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FURTHER CONFIRMATION OF THE PRESENCE OF INDOLYLACRYLIC ACID IN LENTIL SEEDLINGS AND IDENTIFICATION OF HYPAPHORINE AS ITS PRECURSOR

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Key Word Index—*Lens culinaris*; Leguminosae; indolylacrylic acid; biosynthesis; hypaphorine.

Abstract—The physiologically active indolylacrylic acid previously found in the lentil seedlings was further identified. Its natural formation in the same plant was demonstrated and the identification of its precursor was found to be hypaphorine, *N*-trimethyl-L(−)-tryptophan.

INTRODUCTION

The occurrence of indolylacrylic (IACRA) acid has been demonstrated in ethereal and ethyl acetate extracts of lentil seedlings (*Lens culinaris* Med.) by means of paper and thin layer chromatography [1].

The methanolic extract of lentil seedlings was found to contain another indole which was hydrophilic [2] and was called lenticin [3]. IACRA was not detected in the methanolic extract but was found to be enzymatically formed from lenticin [2] which was found to arise from ^{14}C-labelled L(−)-tryptophan [3]. This data raised the following questions which are reported on here: is IACRA a natural substance or is it only an artifact of extraction; and what is the chemical structure of lenticin?

RESULTS

Identification of IACRA by GLC

Methyl esters from eight pure commercial indolic acids were prepared and examined by GLC. The CH_2Cl_2 soluble acidic fraction of an ethyl acetate extract of lentil seedlings was similarly treated. Among the peaks in the GLC trace of the product from the lentil extract was a substance at R_t 7·10 exactly corresponding with that of the methyl ester of IACRA.

Identification of lenticin

The IACRA precursor (lenticin), $C_{14}H_{18}O_2N_2$ has an indolic chromophore and gives a positive reaction to alkaloid reagents. Its IR spectra and that of its nitrate derivative give peaks characteristic of betaines [4]. The free base gave a strong band at 1635 cm^{-1} and a narrow band at 1410 cm^{-1} (COO^- assym and sym). The nitrate, on the other hand, shows a strong band of COOH at 1735 cm^{-1}.

The NMR spectra of the free base confirms the betaine nature of lenticin: the presence of a singlet-$N(Me)_3$ at 3·20 and a multiplet of five aromatic protons at 7·5. In addition, the spectra shows a doublet at 3·25 of a proton partially hidden by the signal at 3·20 and a multiplet at 3·95 of two protons.

The MS were not very informative. The molecular weight was confirmed M^+·246 and a few characteristic ion fragments observed: *m/e* (%), 201 (1·4) [M–(Me)$_3$], 187 (1·5) (M–N–(Me)$_3$, 143 (6) (187-COO^-), 130 (5·4) (143-CH), 116 (5) (130-CH_2). Other spectra of the same sample taken at the same temperature (165°) gave two additional unexplained peaks at *m/e* 271 and 286. From the above data, lenticin is identified as hypaphorine (*N*-trimethyltryptophan). This substance has been

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isolated from the seeds of various varieties of *Erythrina* [5] and *Abrus precatorius* L [6] and its structure and configuration proved [7, 8]. The identification of lenticin and hypaphorine and that of their nitrate derivatives were confirmed by comparison of their melting points and molecular rotation and by the NMR spectra of a synthetic sample of the betaine of (±)tryptophan.

Biosynthesis of IACRA from lenticin-[^{14}C]

Lenticin-[^{14}C] was synthesized as described in the experimental section and added to the culture medium of 4-day-old lentil seedlings.

The ether-soluble acidic fraction from a methanolic extract of the seedlings was chromatographed on MN 261 paper. The radiochromatogram showed several unidentified substances and a strong peak at R_f 0·76 the same as synthetic IACRA. The peak was eluted and rechromatographed in a second solvent. The scanned chromatogram showed a peak at R_f 0·34 again corresponding to IACRA.

CONCLUSION AND DISCUSSION

The presence of indolylacrylic acid in lentil seedlings has been confirmed by GLC. It has been also shown that it is not an artifact of extraction but it is formed naturally in lentil tissues. The natural precursor of this acid, previously called lenticin [3], is shown to be hypaphorine, the betaine of L-(−)tryptophan, a compound previously extracted from the seeds of *Erythrina* [5] div. sp. and *Abrus precatorius* L [6]. *Erythrina* and *Abrus* belong to the Leguminosae as does *Lens culinaris*. It would be interesting to investigate the presence of IACRA and its precursor hypaphorine in related plants. However the sequence L(−)-tryptophane → hypaphorine → IACRA has not been previously described as a pathway of indole metabolism.

EXPERIMENTAL

Mp's are corrected. Molecular rotations are in H_2O at 20°. UV Spectra are in 96% EtOH and IR spectra were recorded in KBr discs. NMR spectra were determined in D_2O with tetramethylsilane as reference: the chemical shifts are in ppm values and coupling constants as Hz. (Relative intensities of ions in MS are given in % of Σ 40). GLC was on a 1·8 × 6mm glass column, packed with 4% SE-30 on Gas chrom Q, 100–120 mesh: He flow 90 ml/min; oven temp. 205°, injection and detection were respectively 250° and 285°. PC was on MN 214 or MN 261 paper in (**1**) i-BuOH–MeOH H_2O (8:0·5:1·5). (**2**) i-PrOH–NH_3 25% H_2O (10:1:1). TLC used silica gel in solvent (**3**) NH_3–EtOH–CH_2Cl_2 (15–40–60). Reagents: Dragendorf, Ehrlich.

IACRA extraction for GLC. 20 g of *Lens culinaris* Med. seedlings grown for 5 days in the dark at 27° on moistened vermiculite, separated from the cotyledons, and ground with sand and solid CO_2. The frozen powder was covered with EtOAc (10 ml/g fr wt.) and left in the dark at 2° for 24 hr. The extract was evaporated to dryness *in vacuo*, taken into CH_2Cl_2 and fractionated by N NH_4OH. The basic fraction was acidified to pH 3·0 (HCl) and re-extracted with CH_2Cl_2, and repurified as before. The final CH_2Cl_2 fraction was evaporated to dryness and methylated by CH_2N_2 in Et_2O. The esters were examined by GLC. The R_t (min), of the reference methyl esters are: indolylcarboxylic acid 1·15; indolylacetic acid 2·35; indolylglyoxylic acid 2·65; indolylpropionic acid 3·15; indolyllactic and indolylbutyric acids 4·35; indolylacrylic acid 7·10; indolylglycolic acid gives two peaks at 1·35 and 3·75. The R_t of the lentil extract are 1·25, 1·85, 2·15, 3·65, 5·7, 6·4 and 7·1.

Purification of lenticin. 400 g Of fr. lentil seedlings grown for 5 days in the dark at 25° without cotyledons were extracted 3 × boiling MeOH for 2–3 min. The MeOH was evaporated *in vacuo* and the remaining aqueous extract lyophilized. The lyophilized powder was extracted by EtOH and the solvent evaporated *in vacuo*. The extract was dissolved in min H_2O, washed (hexane and by Et_2O) and chromatographed on Bio-Gel P_2. The fractions containing the precursor (Ve/Vo, 2·8) were evaporated, and taken into 1 ml H_2O and chromatographed on a phosphocellulose column. The active fractions (detected by UV), were re-chromatographed on MN 214 paper, eluted with MeOH, evaporated and crystallised from Et_2O (12 mg).

Nitrate of the IACRA precursor. Lenticin in min. H_2O was treated with HNO_3 and the crystals formed rapidly washed with EtOH, vacuum dried, and recrystallised from 96% EtOH, m.p. 224° $[\alpha]_D^{25}$ +94 (C = 0·50%). IR (ν): 1730 cm^{-1} (COOH), 750 cm^{-1} disubstituted benzene.

IACRA precursor. The nitrate was dissolved in MeOH–H_2O (80–20) and passed through Amberlite IRA 400 (OH^-). The solution was evaporated and vacuum dried. Crystals of the free base were obtained from EtOH 96%, m.p. 235°, $[\alpha]_D$ +113 (C = 0·4%). (Anal. calc. for $C_{14}H_{18}O_2N_2$ 1/4 H_2O: C, 67·20; H, 7·42; N, 11·14; O, 14·29. Found C, 67·25; H, 7·27; N, 10·67; O, 14·52%); UV λ max 272 (log ε 3·74) 280 (3·79) and 288 nm (3·69). IR spectra: (ν) 1635 cm^{-1} (COO^- asy), 1410 cm^{-1} (COO^- sym) 750 cm^{-1} disubstituted benzene. NMR spectra: (solvent D_2O)s, 3·20 $N(Me)_3$ *d*, 3·25 (Cα H) *J* 3 (partly masked by the 3·20 peak) *m*, 3·95 ($C\beta H_2$), *m*, 7·5 (Ar.) Chromatoplaque TLC: solvent 3, R_f = 0·50, tryptophan R_f = 0·45 (ninhydrin).

Betaine of (±) tryptophan. 200 mg Of the nitrate of betaine of (±) tryptophan were synthesized [7] (m.p. = 212°, $[\alpha]_D$ = 0° (C = 1%) IR 1735 cm^{-1} (COOH)). The free base was obtained after passage through Amberlite IRA 400 (form OH^-). The solution was evaporated and vacuum dried, m.p. = 235°, $[\alpha]_D$ = 0°, IR 1612 cm^{-1} (COO^-), 1410 cm^{-1} (COO^-). The UV and NMR spectra are the same as for lenticin.

Biosynthesis of hypaphorine-[^{14}C]. 10 g Of etiolated lentil seedlings (4-day-old) were incubated in a Petri dish in an aqueous solution of L(−)tryptophan-3-[^{14}C] (Radiochemical Center, Amersham CFA 289, spec. activ. 58 mC/mM) 10 μC/20 ml. After 21 hr incubation, the seedlings were washed with H_2O and transferred to moistened filter paper for 24 hr. The seedlings were extracted 3 × 50 ml boiling MeOH. The mixed extracts were evaporated *in vacuo* (35°), and submitted to the purification procedure described above. The pure radioactive

hypaphorine ($1{\cdot}798 \times 10^6$ dpm) was used as the IACRA precursor.

In vivo *biosynthesis of IACRA*-3-[^{14}C]. 10 g Of etiolated lentil seedlings (4-day-old) were incubated with hypaphorine-^{14}C during 40 hr in the same conditions as above. The evaporated methanolic extract was acidified to pH 3·0 and extracted by Et_2O. The Et_2O was fractionated with 2% $NaHCO_3$, the aqueous phase washed with Et_2O, then acidified to pH 3·0 and extracted by Et_2O, to obtain the acid fraction. This was chromatographed on paper as described.

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