SYNTHESIS OF ($^2H_5$)INDOLE-3-PYRUVIC ACID

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SUMMARY

A method for the synthesis of deuterium-labelled tryptophan and its enzymatic conversion to indole-3-pyruvic acid (($^2H_5$)IPyA) using amino acid oxidase is described. ($^2H_5$)IPyA can be used as an internal standard for the quantitation of IPyA by mass spectrometry in plant extracts and for estimating the breakdown of IPyA to indole-3-acetic acid (IAA) during sample extraction and purification. When used in conjunction with ($^2H_2$)IAA, ($^2H_5$)IPyA should allow more accurate quantitation of IAA in plant extracts.

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INTRODUCTION

A proposed pathway for the biosynthesis of the phytohormone, indole-3-acetic acid (IAA), from tryptophan in higher plants involves the formation of indole-3-pyruvic acid (IPyA) as an intermediate (1,2). Evidence for this is based largely on the chromatographic detection of putative radiolabelled IPyA or of the more stable dinitrophenylhydrazone of IPyA from plant tissues following their incubation with radiolabelled tryptophan (see 3). There is also reasonable evidence that plant tissues contain enzymes that convert tryptophan to IPyA and then to IAA (see 3,4) and IPyA has recently been unequivocally identified in a higher plant tissue by gas chromatography-mass spectrometry (GC-MS) (5). However, no accurate quantitative data on IPyA levels in higher plant tissues are available.

Since IPyA is a highly unstable compound (6,7,8,9,10,11), its breakdown during sample extraction and purification (with the resultant formation of IAA, among other compounds (6,7,8,9,11)) may result in an overestimation of sample IAA content. We therefore report the synthesis of pentadeuterium-labelled IPyA ($(^2H_5)$IPyA) for use as an internal standard for accurate quantitative analysis of IPyA by mass spectrometry. Further, if $(^2H_5)$IPyA was used in conjunction with $(^2H_2)$IAA, it would be possible, by mass spectrometric methods, to estimate the amount of IAA formed by the degradation of endogenous IPyA during sample extraction and purification, and therefore to correct the estimate of endogenous IAA content for this contribution from IPyA breakdown.

EXPERIMENTAL

($^2H_5$)IPyA was synthesized from ($^2H_5$)tryptophan. The latter was made as follows: 5 mg L-tryptophan was dissolved in 200 ml ^2H_2O and 2 ml ^2HCl. Approximately 100 mg Raney nickel was added as a catalyst. The solution was heated under reflux and with stirring at approximately 100°C for 10 days. The ^2H_2O was then evaporated and the above procedure was repeated once to ensure maximum incorporation of deuterium. The product was purified by ion-exchange chromatography using "Zeo-Karb" 225 cation exchange resin, 100-200 mesh (Permutit Company Ltd., Isleworth, Middlesex, U.K.) (a currently available equivalent is 'Dowex' 50-X8(H), 100-200 mesh (BDH Chemicals Ltd., Poole, England)). The procedure used was essentially that of Gaitonde (12).

The ($^2H_5$)tryptophan was converted to ($^2H_5$)IPyA by incubation with L-amino acid oxidase as follows: approximately 10 mg ($^2H_5$)tryptophan was dissolved in 10 ml 0.2 mM phosphate buffer (pH 7.6) containing 0.2 units ml^{-1} catalase (from canine liver; Sigma Chemical Company, St. Louis, Missouri, U.S.A.). L-amino acid oxidase (from snake venom; Boehringer Mannheim GmbH, W. Germany) (2 units) was added and the mixture was incubated at 25°C for 1.5 hr. The ($^2H_5$)IPyA was immediately purified by solvent partitioning and high performance liquid chromatography (HPLC). The incubation mixture was adjusted to pH 3.0 and extracted 3 times with an equal volume of redistilled ethyl acetate. The ethyl acetate phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure at a temperature of less than 30°C. The sample was then subjected to HPLC on a

μBondapak C_{18} column (300 mm x 7.9 mm) (Waters Associates, Milford, Massachusetts, U.S.A.) for the isocratic separation of ($^2H_5$)IPyA from its degradation products, particularly IAA. Details of the HPLC apparatus were as reported by Summons et al. (13). The mobile phase consisted of ethanol : water (30 : 70 (v/v)), 0.2M with respect to acetic acid, at a flow rate of 3.5 ml min^{-1}. A peak which eluted at the retention time of authentic IPyA and which exhibited an ultraviolet absorbance ratio (A_{254}/A_{280}) similar to that of IPyA was collected. The concentration of the ($^2H_5$)IPyA in the HPLC eluate was immediately determined by ultraviolet absorbance and, when used for further experiments, an appropriate amount of the eluate was immediately added to plant extracts. A further aliquot of the eluate was analysed immediately by GC-MS, following evaporation under reduced pressure at less than 30°C. Identity of both the ($^2H_5$)tryptophan and the ($^2H_5$)IPyA was confirmed by GC-MS, which also established their isotopic composition: $^2H_5$ - 56.1%, $^2H_4$ - 86.3%, $^2H_3$ - 47.9%, $^2H_2$ - 10.0%, $^2H_1$ - 2.6% and $^2H_0$ - 2.6%. Trimethylsilyl derivatives of these compounds were prepared as described previously (14). Electron impact mass spectra were obtained using a DuPont 21-491B instrument interfaced with a VG.2025 data system and using a Finnigan 4500 instrument under the conditions described previously (14,15). The ($^2H_5$)IPyA was shown by GC-MS to be essentially pure, containing only a trace (less than 1%) of ($^2H_5$)IAA. The final yield of IPyA was approximately 15%.

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