiling tetrahydrofurane (30 ml). Precipitation with diethylether (60 ml) afforded 2.34 g (71%, mp 156–158°C) of crystalline IAA-αAib methyl ester sufficiently pure (TLC, solvent A, Rf = 0.5) for preparation of the title compound. The mother liquors were passed through a column (56 × 2.5 cm) of silica gel H (56 g) mixed with celite (40 g), eluted with dichloromethane:methanol (9:1, vol/vol) to yield a further 158 mg of the ester which was used for preparation of the analytical sample, as it was less contaminated by dicyclohexylurea. Further purification was by crystallization from tetrahydrofurane:diethylether, preparative TLC (silica gel PF, dichloromethane:methanol, 9:1, vol/vol), and further crystallization from dichloromethane:petrol and from methanol, to yield white needles, mp 160–162°C. Analysis: Calculated for $C_{15}H_{18}N_2O_3$: C, 65.68; H, 6.61; N, 10.21 %. Found: C, 65.91; H, 6.75; N, 10.23 %. ^{1}H NMR [$(CD_3)_2SO$]: δ; 10.93 (broad s, 1 H, indole NH); 7.69–7.00 (m, 5 H, indole ring); 3.62 (s, 2 H, $ArCH_2$); 8.48 (s, 1 H, amide NH); 1.47 (s, 6 H, C-CH_3); 3.64 (s, 3 H, OCH_3) ppm. ^{13}C NMR [$(CD_3)_2SO$]: δ; 136.23, 127.32, 123.59, 121.00, 118.74, 118.29, 111.34, 108.92 (indole ring); 174.55 (amide CO); 170.37 (ester CO); 55.02 (C-NHCO), 51.75 (OCH_3), 32.34 ($ArCH_2$); 25.00 (C-CH_3) ppm.

An aliquot of the above ester (1.37 g, 5 mmol) was hydrolyzed overnight with KOH (5 g) in 95% ethanol (100 ml) at room temperature (Findlay and Dougherty 1948). Water (100 ml) was added to the hydrolyzate, and the alcohol and unreacted ester were extracted with diethylether (5 × 100 ml). The aqueous phase was acidified to pH 2.2 and residues of organic solvents were removed by a stream of nitrogen. The title compound crystallized on seeding at 3°C. (Seed crystals were from a previous preparation in which the conjugate was extracted with ethyl acetate and the extract evaporated.) A further crop of crystals was obtained from the concentrated (40 ml) mother liquors. Further purification by two crystallizations from 5% (vol/vol) aqueous ethanol afforded 823 mg (63%) of the pure title compound as long

Table 2. Analytical data for *N*-(indol-3-ylacetyl)amino acids.

	Calculated %			Found %		
Compound[a]	C	H	N	C	H	N
IAA-αAbu	64.60	6.20	10.76	64.81	6.34	10.86
IAA-αAib	64.60	6.20	10.76	64.85	6.23	10.79
IAA-Nva	65.68	6.61	10.21	65.66	6.88	10.30
IAA-Val	65.68	6.61	10.21	65.42	6.66	10.18
IAA-Nle	65.65	6.99	9.72	66.43	6.71	9.89
IAA-Leu	66.65	6.99	9.72	66.48	6.98	10.00
IAA-Ile	66.65	6.99	9.72	66.89	7.12	9.70
IAA-βAla	63.40	5.73	11.38	63.23	5.88	11.52

[a] Abbreviations are defined in Table 1.

pink needles. Physicochemical and analytical data are presented in Tables 1–3.

Nutrient Media

Tomato hypocotyl sections, *Oenothera* leaf explants, and *Solanum nigrum* callus were grown on a medium containing the mineral salts of Murashige and Skoog (1962) and the following additives (mg/l): thiamine · HCl (1), pyridoxine · HCl (0.5), nicotinic acid (0.5), *myo*-inositol (100), sucrose (30,000), agar (9000), as well as 2-(*N*-morpholino)ethanesulfonic acid (976) and sufficient KOH solution to adjust the pH to 6.0 (before the addition of agar). Benzyladenine was added for tomato (10 μM) and *Oenothera* (5 μM), and kinetin (1.2 μM) for *Solanum nigrum*. The sources of auxin specified below were generally tested at concentrations from 0.01–1000 μM. The liquid medium for tomato cell-suspension culture contained (mg/l): $Ca(NO_3)_2 \cdot 4\,H_2O$ (242), KNO_3 (85), KCl (61), $MgSO_4 \cdot 7\,H_2O$ (42), KH_2PO_4 (20), $FeCl_3 \cdot 6\,H_2O$ (25), sucrose (20,000), 70%-ethanol-soluble yeast extract (12.5 ml/l of medium), benzyladenine and/or a source of auxin as specified below, and NaOH solution to adjust the pH to 6.0. Seventy percent ethanol-soluble yeast extract was prepared by dissolving 100 g of Bacto yeast extract (Difco) in water (300 ml), adding ethanol (700 ml), cooling (3°C), and filtering. The filtrate was evaporated in vacuo and the residue dissolved in water (1 l). The solution was frozen and stored at −20°C.

Media were sterilized at 120°C for 15 (100 ml batches) to 20 (250 and 500 ml batches) minutes. Agar-containing media were then dispensed into 9 cm (30 ml) or 4.5 cm (15 ml) disposable polystyrene Petri dishes.

Solanum nigrum *Callus*

The callus was grown in the dark at 27°C. Stock cultures were maintained in the presence of 100 μM IAA and transferred to fresh medium every 4 weeks. The experiments with IAA conjugates were performed in 9 cm Petri dishes (sealed with parafilm after inoculation), using four explants per dish and five replicates per treatment. Relatively large (0.3–0.5 g) inocula were necessary to obtain uniform callus growth. Callus weights, recorded 6 weeks after planting, were directly used to compare the growth-promoting activities of the IAA conjugates tested.

Tomato Hypocotyl Explants

Seeds of *Lycopersicon esculentum* Mill. cv. Marglobe were sterilized (20 min) in 0.5% sodium hypochlorite solution, containing 0.01% of sodium dodecyl sulfate, rinsed with sterile water, and planted on water agar (0.8%) containing sucrose (0.5%). After germination in the dark at 27°C for 6 days, the hypocotyls were cut into sections about 5 mm in length (weight: 2–3 mg), excluding the apical and basal 5 mm. Test media were prepared in 4.5 cm Petri dishes, using four explants per dish and five replicates per treatment. The Petri dishes were sealed with Parafilm and kept at 22°C under continuous illumination (GE Deluxe "cool-white" fluorescent tubes), applying 40–60 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetically active radiation (400–700 nm) at the level of the cultures. The relative growth-promoting activity of an IAA conjugate was assessed visually 6 weeks after planting. Explants of the same age and lot planted on media containing a series of dilutions of IAA-alanine were used as the reference. In the same fashion, the growth-promoting activity of 2,4-D was compared to that of the more active IAA conjugates.

Oenothera *Leaf Explants*

Seeds of *Oenothera johansen* L. (kindly supplied by Dr. B. Sears) were planted, seedlings were grown, and leaf explants were maintained under essentially the same conditions as described for tomato hypocotyl explants. Germination was in the light, however, and seed sterilization required presoaking in water and treatment with hypochlorite-dodecyl sulfate for 40 min. Several subtransfers were necessary after planting to remove contaminated seedlings. When the leaves of the young plants were several centimeters long, they were divided into sections, about 5 mm in length, which were used as the explants. Callus induction and development were assessed visually, during a period of 2 months after planting the leaf explants.

Tomato Cell Suspension Culture

The tomato cell line used was obtained through the courtesy of Dr. D. T. A. Lamport. It had been maintained in the medium specified above, supplemented with 9 μM 2,4-D, and transferred to fresh medium in about 10-day intervals. Experiments were performed in 100–500 ml batches, to which 10–20% by volume of

Table 3. ^{13}C-NMR chemical shift values[a] (δ; ppm) for *N*-(indol-3-ylacetyl) amino acids [in $(CD_3)_2SO$; c = 0.4 mmol/ml] used in this study.

	Chemical shift (δ, ppm)							
	Amino acid moiety						IAA moiety	
Compound[b]	COOH	C_α	C_β	C_γ	C_δ	C_ϵ	CH_2	CONH
IAA-Gly	171.4	41.1					32.6	171.3
IAA-Ala	174.2	47.8	17.5				32.5	170.5
IAA-αAbu	173.6	53.4	24.7	10.6			32.5	171.0
IAA-αAib	175.8	55.0	25.1(2C)				32.6	170.3
IAA-Nva	173.8	51.7	33.4	18.9	13.7		32.5	170.8
IAA-Val	173.1	57.3	30.1	19.4 18.2			32.4	171.0
IAA-Nle	174.0	52.0	31.0	27.7	21.8	13.9	32.5	170.9
IAA-Leu	174.2	50.5	40.2	24.5	23.1 21.5		32.5	170.7
IAA-Ile	173.1	56.4	36.7	24.9 15.8	11.5		32.4	170.9
IAA-βAla	173.1	34.1	35.2				32.8	171.1
IAA-γAbu	174.5	31.3	24.9	37.8			33.0	171.2
IAA-δAva	174.4	33.5	22.1	28.9	38.5		32.9	170.6
IAA-εAhx	174.5	33.9	24.5	26.2	33.9	38.8	33.0	170.6
εIAA-Lys[c,d]	175.2	56.2	29.8	23.7	31.8	40.4	34.2	174.7
$(IAA)_2$-Lys[d]	173.8	52.1	29.0	23.1	31.0	NO[e]	32.5(α) 32.9(ε)	170.5 170.8
δIAA-Orn[c,d]	175.2	55.9	29.6	26.2	40.3		34.2	175.0

For most compounds the indole ring-carbon resonances appeared at the following δ-values (in ppm) ± 0.2: 123.7 (C-2); 108.9 (C-3); 127.2 (C-3a); 118.7 (C-4); 118.3 (C-5); 121.0 (C-6); 111.3 (C-7); 136.1 (C-7a). *Exceptions:* εIAA-Lys and δIAA-Orn; 119.7, 120.2, 122.7, 125.7 (C-2, C-4, C-5, C-6); 109.3 (C-3); 128.3 (C-3a); 112.9 (C-7); 137.5 (C-7a; under solvent signal for εIAA-Lys).

[a] The central peak of the $(CD_3)_2SO$ resonance (δ = 39.6 ppm) was used as the reference.

[b] Abbreviations are listed in Table 1.

[c] In pyridine-d_5:D_2O (1:1, vol/vol) containing $(CD_3)_2SO$ as a reference.

[d] c = 0.2 mmol/ml.

[e] NO, Not observed (under solvent signal).

a 10-day-old culture were added. The larger inoculum gave more uniform growth, but only slightly increased cell yields. The solutions were incubated, in Erlenmeyer flasks of about fourfold volume, on a gyratory shaker (about 100 rpm) at 27°C. Ten to 12 days after inoculation, samples were withdrawn (in duplicate) with a wide-mouth pipette and centrifuged in graduated test tubes, in a low-speed table top centrifuge. The volume of the sediment was taken as the "packed cell volume."

Results and Discussion

Chemical Characterization of N-(IAA)Amino Acids

The IAA amino acid conjugates studied in this work are listed in Table 1. All were purified to chromatographic homogeneity—in particular, to complete absence of free IAA (checked by TLC; detection limit approximately 0.1%). For compounds which have previously been prepared and fully characterized (Hutzinger and Kosuge 1968, Wieland and Hörlein 1955), our melting points corresponded closely to the literature values. There were, however, discrepancies for conjugates which have been reported as the DL-racemates (Wieland and Hörlein 1955) or without complete analytical data (e.g., Feung et al. 1975). Therefore, we characterized those and most new conjugates by elemental analysis and/or ^{13}C NMR spectra, as far as possible with the quantities obtained. The results are presented in Tables 2 and 3. The NMR data are in accord with chemical shift rules and with published values for the corresponding amino acids, with plausible *N*-acylation shifts (Pretsch et al. 1981, Wüthrich 1976). The structures of IAA-γAbu, IAA-δAva, and IAA-εAhx were confirmed by x-ray diffraction analysis (Kojić-Prodić et al. 1991 and unpublished data).

Except for IAA-Nle, the conjugates of chiral amino acids listed in Table 1 showed optical rotation when measurements were performed using light of a wavelength of 546 nm (sodium D line). In

Table 4. Growth-promoting activity of *N*-(indol-3-ylacetyl)amino acids in *Solanum nigrum* callus (explants from stock cultures on 100 μM IAA).

Compound[a]	Optimal Callus weight (g)	Optimal Concentration (μM)	Optimal range[b] of concentrations (μM)	Half-optimal concentration (μM)
2,4-D	3.9 ± 0.4	1–10[f]	0.07–20	0.02
IAA-Gly	4.5 ± 0.3	100[c]	10 . . .[g]	0.8
IAA-Ala	4.0 ± 0.3	10	0.2–400	0.03
IAA-αAbu	4.4 ± 0.3	100	0.9–300	0.2
IAA-αAib	3.9 ± 0.2	1000[c]	300 . . .[g]	30[d]
IAA-Nva	4.0 ± 0.2	100	10–300	0.9
IAA-Val	4.4 ± 0.4	100	20 . . .[g]	4
IAA-Nle	3.5 ± 0.4	100	20–300	3
IAA-Leu	3.6 ± 0.2	100	20–200	2
IAA-Ile	4.6 ± 0.3	1000[e]	20 . . .[g]	2
IAA-γAbu	4.1 ± 0.3	100	1–500	0.3
IAA-δAva	5.1 ± 0.3	100	5–500	0.8
IAA-εAhx	4.8 ± 0.3	100	4 . . .[g]	0.7
εIAA-Lys	3.7 ± 0.2	1–10[f]	0.2–200	0.01
εIAA-D-Lys	4.3 ± 0.4	10	0.8–200	0.03
αIAA-Lys	4.0 ± 0.3	100	3 . . .[g]	0.5
$(IAA)_2$-Lys	3.8 ± 0.3	10	0.3–1000	0.03
δIAA-Orn	4.3 ± 0.5	100	5–400	2

[a] Abbreviations are defined in footnote 1 and Table 1.
[b] Callus weight 75% of maximum value or above.
[c] Highest concentration tested. The dose–response curve did not level off, although callus weights were in the range observed with optimal concentrations of the other amino acid conjugates tested in this system.
[d] Tentative estimate because of the reasons explained in footnote c.
[e] Highest concentration tested; no steep increase of the dose–response curve between 100 and 1000 μM.
[f] Almost equal callus weights at these concentrations, as shown in Fig. 1.
[g] Upper limiting concentration not reached.

a more quantitative manner, the IAA conjugates synthesized from D- and L-alanine were analyzed for enantiomeric purity by HPLC on a chiral column. As the mixed-anhydride method used for their preparation has never been observed to cause complete inversion at the reaction center, only partial racemization may be suspected. However, the products obtained from either isomer of alanine showed a single peak in the above HPLC system. Retention times were 5.0 min for IAA-D-Ala and 6.2 min for the L-isomer; a mixture of the two clearly separated. X-ray diffraction patterns in monocrystals (Kojić-Prodić et al. 1991 and unpublished data) confirmed enantiomeric purity for all the conjugates of chiral α-amino acids listed in Table 1, including IAA-Nle (optical rotation of chiral compounds is wavelength-dependent and may be zero at 546 nm). Such structural data are not yet available for δIAA-Orn and εIAA-Lys. These amino acids were subjected to alkali during protection of their α-amino groups by complexation with Cu^{2+}; and, after coupling with IAA, they were exposed to strong acid for deprotection (Hutzinger and Kosuge 1968). This and the fact (Table 4) that the N_ϵ-(IAA) conjugates prepared from either D- or L-lysine showed about the same biological activity, while IAA conjugates of other D-amino acids are practically inactive (Bialek et al. 1983, Hangarter et al. 1980), would indeed be good reasons to suspect major racemization. Unfortunately, the small quantities of the "D-lysine" conjugate were completely spent in biological tests, and thus were not available for examination on the above chiral column. Two samples independently prepared from L-lysine did, however, give a single peak, under conditions which clearly separated IAA-D-Ala from its L-isomer.

Solanum nigrum Callus

This callus line was chosen as a test system which responds to *N*-(IAA) amino acids by unorganized growth. Representative dose–response curves are shown in Figure 1 for εIAA-Lys, as an example. The results of two independent experiments are presented to illustrate the degree of reproducibility obtained, and the approach adopted to construct an "average" dose–response curve. This was then

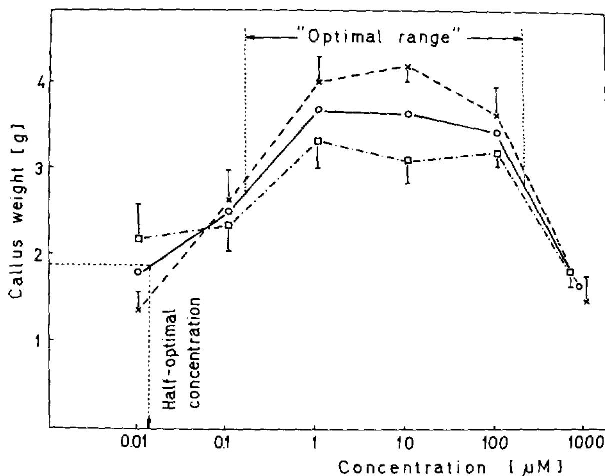

Fig. 1. Growth of *Solanum nigrum* callus on media containing various amounts of N_ϵ-(IAA)lysine. The original data obtained in two experiments (x---x and □-·-·-□) are shown; bars represent standard errors of the mean (N = 20). The average response (O—O) calculated from mean callus weights (arithmetic means) at fixed substrate concentrations was used to estimate the half-optimal concentration and the "optimal range" of concentrations (response at least 75% of the maximal).

used to estimate the parameters employed to compare the growth-promoting activity of εIAA-Lys to that of other amino acid conjugates: the optimal concentration and callus weight, the "optimal range" of concentrations, and the half-optimal concentration.

The ascending parts of the dose–response curves were of a similar, sigmoidal to roughly log-linear shape for 2,4-D and all the *N*-(IAA)amino acids tested in this system. An "optimal range" of concentrations, narrower than that for εIAA-Lys (Table 4), indicates an increasingly skewed dose–response curve declining more steeply at supraoptimal concentrations.

Optimal concentrations for the compounds studied varied over three orders of magnitude. An extremely narrow "optimal range" of concentrations (ratio of limiting concentrations), as observed for IAA-Nle and IAA-Leu, is probably a more reliable indicator of toxicity than the respective, low, optimal callus weights, as strictly constant properties of the inoculum cannot be taken for granted. Among the IAA conjugates tested in this work, IAA-Ala, εIAA-Lys (D and L), and $(IAA)_2$-Lys, for which the "optimal range" covers three orders of magnitude, appear to be the least inhibitory at high concentrations. They were also the most active ones, approaching the activity of 2,4-D, as judged by comparison of the respective optimal and half-optimal concentrations given in Table 4.

Before ranking the activities of all the IAA conjugates listed in Table 4, we attempted to assess possible effects of residual auxin from the stock cultures in the relatively large explants which had to be used to obtain a reproducible growth response. This was done by comparing the response of explants derived from stock cultures grown on 100 μM IAA, 100 μM IAA-Ala, or 9 μM 2,4-D. Calli were then repeatedly subcultured on medium containing various concentrations of IAA-Gly (from stock on IAA and IAA-Ala) and IAA-Ala (from stock on IAA and 2,4-D). Despite the different provenance of the original explants, optimal callus weights did not change in a systematic fashion during the series of subcultures, and optimal concentrations remained the same as stated in Table 4. Thus, auxin residues in the explants do not affect the response to *optimal* levels of a different source of auxin in the test media. However, the callus growth in response to *sub-optimal* levels of IAA-Gly and IAA-Ala declined for a number of subcultures (four for stock on 9 μM 2,4-D and two for stock on 100 μM IAA or IAA-Ala) and then stabilized. Due to the resulting changes in the initial slopes of the dose–response curves, half-optimal concentrations shifted by a factor of 5–10 with respect to the values in Table 4 (i.e., to 4 μM for IAA-Gly and to 0.3 μM for IAA-Ala). To what extent these changes reflect depletion of the pool of residual auxin within the explants is a matter of discussion. Gradual loss of viability of tissues repeatedly subcultured at low auxin concentrations (indicated by accumulation of dark pigments) may also contribute to the observed effects. Thus, it should be permissible to use half-optimal concentrations to compare the growth-promoting properties of the IAA conjugates listed in Table 4, as long as small differences are not overinterpreted, and as long as the activity scale obtained is corroborated by differences in optimal concentrations. According to these criteria, the activity of IAA conjugates of aliphatic, straight-chain, α-monoamino acids decreased as the molecular weight of the amino acid moiety increased. Branching of the amino acid backbone at C-β or C-γ had a minor effect, while substitution of H-α, as in IAA-αAib, drastically reduced auxin activity. Differences between the growth-promoting properties of conjugates of the ω-amino acids examined and of their α-L-isomers were not very pronounced.

Solanum nigrum callus weakly responded to free IAA (optimal callus weight, approximately 3 g). Under the experimental conditions routinely used, the differences in final callus weights obtained with a series of IAA concentrations were too small with respect to the sampling variance to define a reliable dose–response curve. However, when explants

from stock cultures on 9 μM 2,4-D were dispensed to a range of IAA concentrations and then repeatedly transferred, in 6-week intervals, to fresh media containing identical IAA levels, the optimal concentration stabilized at 100 μM, and the half-optimal concentration at 2 μM.

Tomato Hypocotyl Explants

This system responds to auxin by callus formation and organogenesis. The effect of IAA-Ala has been described in detail (including color photographs) by Hangarter et al. (1980). Within a short time, the shoot formation occurring to some extent in no-auxin controls is progressively suppressed with increasing IAA-Ala concentrations. Callus growth peaks at concentrations around 10 μM, and is progressively inhibited at conjugate levels above 100 μM. Roots may be formed, in a somewhat irregular fashion, if the explants are kept longer than 1 month. Essentially, the same pattern of morphogenetic effects was observed for the IAA conjugates studied in this work, except that the entire dose–response curves could be shifted to higher concentrations. Details are presented in Table 5, which includes several conjugates already tested by Hangarter et al. (1980) in a more qualitative fashion. Apart from the fact that the response of the tomato system is complex, and thus is more difficult to describe numerically, the relative activities of the IAA conjugates examined were quite similar to those observed in *Solanum nigrum* callus. Some of the more interesting differences will be discussed below. Free IAA preferentially induces organogenesis in tomato hypocotyl explants (Hangarter et al. 1980), so its effect is *qualitatively* different from that of the conjugates studied in this work. IAA concentrations optimal for shoot and root growth were, respectively, 10 and 100 μM; callus growth was modest and showed no pronounced concentration dependence.

Table 5. Relative growth-promoting activities of *N*-(indol-3-ylacetyl)amino acids in tomato hypocotyl explains cultured *in vitro*.

Compound[a]	Relative activity[b]
2,4-D	~10
IAA-Gly	~1[c]
IAA-Ala	1
IAA-αAbu	1
IAA-αAib	—[d]
IAA-Nva	0.1
IAA-Val	0.01–0.1
IAA-Nle	0.01–0.1
IAA-Leu	0.01–0.1
IAA-Ile	0.01
IAA-βAla[e]	
IAA-γAbu	0.1
IAA-δAva	0.1
IAA-εAhx	0.1
εIAA-Lys	1
αIAA-Lys	0.001–0.01
$(IAA)_2$-Lys	0.01
δIAA-Orn	0.1–1

[a] Abbreviations are explained in Table 1.
[b] Ratio of biologically equivalent (suboptimal to optimal) concentrations of the respective conjugate and that of IAA-Ala.
[c] Quoted for comparison from the work of Hangarter et al. (1980).
[d] No definite activity at concentrations up to 1000 μM.
[e] Activity about the same as for IAA-Val (determined in a different set of experiments under essentially the same conditions as used in this work).

Other Systems

In a less systematic fashion than above, some of the more active *N*-(IAA)-amino acids were tested in two additional in vitro systems. εIAA-Lys and IAA-Ala, at concentrations of 100 and 1000 μM, induced fast-growing callus on *Oenothera* leaf explants. IAA-γAbu and IAA-δAva were less satisfactory. As a member of the Onagraceae (Oenotheraceae) family, *Oenothera* is only remotely related to the solanaceous species from which the other in vitro systems studied in this work were derived.

The performance as sources of auxin in tomato cell suspension culture was tested for IAA-Ala and εIAA-Lys. The cell line employed was routinely grown in the presence of 9 μM 2,4-D to yield, within a culture period of about 10 days, a packed cell volume of 20–30%. It was suspected that in this system 2,4-D acts: (1) as a source of auxin; and (2) as a pharmacon, causing independence of an external source of cytokinin, an incompletely understood effect frequently observed in plant tissue culture. If so, less than 9 μM 2,4-D might be required for function (2), function (1) being performed by an IAA conjugate. Indeed, when 9 nM 2,4-D was added to the medium, along with 100 μM IAA-Ala, packed cell volumes of approximately 20% were consistently obtained. Increasing the concentration of the IAA conjugate to 1000 μM did not markedly affect yields, while concentrations lower than 100 μM were progressively less effective. The same tendencies were observed with εIAA-Lys as the source of auxin, although limited supplies precluded detailed studies. In a second set of experiments, the level of the alanine conjugate was kept at 100 μM, and the 2,4-D was replaced with a range of concentrations of benzyladenine. A benzyladenine level of 3 μM

resulted in packed-cell volumes of about 25%, which are within the range normally attained with 9 μM 2,4-D as the only plant growth regulator added.

Concluding Remarks

We have tested the growth-promoting properties, in plant tissue culture, of IAA conjugates of aliphatic amino acids with a 2- to 6-carbon backbone, including all straight-chain α-L- and ω-amino acids, a number of branched α-L-amino acids, and the α,ω-diamino acids, ornithine and lysine. In this array of compounds, the most active ones were located in two subsets centered by IAA-Ala and εIAA-Lys, which showed about the same growth-promoting properties in the different systems studied here. The activity of closely related conjugates was species-dependent: in tomato hypocotyl explants, IAA-Gly, IAA-Ala, and IAA-αAbu had about the same effect, while the alanine conjugate was considerably more active in *Solanum nigrum* callus. Conversely, $(IAA)_2$-Lys was about as active as εIAA-Lys in *Solanum nigrum*, but about a 100 times less active in tomato. Both IAA-Ala and εIAA-Lys were about as effective as 2,4-D in *Solanum nigrum*, but about 10 times less active in tomato. These interspecific variations are difficult to explain, as long as the reasons for the different activity of individual amino acid conjugates in the same tissue are incompletely understood. Differences in susceptibility to degradation by peroxidases (Park and Park 1987) and to hydrolysis by enzymes liberating free IAA as the physiologically active component (Hangarter and Good 1981, Bialek et al. 1983) have been shown; however, differences in uptake, distribution between cell compartments, and toxicity of the amino acid moieties also deserve consideration.

Further searching for highly active and easy to prepare *N*-(IAA)amino acids should probably be focused on derivatives closely related to IAA-Ala and εIAA-Lys. At present, these two conjugates can themselves be recommended as sources of auxin in plant tissue culture, if undifferentiated cell proliferation is desired. They showed reasonable activity even in "difficult" systems, such as *Oenothera* leaf callus and tomato cell suspension culture. One of their practical advantages, as compared to 2,4-D, is the wide "optimal range" of concentrations. This would reduce the number of individual auxin concentrations that would have to be tested with new tissue sources.

Acknowledgments. This work was supported by U.S.-Yugoslav research agreement JF-813 (USDA), the Research Council of the Republic of Croatia, and postdoctoral fellowships (V.M., R.P.H.) of Michigan State University. We are grateful to J. D. Cohen for being able to use his HPLC equipment and to R. Herman for microanalyses.

References

Andersson B, Sandberg G (1982) Identification of endogenous *N*-(3-indoleacetyl)-aspartic acid in Scots pine (*Pinus sylvestris* L.) by combined gas chromatography-mass spectrometry, using high-performance liquid chromatography for quantification. J Chromatogr 238:151–156

Bialek K, Meudt WJ, Cohen JD (1983) Indole-3-acetic acid (IAA) and IAA conjugates applied to bean stem sections: IAA content and the growth response. Plant Physiol 73:130–134

Cohen JD (1982) Identification and quantitative analysis of indole-3-acetyl-L-aspartate from seeds of *Glycine max* L. Plant Physiol 70:749–753

Duddeck H, Hiegemann M, Simeonov MF, Kojić-Prodić B, Nigović B, Magnus V (1989) Conformational study of some amino acid conjugates of indol-3-ylacetylacetic acid (IAA) by ^{1}H-NOE-difference spectroscopy: structure/auxin activity relationships. Z Naturforsch 44c:543–554

Ehmann A (1977) The van Urk-Salkowski reagent: a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. J Chromatogr 132:267–276

Epstein E, Baldi BG, Cohen JD (1986) Identification of indole-3-acetylglutamate from seeds of *Glycine max* L. Plant Physiol 80:256–258

Evidente A, Surico G, Iacobellis NS, Randazzo G (1986) α-*N*-Acetyl-indole-3-acetyl-ε-L-lysine: a metabolite of indole-3-acetic acid from *Pseudomonas syringae* pv. *savastanoi*. Phytochemistry 25:125–128

Feung CS, Hamilton RH, Mumma RO (1975) Indole-3-acetic acid: mass spectra and chromatographic properties of amino acid conjugates. J Agric Food Chem 23:1120–1124

Feung CS, Hamilton RH, Mumma RO (1977) Metabolism of indole-3-acetic acid. IV. Biological properties of amino acid conjugates. Plant Physiol 59:91–93

Findlay SP, Dougherty G (1948) The synthesis of certain substituted indoleacetic acids. J Org Chem 13:560–569

Fuchs S, Haimovich J, Fuchs Y (1971) Immunochemical studies of plant hormones. Eur J Biochem 18:384–390

Good NE (1956) The synthesis of indole-3-acetyl-D,L-aspartic acid and related compounds. Can J Chem 34:1356–1358

Hangarter RP, Good NE (1981) Evidence that IAA conjugates are slow-release sources of free IAA in plant tissues. Plant Physiol 68:1424–1427

Hangarter RP, Peterson MD, Good NE (1980) Biological activities of indoleacetyl-amino acids and their use as auxins in tissue culture. Plant Physiol 65:761–767

Hutzinger O, Kosuge T (1968) Microbial synthesis and degradation of indole-3-acetic acid. III. The isolation and characterization of indole-3-acetyl-ε-L-lysine. Biochemistry 7:601–605

Klieger E, Gibian H (1961) Über Peptidsynthesen. VIII. Weitere Synthesen von Glutamylpeptiden mit Carbobenzoxy-L-pyroglutaminsäure. Liebigs Ann Chem 649:183–202

Kojić-Prodić B, Nigović B, Horvatić D, Ružić-Toroš Ž, Magnus

V, Duax WL, Stezowski JJ, Bresciani-Pahor N (1991) Comparison of the structures of the plant growth hormone indole-3-acetic acid, and six of its amino-acid conjugates. Acta Cryst. B47:107–115

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nefkens GHL, Tesser GI, Nivard RJF (1960) A simple preparation of phthaloyl amino acids via a mild phthaloylation. Rec Trav Chim Pays Bas 79:688–698

Park RD, Park CK (1987) Oxidation of indole-3-acetic acid-amino acid conjugates by horseradish peroxidase. Plant Physiol 84:826–829

Percival FW (1986) Isolation of indole-3-acetyl amino acids using polyvinylpolypyrrolidone chromatography. Plant Physiol 80:259–263

Pretsch E, Clerc T, Seibl J, Simon W (1981) Tabellen zur Strukturaufklärung organischer Vebindungen mit spektroskopischen Methoden, 2nd edn. Springer-Verlag, Berlin Heidelberg New York, pp C5–C265

Schwyzer R, Costopanagiotis A, Sieber P (1963) Zwei Synthesen des α-Melanotropins (α-MSH) mit Hilfe leicht entfernbarer Schutzgruppen. Helv Chim Acta 46:870–889

Sembdner G, Gross D, Liebisch H-W, Schneider G (1980) Biosynthesis and metabolism of plant hormones. In: Macmillan J (ed) Encyclopedia of plant physiology, new series, vol. 9, Springer-Verlag, Berlin Heidelberg, pp 281–444

Sonner JM, Purves WK (1985) Natural occurrence of indole-3-acetylaspartate and indole-3-acetylglutamate in cucumber shoot tissue. Plant Physiol 77:784–785

Wang SS, Tam JP, Wang BSH, Merrifield RB (1981) Enhancement of peptide coupling reactions by 4-dimethylaminopyridine. Int J Peptide Protein Res 18:459–467

Wieland T, Hörlein G (1955) Synthesen einiger β-Indolacetyl-aminosäuren und -peptide. Liebigs Ann Chem 591:192–199

Wüthrich K (1976) NMR in biological research: peptides and proteins. North-Holland/American Elsevier, Amsterdam Oxford New York