BIOSYNTHESIS OF ERGOT ALKALOIDS

Cell-free formation of 4-(E-4′-hydroxy-3′-methyl-but-2′-enyl)-L-tryptophan

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1. Introduction

The enzymatic synthesis of 4-dimethylallytryptophan (DMAT) from γ, γ-dimethylallylpyrophosphate and L-tryptophan with a crude extract from mycelia of a clavine alkaloid-producing strain of *Claviceps purpurea* was reported by Heinstein et al. [1] and Lee et al. [2]. These authors reported the formation of only this single product in their enzyme incubations. In this paper, we wish to report the enzymatic synthesis of 4-(E-4′-hydroxy-3′-methyl-but-2′-enyl)-L-tryptophan (HDMAT) from isopentenylpyrophosphate (IPP) and L-tryptophan with a crude extract from mycelia of a lysergic acid alkaloid-producing strain of *Claviceps paspali* [3]. The isolation of 4-(4′-hydroxy-3′-methyl-but-2′-enyl)-tryptophan (undetermined stereochemistry about the isoprenoid double bond) from cultures of *Claviceps purpurea* was previously reported by Anderson and Saini [4].

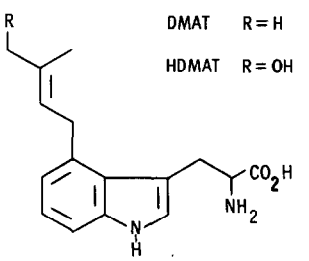
2. Materials and methods

2.1. *Materials*

Authentic DMAT and HDMAT were synthesized by the procedures of Plieninger et al. [5–7]. Lysergic acid amide was isolated by the procedure of Arcamone et al. [8]. D-Amino acid oxidase and L-amino acid oxidase were purchased from Worthington Biochemicals Inc, [1-^{14}C]IPP (61 mCi/mmol) was purchased from Amersham Searle Corporation.

2.2. *Analytical methods*

Protein was determined by the method of Brahmall et al. [9] and expressed as mg/ml bovine serum albumin. Total alkaloid was determined by the modified Van Urk procedure of Michelon and Kelleher [10].



The following solvent systems were used for the chromatography of DMAT and HDMAT: (A) ethanol–conc. NH_4OH–water (20:1:2), (B) *n*-butanol saturated with water and (C) *n*-butanol–acetic acid–water (12:3:5) for paper chromatography on Whatman No. 1 and D) *n*-butanol–acetic acid–water (4:1:1) for thin-layer chromatography (tlc) on silica gel. Spots on chromatograms corresponding to DMAT or HDMAT were revealed by spraying with a 0.25% solution of ninhydrin in acetone and then heating in a current of warm air.

Radioactivity determinations were made with a Packard Tri-Carb Liquid Scintillation Spectrometer (Model No. 3375) using a cocktail prepared by dissolving 2,5-dipenyloxazole (2.5 g) and naphtalene (50 g) in 500 ml, scintillation grade, 1,4-dioxane. Radioactivity on chromatograms was located by scanning with a Vanguard Autoscanner.

2.3. *Enzyme preparation*

An isolate of *Claviceps paspali*, number 31 of the fifth selection [3], was grown on the 8-salt mannitol-

succinate medium according to the 3-stage submerged culture procedure of Mary et al. [3]. All subsequent operations involved in the preparation of cell-free extracts were conducted at 2–4°C. Mycelia from 600 ml of 4-day-old alkaloid-producing cultures were filtered from the broth and washed with cold 0.01 M Tris buffer, pH 8.0, containing 0.02 M each of diethyldithiocarbamate, thioglycolic acid and mercaptoethanol. The washed mycelia were suspended in 6 vol. above buffer (60 ml) and ruptured by hand-grinding with washed sea sand. The resulting slurry was centrifuged at 10 000 × *g* for 30 min. The supernatant was centrifuged at 105 000 × *g* for 90 min to give a clear pale-yellow solution that was used in the incubations.

2.4. *Enzyme incubations*

The synthetase reaction mixture consisted of 100 μl enzyme solution (0.5 mg protein/ml), [1-^{14}C]IPP (0.0078 μmol, 1.06 × 10^6 dpm), L-tryptophan (0.0156 μmol), ATP (0.05 μmol), $MgSO_4 \cdot 7\ H_2O$ (0.05 μmol), L-methionine (0.05 μmol), liver concentrate (20 μg) and the above buffer, all in total vol. 160 μl. After incubation for 1 h at 30°C the reaction was stopped by holding in a boiling water bath for 2 min. The total incubation mixture was chromatographed on paper using solvent system A. The product at R_F 0.61 was cut from the chromatogram, eluted with methanol, and rechromatographed on paper using solvent system B.

The configuration of the enzymatically formed HDMAT was determined by incubating enzymatically formed HDMAT (6000 dpm/incubation) mixed with synthetic HDMAT (0.5 mg) with both D- and L-amino acid oxidase. The L-amino acid oxidase reaction mixture consisted of HDMAT (0.5 mg, 6000 dpm), catalase (1.0 mg, 5550 units) and L-amino acid oxidase (4 units) in total vol. 0.4 ml, 0.20 M Tris buffer, pH 7.8, containing 0.10 M KCl. The D-amino acid oxidase reaction mixture consisted of HDMAT (0.5 mg, 6000 dpm), catalase (1.0 mg, 5550 units) and D-amino acid oxidase (1.0 mg, 3.7 units) in total vol. 0.4 ml, 0.02 M sodium pyrophosphate buffer, pH 8.3. Incubations were conducted aerobically at 37°C for 3 h. The total incubation mixtures were freeze-dried to solid residues. The residues were separately extracted with absolute ethanol (0.5 ml) and the ethanolic extracts were chromatographed, separately, on paper with solvent system B.

3. Results and discussion

When the total synthetase incubation mixture was chromatographed on paper with solvent system A, a radioactive band centered at R_F 0.61 appeared. This band was barely detectable on chromatograms of incubation mixtures lacking added L-tryptophan and not detected at all in the boiled enzyme control. When this band was cut from the chromatogram, eluted with methanol, and rechromatographed on paper using solvent system B, three radioactive bands appeared: these were designated 1̃, 2̃ and 3̃.

Each of these synthetase reaction products was purified to chromatographic homogeneity by successive paper chromatography with systems A and B and elution with methanol.

Compound 3̃ was identified as the previously reported DMAT [1,2] by co-chromatography on paper with solvent systems A, B and C.

Compound 2̃ was tentatively identified as HDMAT by co-chromatography on paper with solvent systems A, B and C and tlc on silica gel with solvent system D.

In order to confirm the identity of 2̃ as HDMAT, synthetic HDMAT (30 mg) was mixed with enzymatically formed 2̃ (27 000 dpm partially purified material) and recrystallized to constant specific activity.

Initially, methanol–ether was used as a crystallization solvent. Because of low yield encountered in the first crystallization, a second crop of crystals was collected and added to the first crop and the crystallization solvent was changed from methanol–ether to methanol for subsequent crystallizations. The results are shown in table 1.

Table 1
Co-crystallization of 2̃ with synthetic 4-(E-4′-hydroxy-3′-methyl-but-2′-enyl)-tryptophan

Crystallization no.	Specific activity (dpm/mg)
1	705[a]
2	792[b]
3	583
4	578

[a] First crop of crystals from methanol–ether
[b] First crystallization, from methanol, of the combined first and second crops of crystals from methanol–ether

The constancy of specific activity after the third crystallization, together with the identical chromatographic behavior in four different systems, established the identification of the enzymatically produced 2 as HDMAT. Upon incubation of enzymatically formed 2, separately with D- and L-amino acid oxidase, a new product was formed only in the L-amino acid oxidase incubation; only starting substrate was seen on chromatograms of the D-amino acid oxidase incubations. Compound 1 has not yet been identified.

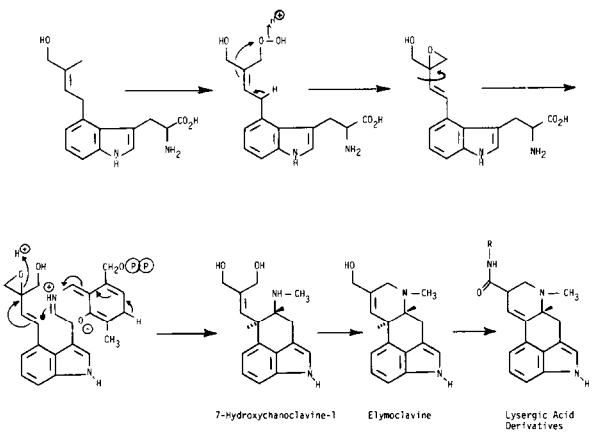
Separate controls with boiled and unboiled liver concentrate (No. 202-20 from Sigma Chem. Corp.) and enzyme showed that neither 1 nor 2 were formed from DMAT in the absence of active *Claviceps* enzymes.

Each of the three synthetase reaction products was administered, separately, to growing cultures of *Claviceps paspali* and incorporated into lysergic acid amide (recrystallized to constant specific activity). Percent total incorporations for 1, HDMAT, and DMAT into lysergic acid amide were 0.83, 1.52 and 8.71, respectively.

Because HDMAT has been demonstrated to occur in culture filtrates from *Claviceps* fermentations (undetermined stereochemistry about the isoprenoid double bond) [4] and has now been shown to be produced enzymatically by an extract of an alkaloid-producing isolate of *C. paspali* and incorporated into lysergic acid amide, it must be considered to be a natural intermediate in a biosynthetic pathway leading to ergot alkaloids. A plausible mechanism for its incorporation into lysergic acid alkaloids is shown in Scheme 1 which is adapted from a proposal by Pachlatko et al. [11] for the incorporation of HDMAT into elymoclavine but not into agroclavine.

Scheme 1

Proposal for the conversion of 2 to lysergic acid derivatives via 7-hydroxychanoclavine-I



Acknowledgements

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