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SYNTHESIS OF *N*-ε-(INDOLE-3-ACETYL)-L-LYSINE

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Key Word Index—*Pseudomonas syringae* subsp. *savastanoi*; olive and oleander knot disease; auxins; synthesis; *N*-ε-(indole-3-acetyl)-L-lysine and its *N*-α-acetyl derivative; *N*-α-(indole-3-acetyl)-L-lysine and its *N*-ε-acetyl derivative.

Abstract—*N*-ε-(indole-3-acetyl)-L-Lysine (ε-IAA-Lys) and its *N*-α-acetyl derivative (Ac-ε-IAA-Lys), products of the metabolism of indole-3-acetic acid (IAA) in *Pseudomonas syringae* subsp. *savastanoi*, have been synthesized by a conjugation of IAA with the *N*-α-CBz-L-lysine *p*-nitrophenylester. The same synthetic strategy was used to obtain the unnatural conjugate, *N*-α-(indole-3-acetyl)-L-lysine (α-IAA-Lys) and its *N*-ε-acetyl derivative (Ac-α-IAA-Lys). Comparative bioassay for auxin activity on wheat coleoptiles proved that ε-IAA-Lys and Ac-ε-IAA-Lys significantly stimulated their growth, although their activity was considerably less than IAA. No activity was shown by α-IAA-Lys and Ac-α-IAA-Lys.

INTRODUCTION

Virulent strains of *Pseudomonas syringae* subsp. *savastanoi* cause overgrowths (knots, galls) on the host plants through the production of phytohormones, auxins and cytokinins, which alter the physiological phytohormone balance and induce tissue proliferation. However, although several studies have confirmed that indole-3-acetic acid (IAA, **1**) is the main virulence factor in the olive and oleander knot diseases and that cytokinins play a secondary role [1–4], no definitive data are available on the altered phytohormone balance in plants.

Pseudomonas syringae subsp. *savastanoi* converts L-tryptophan in IAA in two steps, catalysed by tryptophan 2-mono-oxygenase and indolacetamide hydrolase [5, 6]. However, IAA is not the end product of tryptophan metabolism, since in oleander, but not in olive strains, the auxin is converted into *N*-ε-(indole-3-acetyl)-L-lysine (ε-IAA-Lys, **5**) [7] or its *N*-α-acetyl derivative (Ac-ε-IAA-Lys, **6**) [8]; two amino acid conjugates with auxin activity less than IAA [7–9]. This metabolic trait has been suggested to be an important feature for the regulation of IAA pool size and of the virulence of the pathogen [10].

The above considerations prompted us to investigate IAA content and its metabolism during the pathogenesis. However, a limiting factor in these studies is that the metabolites of interest may occur at very low levels. To overcome this difficulty, we have synthesized the naturally occurring IAA metabolites, **5** and **6** and their corresponding unnatural analogues α-IAA-Lys (**9**) and Ac-α-IAA-Lys (**10**), which may be useful as standard substances during the isolation and identification of metabolites of interest.

RESULTS AND DISCUSSION

The synthetic method used in this work, to prepare the IAA-Lys adduct derivatives (**5**, **6**, **9**, **10**) starts from the amide bond formation between the commercially available IAA (**1**) and the appropriately protected L-lysine derivatives (**2**, **7**). Thus, by reaction of IAA (**1**, Scheme 1) with *N*-α-CBz-L-lysine-4-nitrophenylester (**2**) in the presence of dicyclohexylcarbodiimide (DCCI), as condensing agent, the protected intermediate **3** was obtained (86% yield). Hydrolysis of **3**, carried out with NaOH in dioxane–water (1:1) at 0°, followed by treatment with a cation exchange resin (H^+ form), yielded the corresponding acid **4** (87% yield). Finally, the deprotection of the α-amino group of **4**, by hydrogenation, afforded the expected **5** (90% yield). Successive acetylation of **5**, performed by reaction with Ac_2O–NaOAc, and the usual work-up, gave **6** in 85% yield. Both synthetic **5** and **6** resulted in products which were identical by comparison of physico-chemical and spectral properties, to the naturally occurring metabolites. In our opinion this synthetic strategy, which requires only three steps (Scheme 1) to prepare **5** (57% overall yield), represents an improved method with respect to the previously reported procedure [7]. In fact, the preceding synthesis (45% overall yield) provided a very complex method for the preparation of

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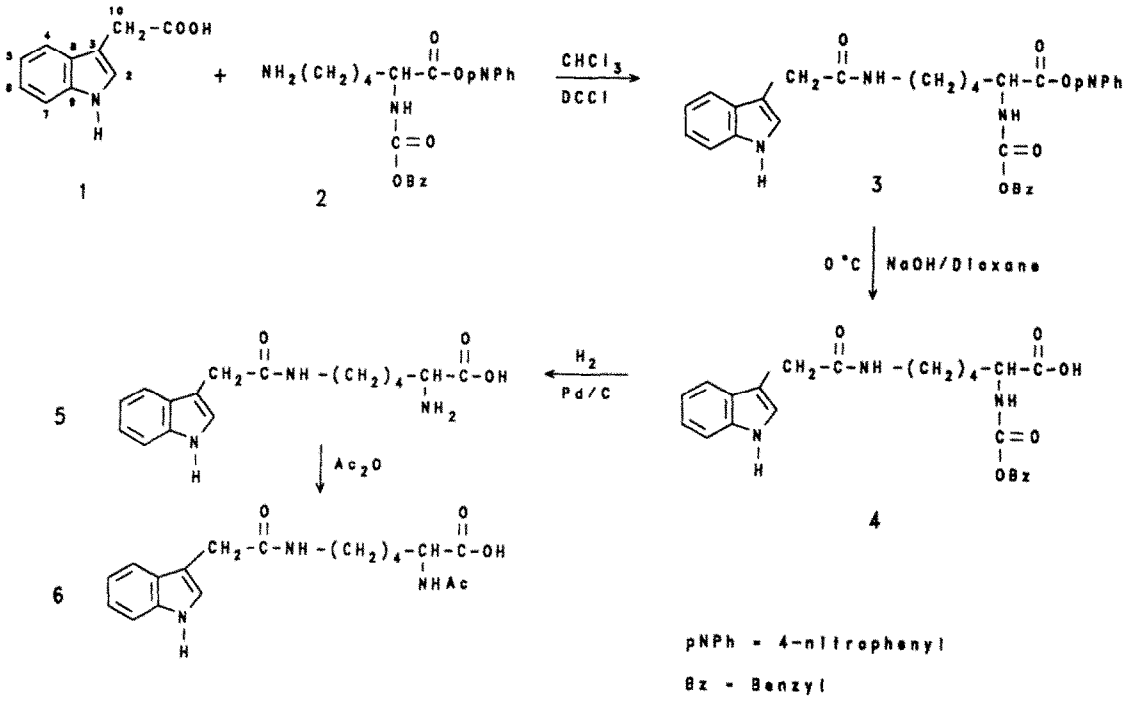
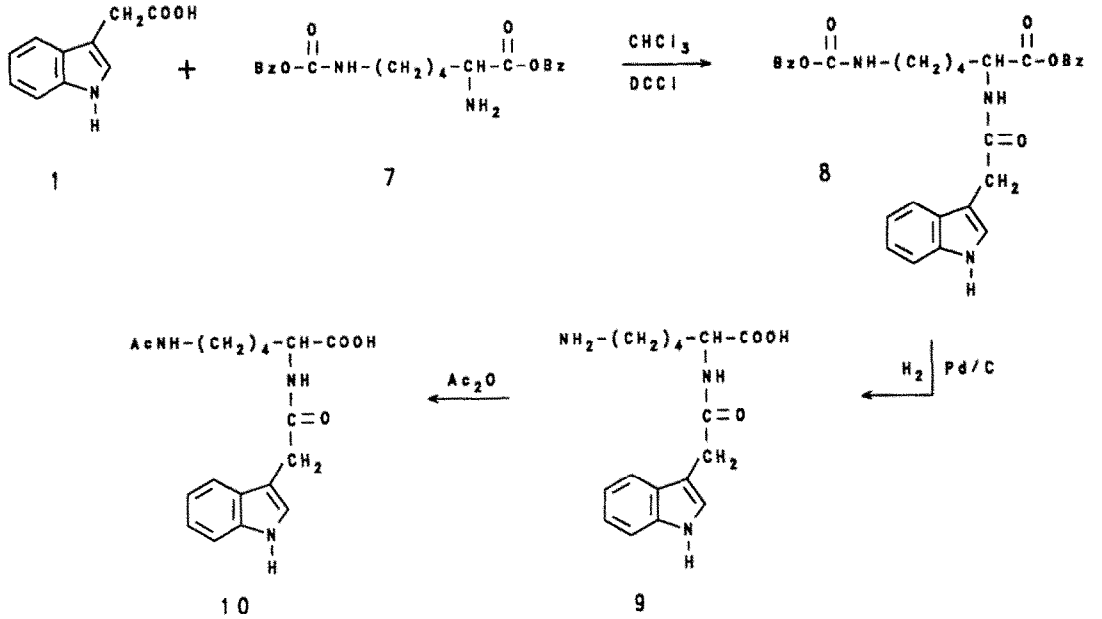


Scheme 1.



Scheme 2.

the *N*-α-protected-L-lysine derivative and a laborious procedure to realize its conjugation with IAA. In addition, the above-cited procedure did not consider the crucial problem of the probable occurrence of α-racemization in the final product. Considering the well-known peptide synthetic strategy, it is possible to use appropriately protected amino acids and controlled reaction conditions to minimize the α-racemization. Nevertheless, a final examination of the optical purity of the synthetic products is required.

Therefore, we tested the optical purity of the synthesized compound **5** using Marfey's procedure [11], which allows the detection of the percentage of both of the enantiomers by their conversion to a diastereoisomeric mixture, followed by quantitative HPLC analyses. For compound **5**, we found an optical purity of 96%.

To synthesize **9** and its *N*-ε-acetyl derivative **10**, the same synthetic pathway was used, but employing as starting product the *N*-ε-CBz-L-lysine benzylester (**7**). In this case, only two steps (Scheme 2) were required to obtain **9**. In fact, the catalytic hydrogenation of the intermediate **8** (84% yield) provided its complete deprotection, thus affording **9** (88% yield). Acetylation of **9**, performed as described to obtain **5**, furnished its acetyl derivative **10** (78% yield). The optical purity of **9**, measured as described above was 98%. ^1H and ^{13}C NMR (Experimental) confirmed the structures of all the synthesized compounds.

Comparative bioassay on wheat coleoptiles proved that 10 μM **5** and **6** solutions significantly stimulated their growth, while no effect was observed with less concentrated solutions. In particular, **6** and **5** were different from the controls at $P=0.05$ and $P=0.01$, respectively. However, their activity was 19.8 and 36.4%, respectively, of that determined by an equimolar IAA solution (100%; Table 1). In this regard, however, the relative activity of two IAA–lysine conjugates seems to be overestimated, since we have observed that in the assay conditions 0.1 μM IAA induced a significant effect, while 1–10 μM IAA showed a saturation of the effect (data not shown). No activity was shown (Table 1) by the unnatural derivatives **9** and **10**.

Table 1. Effect of 10 μM IAA–lysine conjugates on wheat coleoptile growth*

Treatment†	Length‡ (mm)	Increment of growth (% of IAA)§
Control	9.47 aA	—
Ac-α-IAA-Lys (**10**)	9.93 abB	12.1
α-IAA-Lys (**9**)	10.17 abc	18.5
Ac-ε-IAA-Lys (**6**)	10.22 Abc	19.8
ε-IAA-Lys (**5**)	10.85 Bc	36.4
IAA (**1**)	13.28 D	100.0

* Wheat coleoptile sections of 5 mm were used.

† Solutions containing 0.1% methanol were used.

‡ Values in the columns scored by the same letters, are statistically different at the $P=0.05$ (lower case letters) or $P=0.01$ (capital letters) level in Duncan's multiple range test.

§ In the calculation of the percentage of growth, the final coleoptile length of control was subtracted.

EXPERIMENTAL

General. Optical rotations and UV: MeOH; ^1H and ^{13}C NMR: CD_3OD, unless otherwise noted, at 270 and 67.92 MHz, respectively. FAB-MS: glycerol–thioglycerol using Xe as bombarding atoms, at 9.5 kV. Analytical and prep. TLC: silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively). Eluent systems: (A) *n*-BuOH–HOAc–H_2O (60:15:25); (B) $CHCl_3$–MeOH (6:4). The spots were visualized by UV light or I_2 vapour.

Biological assay. Auxin activity was assessed by wheat coleoptile straight-growth assay. The method described in ref. [12], with minor modifications, was followed. Compounds were dissolved in a small amount of MeOH and brought up to the required concn with buffer (10 mM K_2PO_4, 5 mM citric acid, 2% sucrose, pH 5). Buffer containing the same amount of MeOH (0.1%) was used for comparison.

N-α-*Carbobenzyloxy*-N-ε-(*indole*-3-*acetyl*)-L-*lysine*-p-*nitrophenyl-ester* (**3**). To a soln of IAA (**1**, 174 mg, 1 mmol) and amino acid derivative **2** (438 mg, 1 mmol) in dry $CHCl_3$ (12 ml) were added DCCI (824 mg, 4 mmol) and pyridine (121 μl, 1.5 mmol) and the final soln was stirred at room temp. After 3.5 hr the reaction was quenched by addition of H_2O (1 ml) and kept at room temp. with stirring, for 1 hr. The filtrate, dried *in vacuo*, was chromatographed on a silica gel column using increasing amounts of MeOH in $CHCl_3$. The fractions eluted with $CHCl_3$–MeOH (9:1) yielded 480 mg of pure **3** (86% yield). ^1H NMR ($CDCl_3$) δ: 8.25 (2H, *d*, $J=6.8$ Hz, *ortho*-H NO_2 in *p*Nph); .7.53 (1H, *dd*, H-4); 7.47–7.07 (11H, other aromatic protons), 5.75 (1H, *bt*, $J=6.0$ Hz, ε-NH); 5.46 (1H, *bd*, $J=7.7$ Hz, α-NH); 5.14 (2H, *s*, CH_2 of benzyl group); 4.46 (1H, *ddd*, α-CH); 3.72 (2H, *s*, CH_2-10); 3.19 (2H, *m*, ε-CH_2); 2.00–1.22 (6H, complex signal, β and δ-CH_2).

N-α-*Carbobenzyloxy*-N-ε-(*indole*-3-*acetyl*)-L-*lysine* (**4**). To solid **3** (450 mg, 0.81 mmol) was added, 0°, a soln (10 ml) of dioxane–NaOH 0.1 M (1:1) and the mixt. was kept, with stirring, at 0° for 1 hr. The resulting soln was neutralized through addition of Dowex AG 50WX8 resin (H^+ form) and after filtration, dried *in vacuo*. The residue, dissolved in $CHCl_3$–MeOH (99:1) was chromatographed on a silica gel column using increasing amounts of MeOH in $CHCl_3$. The fractions eluted with $CHCl_3$–MeOH (8:2) yielded 308 mg of pure **4** (87% yield). ^1H NMR differed from that of **3** for the following signal system δ: 7.75 (1H, *dd*, H-4); 7.44–7.26 (6H, complex signal, aromatic protons of benzyl and H-7); 7.19 (1H, *s*, H-2); 7.16 (1H, *ddd*, H-6); 7.03 (1H, *ddd*, H-5); 4.03 (1H, *dd*, α-CH).

N-ε-(*indole*-3-*acetyl*)-L-*Lysine* (**5**). A solution of **4** (300 mg, 0.69 mmol) in EtOH (12 ml) was hydrogenated over Pd charcoal (10% Pd, 400 mg) with stirring at room temp. and atmos. pres. After 15 hr, TLC analysis (0.25 mm, eluent A) showed the almost complete conversion of **4** in a product at R_f 0.35. The catalyst was removed by filtration and the filtrate evaporated *in vacuo*, thus obtaining almost pure **5** (188 mg, 90% yield). $[\alpha]_D^{25}$ +20° ($c=0.7$); UV λ_{max} nm: 289, 280, 273 and 219 (log ε: 3.49, 3.62, 3.58 and 4.38). FAB-MS *m/z*: 304 $[MH]^+$, 130 (quinolinium cation). ^1H NMR, δ: 7.54 (1H, *dd*, H-4); 7.35 (1H, *dd*, H-7); 7.18 (1H; *s*, H-2); 7.10 (1H, *ddd*, H-6); 7.02 (1H, *ddd*, H-5); 3.65 (2H, *s*, CH_2-10); 3.50 (1H, *dd*, α-CH); 3.18 (2H, *m*, ε-CH_2); 1.80 (2H, *m*, β-CH_2); 1.48 (2H, *m*, δ-CH_2); 1.39 (2H, *m*, γ-CH_2, partially overlapped with δ-CH_2). ^{13}C NMR, δ: 178.5 (*s*, COOH); 175.3 (*s*, CONH); 138.4 (*s*, C-9); 128.9 (*s*, C-8); 125. 3 (*d*, C-2); 122.9 (*d*, C-5); 120.3 (*d*, C-4); 119.7 (*d*, C-6); 112.7 (*d*, C-7); 109.8 (*s*, C-3); 56.6 (*d*, α-C); 40.5 (*t*, ε-C); 34.4 (*t*, β-C); 32.8 (*t*, δ-C); 30.4 (*t*, C-10); 24.0 (*t*, γ-C).

N-α-*Acetyl*-N-ε-(*indole*-3-*acetyl*)-L-*lysine* (**6**). Compound **5** (80 mg, 0.26 mmol) was acetylated with a mixt.

of Ac_2O (6 ml) and NaOAc (80 mg). The reaction was performed at 0°. After 2 hr, the reaction mixt. was poured into iced water. The aq. soln was then acidified, pH adjusted to 2 using 1 M HCl, and extracted with EtOAc (3 × 30 ml). The combined organic extracts were dried (Na_2SO_4) and evapd *in vacuo*. The residue was purified by prep. TLC (eluent B). The band at R_f 0.25, eluted with $CHCl_3$–MeOH (4:6), afforded 76 mg of pure **6** (85% yield). $[\alpha]_D^{25}$ +9.7° (*c* 0.8); UV λ_{max} nm: 289, 280, 272 and 219 (log ε: 3.50, 3.62, 3.58 and 3.98). FAB-MS *m/z*: 346 $[MH]^+$, 130 (quinolinium cation). NMR spectra differed from those of **5** only for the following signals: ^{1}H NMR, δ: 4.21 (1H, *dd*, α-CH); 1.94 (3H, *s*, MeCO); 1.79 (1H, *ddt*, β-CH); 1.62 (1H, *ddt*, β′-CH); ^{13}C NMR, δ: 172.5 (*s*, CO-Me); 22.7 (*q*, Me).

N-ε-*Carbobenzyloxy*-N-α-(*indole*-3-*acetyl*)-L-*lysinebenzylester* (**8**). IAA (**1**; 174 mg, 1 mmol) and amino acid derivative **7** (371 mg, 1 mmol) in dry $CHCl_3$ (12 ml), were converted into **8** according to the method used to yield **3**. After 1 hr, the reaction was stopped and the residue, obtained as described for **3**, was chromatographed on a silica gel column using increasing amounts of MeOH in $CHCl_3$. The fractions, eluted with $CHCl_3$–MeOH (99.5:0.5), yielded 441 mg of pure **8** (84% yield). ^{1}H NMR ($CDCl_3$) δ: 7.44 (1H, *dd*, H-4); 7.32–6.92 (14H, complex signal, aromatic protons); 6.12 (1H, *bd*, J = 7.5 Hz, α-NH); 5.00 and 4.98 (4H, two AB systems, 2 OCH_2–Ph); 4.55 (1H, *ddd*, α-CH); 3.64 (*s*, CH_2-10); 2.88 (2H, *m*, ε-CH_2); 1.62 (1H, *ddt*, β-CH); 1.41 (1H, *ddt*, β′-CH); 1.20 (2H, *m*, δ-CH_2); 0.96 (2H, *m*, γ-CH_2).

N-α-(*indole*-3-*acetyl*)-L-*Lysine* (**9**). Compound **8** (400 mg, 0.76 mmol), was hydrogenated using the same conditions used to convert **4** into **5**. After 5 hr, TLC analysis (silica gel, eluent B) showed the complete conversion of **8** in a product at R_f 0.3. The mixt. was filtered, and the filtrate, dried *in vacuo*, furnished 202 mg of almost pure **9** (88% yield). $[\alpha]_D^{25}$ +0.5° (*c* 0.55). UV λ_{max} nm: 289, 280, 274 and 220 (log ε: 3.57, 3.65, 3.60 and 4.36). FAB-MS *m/z*: 304 $[MH]^+$, 130 (quinolinium cation). ^{1}H NMR, δ: 7.55 (1H, *dd*, H-4); 7.36 (1H, *dd*, H-7); 7.21 (1H, *s*, H-2); 7.10 (1H, *ddd*, H-6); 7.01 (1H, *ddd*, H-5); 4.30 (1H, *dd*, α-CH); 3.71 (2H, AB system, CH_2-10); 2.66 (2H, *t*, J = 7.1 Hz, ε-CH_2); 1.79 (1H, *ddt*, β-CH); 1.64 (1H, *ddt*, β′-CH); 1.50 (2H, *m*, δ-CH_2); 1.20 (2H, *m*, γ-CH_2). ^{13}C NMR, δ: 178.5 (*s*, COOH); 174.6 (*s*, CONH); 138.4 (*s*, C-9); 128.9 (*s*, C-8); 125.5 (*d*, C-2); 122.9 (*d*, C-5); 120.2 (*d*, C-4); 119.7 (*d*, C-6); 112.8 (*d*, C-7); 109.7 (*s*, C-3); 55.4 (*d*, α-C); 40.7 (*t*, ε-C); 34.4 (*t*, β-C); 33.2 (*t*, δ-C); 28.2 (*t*, C-10); 23.6 (*t*, γ-C).

N-ε-*Acetyl*-N-α-(*indole*-3-*acetyl*)-L-*lysine* (**10**). Compound **9** (100 mg, 0.29 mmol) was acetylated according to the conditions reported for converting **5** into **6**. After extractions with EtOAc (3 × 40 ml), the organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by prep. TLC (eluent B). The band at R_f 0.24, eluted with $CHCl_3$–MeOH (4:6), afforded 78 mg of pure **10** (78% yield). $[\alpha]_D^{25}$ +3.1° (*c* 0.43); UV λ_{max} nm: 289, 280, 274 and 219 (log ε: 3.52, 3.61, 3.55 and 4.10). FAB-MS *m/z*: 346 $[MH]^+$, 130 (quinolinium cation). NMR spectra differed from those of **9** only for the following signals: ^{1}H NMR, δ: 3.00 (2H, *t*, J = 6.9 Hz, ε-CH_2), 1.88 (3H, *s*, Me-CO); 1.37 (2H, *m*, δ-CH_2); 1.16 (2H, *m*, γ-CH_2); ^{13}C NMR, δ: 173.4 (*s*, CO-Me); 56.3 (*d*, α-C); 34.0 (*t*, δ-C); 30.2 (*t*, C-10); 24.1 (*t*, γ-C); 22.8 (*q*, Me).

Analysis of optical isomers. To 10 μmol (2.7 mg) of **5** (or **9**), dissolved in 0.2 ml of H_2O, was added a soln of 15 μmol (4.1 mg) 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide (FDAA) in 0.4 ml of acetone, and then 80 μl of 1.0 M $NaHCO_3$. The mixt. was kept at 40°. After 1 hr, 40 μl of 2 M HCl was added to the cold soln, and the degassed mixt. was analysed by HPLC, using a Supersphere RP18 column (Merck, 25 cm, 0.4 cm i.d., 4 μm) eluted with a linear gradient from 10 to 100% CH_3CN, in 0.1 M triethylammonium acetate (pH 7) in 50 min. The product peaks relative to the diasterisomeric products of **5** (retention time 24.3 and 25.3 min) and **9** (retention time 27.6 and 28.9 min) indicated a 96 and 98% optical purity, respectively.

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