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IDENTIFICATION OF NICOTINE BIOSYNTHETIC INTERMEDIATES IN TOBACCO ROOTS BY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The occurrence of two nicotine biosynthetic intermediates, putrescine (1,4-diaminobutane) and N-methylputrescine (N-methyl-1,4-diaminobutane), within tobacco roots has been confirmed by combined liquid chromatography–mass spectrometry. Perchloric acid extracts of tobacco roots were derivatized with benzoyl chloride. High-performance liquid chromatography was used to separate polyamines. Compounds resolved by isocratic elution on a reversed-phase C_{18} column were detected by UV absorbance at 254 nm. Column effluents were transferred to the mass spectrometer by a direct liquid introduction interface. Putrescine and N-methylputrescine were identified by selected ion monitoring of ions at m/z 297 and 311, respectively in the positive ion chemical ionization mode. Identification of N-methylputrescine within tobacco roots strengthens the proposed biosynthetic pathway for nicotine.

INTRODUCTION

The biosynthesis of nicotine within *Nicotiana* species has been studied extensively and precursors for both the pyridine and pyrrolidine rings of nicotine have been determined from research with labeled precursors[1,2]. There is considerable evidence that the biosynthesis of the N-methylpyrrolidine ring involves the conversion of putrescine to N-methylputrescine and subsequent oxidation of N-methylputrescine to 4-methylaminobutanal (Fig. 1).

Mizusaki *et al.*[2] reported that a putrescine N-methyltransferase enzyme from tobacco roots catalyzed the conversion of putrescine to N-methylputrescine. They also showed that N-methylputrescine oxidase from tobacco roots converted N-methylputrescine into 4-methylaminobutanal[3]. Saunders and Bush[4] have studied the activities of both enzymes in extracts of burley tobaccos differing in nicotine content. Feth *et al.*[5] utilized high-performance liquid chromatography (HPLC) to assay putrescine N-methyltransferase in roots of *Nicotiana tabacum*. Feth *et al.*[6] have also utilized HPLC to assay N-methylputrescine oxidase in tobacco roots and tissue cultures. These reports, coupled with tracer studies, strengthen the view that

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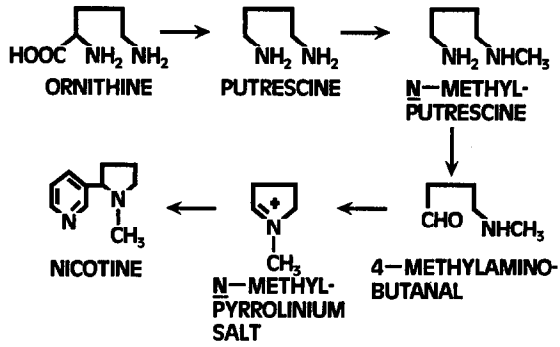

Fig. 1. Biosynthetic pathway of N-methylpyrrolidine ring of nicotine.

N-methylputrescine is involved in nicotine biosynthesis; however, this compound has not yet been reported to occur in tobacco roots. One reason for this may be that the compound is readily utilized for nicotine and does not accumulate within tobacco roots under normal growing conditions.

During investigation of nicotine biosynthesis in stressed tobacco, HPLC profiles of polyamines revealed a compound which co-eluted with authentic N-methylputrescine. A liquid chromatography–mass spectrometry (LC–MS) technique[7] was used to confirm the occurrence of N-methylputrescine in tobacco roots. This report presents our findings on the occurrence of N-methylputrescine in tobacco roots.

EXPERIMENTAL

Chemicals and plant materials

The hydrochloride salt of putrescine and benzoyl chloride were obtained from Sigma (St. Louis, MO, U.S.A.). N-Methylputrescine dihydrochloride was generously provided by Dr. E. Leete of the University of Minnesota. All other chemicals and solvents were of reagent grade or higher.

Seeds of *Nicotiana rustica* var. *pumila* were sown in vermiculite and maintained in an environmentally-controlled chamber at 26°C under a 16-h photoperiod. Three to four weeks after seeding, plants were transferred to nutrient jars containing 3/4 strength Hoagland's solution. Plants with seven or eight leaves were decapitated at the fourth leaf and roots collected at various time intervals after decapitation.

Benzoylation

The procedure for extraction and benzoylation of tobacco roots followed Flores and Galston[8] with slight modifications. Plant root (1 g) was homogenized in 7% perchloric acid and centrifuged. An aliquot of the supernatant (250 μl) was added to 1 ml of 2 *N* sodium hydroxide. A volume of 10 μl of benzoyl chloride was added and the tubes vortexed for at least 30 s to ensure a homogeneous suspension of benzoyl chloride. The benzoylation reaction was carried out for 20 min in a hood at room temperature. A volume of 1 ml of saturated sodium chloride was added, followed by 2 ml of diethyl ether. After centrifugation at 5000 *g* for 2 min, 1 ml of the ether phase

was collected and evaporated to dryness under a stream of dry air. Benzoylamines were dissolved in 0.25 ml of acetonitrile prior to LC–MS identification.

LC–MS analysis

LC was performed with a Waters Assoc. system equipped with two Model 6000A pumps, Model 720 system controller, Model 710B automatic injection system. The column was a Micromeritics C_{18} column, particle size 7.5 μm, 30 cm × 5 mm I.D. (Micromeritics, Norcross, GA, U.S.A.). The solvent was 50% acetonitrile in water maintained at a flow-rate of 1.0 ml/min for 15 min. After elution of the polyamines, the solvent was increased to 100% acetonitrile to remove other derivatized components present in the crude extracts. Typically, 50-μl aliquots of each sample were injected into the HPLC. Standards of authentic polyamines were used for confirmation. The HPLC effluent from the UV detector was passed through a Hewlett-Packard direct liquid introduction (DLI) LC–MS interface (Hewlett-Packard, Palo Alto, CA, U.S.A.). The splitting ratio was 1:99 resulting in a transfer of 0.5 μl of the HPLC effluent to the mass spectrometer. The mass spectrometer was a Hewlett-Packard 5985B GC–MS system. The instrument was scanned at 1 s per scan from 100 to 450 a.m.u. in the positive ion chemical ionization (PICI) mode. The mass spectrometer was operated at a source pressure of 0.5 Torr, a source temperature of 200°C, an electron energy of 230 eV, an emission current of 300 μA, and an electron multiplier of 1800 V.

RESULTS AND DISCUSSION

HPLC profiles of polyamine from roots of tobacco showed the presence of putrescine. In addition, a compound which co-eluted with authentic N-methyl-putrescine was detected. Since putrescine has already been identified as a component of tobacco roots[9], and our HPLC profiles indicated that it was present in our samples, we chose first to test the LC–MS capability by analyzing benzoylated root extracts for putrescine.

Since no peaks could be detected at the elution time for putrescine or N-methylputrescine in the total ion monitoring of a tobacco root extract, the sample was scanned by selected ion monitoring. Fig. 2 indicates a peak with a protonated molecular ion at m/z 297 which matched the elution time for benzoylated putrescine. It appears that putrescine is derivatized with two benzoyl groups. There was no indication of substitution by only one benzoyl group. Fig. 3 shows the PICI mass

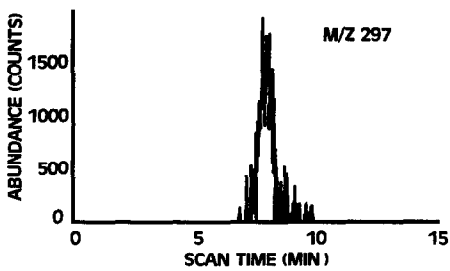

Fig. 2. Selected ion chromatogram of benzoylated putrescine from a tobacco root extract.

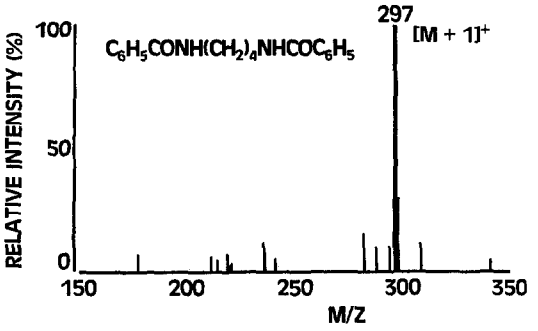

Fig. 3. PICI mass spectrum of benzoylated putrescine.

spectrum of the derivatized putrescine and its chemical formula. LC–MS analysis of authentic putrescine confirmed the ion monitoring patterns shown in the tobacco extracts.

Tobacco root extracts were subsequently scanned for the presence of N-methylputrescine by selected ion monitoring. Fig. 4 shows that monitoring with an ion at m/z 311 revealed a peak matching the elution time for authentic derivatized N-methylputrescine. A PICI mass spectrum of the sample compound is shown in Fig. 5. The

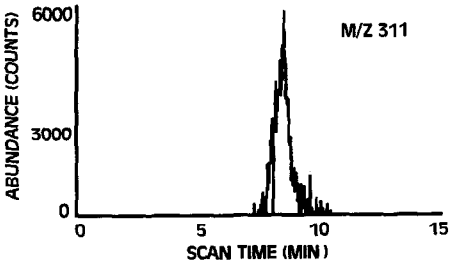

Fig. 4. Selected ion chromatogram of benzoylated N-methylputrescine from a tobacco root extract.

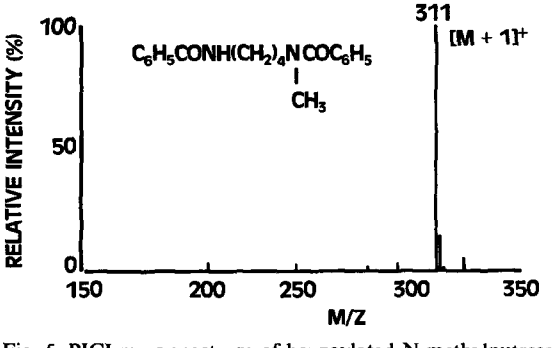

Fig. 5. PICI mass spectrum of benzoylated N-methylputrescine.

compound showed a protonated molecular ion at m/z 311, indicating the presence of two benzoyl groups. There was no indication of the presence of N,N-dimethylputrescine in extracts of tobacco roots.

Although the mass spectrometer is less sensitive than the UV detector due to the 1:99 splitting ratio, it remains the most selective among the LC detectors. We were unable to analyze underivatized samples because the acetonitrile solvent interfered with spectral analyses for N-methylputrescine. Apparently, acetonitrile and water solvate to form an adduct having an isotopic mass the same as the underivatized N-methylputrescine.

The sensitivity of the LC–MS system was adequate to confirm the presence of putrescine and N-methylputrescine in tobacco roots. This evidence strengthens the proposed root locale for nicotine biosynthesis.

CONCLUSIONS

Confirmation of the presence of putrescine and N-methylputrescine in tobacco roots in this report strengthen the proposed pathway for the synthesis of the pyrrolidine ring of nicotine. It is anticipated that LC–MS techniques can be further utilized to confirm and localize other nicotine biosynthetic intermediates.

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