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# FORMATION OF CLAVICIPITIC ACID IN CELL-FREE SYSTEMS OF *CLAVICEPS* SP.

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**Key Word Index**—*Claviceps* sp.; Clavicipitaceae; ergot; enzymatic study; clavine alkaloids; 4-dimethylallyltryptophan; clavicipitic acid.

**Abstract**—4-Dimethylallyltryptophan-[3-$^{14}$C] was converted to clavicipitic acid in cell-free extracts from *Claviceps* sp. SD 58 and *Claviceps purpurea* PRL 1980. Activity was concentrated in the microsomal fraction. Oxygen was required but there was no cofactor requirement. *p*-(Hydroxymercuri)benzoic acid strongly inhibited the conversion. Addition of diethyldithiocarbamate increased conversion 2·5×. Conversion was favored at high pH. Clavicipitic acid [$^{14}$C] added to cultures of *Claviceps* sp. SD 58 was not significantly incorporated into elymoclavine.

## INTRODUCTION

4-Dimethylallyltryptophan (DMAT) is the first intermediate in clavine alkaloid biosynthesis [1, 2]. Clavicipitic acid has been isolated from cultures of *Claviceps* sp [3]. A revised structure has recently been proposed [4]. We report the conversion of DMAT to clavicipitic acid in cell-free systems from *Claviceps* sp. SD 58 and *Claviceps purpurea* PRL 1980 and some of the properties of the systems.

## RESULTS

Clavicipitic acid was identified as a major product of [$^{14}$C]-DMAT in crude extracts from *Claviceps* sp. SD 58 by cochromatography with reference clavicipitic acid in three solvents.

Both the soluble and microsomal fractions catalyzed the conversion of DMAT to clavicipitic acid, although conversion was consistently higher in the microsomal fraction (Table 1). When DMAT was incubated with boiled extracts, there was no spot for clavicipitic acid on the radioautogram and the radioactivity was decreased to a low level. Conversion of DMAT to clavicipitic acid was 24·7% in the presence of air but only 3·1% in an $N_2$ atmosphere. Formation of clavicipitic acid, therefore, requires $O_2$, although $O_2$ is not incorporated into clavicipitic acid in the overall reaction. When the incubation was carried out in a 95:5 CO–$O_2$ atmosphere, conversion was 14·5% compared to 13% in a 95:5 $N_2$–$O_2$ atmosphere. The failure of CO to inhibit conversion of DMAT to clavicipitic acid suggests

Table 1. Effect of various treatments on conversion of 4-dimethylallyltryptophan-[3-$^{14}$C] to clavicipitic acid by cell-free systems from *claviceps* sp. SD 58.

| Treatment* | Protein (mg/ml) | % Clavicipitic acid |
|---|---|---|
| Experiment I | | |
| Crude extract | 15·7 | 15·4 |
| Microsomal fraction | 4·8 | 11·3 |
| Supernatant fraction | 12·0 | 4·5 |
| Experiment II | | |
| Microsomal fraction (M.F.) | 5·4 | 13·3 |
| M.F. + *N*-ethylmaleimide | | 4·5 |
| M.F. + *p*-(Hydroxymercuri)-benzoic acid | | 1·5 |
| M.F. + Diethyldithiocarbamate | | 33·9 |
| M.F. + *o*-Phenanthroline | | 5·1 |

* The incubation mixture contained enzyme, 0·2 mg DMAT (0·2 $\mu$Ci) and 0·02 M KPi buffer pH 7 in a final vol of 2·2 ml. Incubation was for 13 hr at 26°. Concentrations of additional components in Experiment II were 0·02 M.

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that cytochrome P-450 is not involved in the reaction. Addition of catalase or $H_2O_2$ had little effect on the formation of clavicipitic acid. Peroxidase is therefore not involved. The activity was reduced partially by the addition of 0·02 M *N*-ethylmaleimide and completely by 0·02 M *p*-(hydroxymercuri)benzoic acid (Table 1), suggesting that sulfhydryl groups of the enzyme are involved in the reaction.

Diethyldithiocarbamate strongly stimulated conversion of DMAT to clavicipitic acid (Table 1). Diethyldithiocarbamate has an extremely high affinity for certain metals, e.g. cupric ions [5]. The increased conversion in the presence of diethyldithiocarbamate may be due to removal of an inhibitory metal ion. *o*-Phenanthroline, another metal ion chelator, inhibits conversion. This may reflect difference in metal ion specificity of the two chelators.

Results similar to those described above were obtained with *Claviceps purpurea* PRL 1980. Experiments with the 60–80% $(NH_4)_2SO_4$ fraction were also carried out with this strain. The conversion was markedly increased at high pH in all fractions (Table 2). Addition of a mixture of coenzymes or an NADPH-generating system had no effect on conversion at either pH in either the 60–80% $(NH_4)_2SO_4$ fraction or the microsomal fraction. There is therefore no cofactor or cosubstrate requirement for activity.

Incorporation of [$^{14}$C]-clavicipitic acid into elymoclavine was only 1% compared to 35% incorporation of DMAT into elymoclavine (Table 3). This confirms a similar observation by H. G. Floss (private communication). The low incorporation suggests that clavicipitic acid is not a precursor of the clavine alkaloids although it is possible that clavicipitic acid is not able to penetrate the cell membrane.

Table 2. Effect of pH on conversion of 4-dimethylallyltryptophan-[3-$^{14}$C] to clavicipitic acid by cell-free systems from *claviceps purpurea* PRL 1980

| Cellfraction* | Protein (mg/ml) | % Clavicipitic acid (pH 7) | (pH 10·3) |
|---|---|---|---|
| Crude extract | 3·6 | 7·7 | 58·9 |
| Microsomes | 2·5 | 18·4 | 61·4 |
| Supernatant | 3·1 | 4·4 | 22·3 |
| 60–80% $(NH_4)_2SO_4$ | 2·9 | 5·7 | 13·8 |

* The incubation mixture contained 0·27 mg DMAT (0·11 $\mu$Ci), 0·7 M KPi pH 7 or 0·7 M sodium carbonate pH 10·3 and 0·02 M diethyldithiocarbamate in a final volume of 4·2 ml. Incubation time was 45 min at 26°.

Table 3. Incorporation of clavicipitic acid [$^{14}$C] and 4-dimethylallyltryptophan-[3-$^{14}$C] into alkaloids by *claviceps* sp. SD 58

| | Clavicipitic acid | DMAT |
|---|---|---|
| Counts/min fed | 44500 | 43405 |
| Counts/min recovered as alkaloids | 2700 | 19600 |
| % incorporation into alkaloids | 6·1 | 46·2 |
| Counts/min recovered in elymoclavine | 480 | 15180 |
| % incorporation in elymoclavine | 1·1 | 35·0 |
| Counts/min/$\mu$g elymoclavine | 0·4 | 6·1 |
| Specific incorporation (%) | 0·015 | 0·22 |

## DISCUSSION

The conversion of DMAT to clavicipitic acid involves removal of two hydrogens from DMAT. Oxygen is reduced in the process. The enzyme is present both in the supernatant and microsomal fractions, but more activity is present in the microsomal fraction. Baxter and co-workers [6, 7] showed that DMAT had a high affinity for cytochrome P-450 in the microsomal fraction from *Claviceps* sp. However, cytochrome P-450 does not appear to be involved in this reaction, since carbon monoxide is not an inhibitor. It is suggested that the increased activity at high pH is due to increased nucleophilicity of the $\alpha$-amino nitrogen of DMAT which may facilitate formation of the carbon–nitrogen bond in clavicipitic acid.

## EXPERIMENTAL

Claviceps sp. SD 58 was generously provided by Dr. J. E. Robbers and reference clavicipitic acid was a gift from Dr. H. G. Floss of Purdue University, LaFayette, Indiana. 4-Dimethylallyltryptophan-[3-$^{14}$C] was synthesized as described previously [8]. *Claviceps* sp. SD 58 was grown in NL-406 medium [9] with 0·3 g yeast extract per l. for 10–16 days. *C. purpurea* PRL 1980 was grown in mannitol–tryptophan–succinic acid [10] medium supplemented with 3 mM niacinamide for 3–5 days. The culture was filtered on a Buchner funnel and washed cells were homogenized in 0·02 M KPi buffer pH 7 with a Virtis 45 homogenizer for 3–6 min at maximum speed. The mixture was centrifuged at 10000 *g* for 20 min. The supernatant (crude extract) was centrifuged for 1 hr at 105000 *g*. The residue was suspended in 0·02 M KPi buffer pH 7 (microsomal fraction). To the 105000 *g* supernatant 1% protamine sulfate soln in 0·02 M KPi buffer pH 7 was added at the rate of 15 ml/100 ml of supernatant, and the mixture was centrifuged at 10000 *g* for 10 min. $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation and the mixture was centrifuged at 10000 *g* for 10 min

The ppt. was discarded and $(NH_4)_2SO_4$ was added to 80% saturation. The ppt. was collected by centrifugation and dissolved in 2 ml 0·02 M KPi buffer pH 7. The soln was dialyzed for 18 hr against 16 l. in 4-l. portions of 0·02 M KPi buffer pH 7. Mixtures were incubated with shaking. After incubation, the incubation mixture was adjusted to pH 5 with dil HCl and passed through a column of Dowex 50($H^+$) resin (100–200 mesh). Alkaloids and amino acids were eluted from the resin with 5% $NH_4OH$. The eluate was dried under vacuum on a rotary evaporator and the residue was dissolved in 0·5 ml of 95% EtOH containing 1% $NH_4OH$ for TLC. Unless otherwise stated, $CHCl_3$–MCOH–HOAc (75:20:5) was the solvent used for TLC. After TLC, a radioautogram was prepared and the areas on the TLC plate which corresponded to spots on the radioautogram were scraped directly into counting vials. Fifteen ml of 0·5% 2,5-diphenyloxazole in toluene was added and the radioactivity was counted on a liquid scintillation counter. The formation of clavicipitic acid was expressed as the cpm of the clavicipitic acid region divided by the cpm of all regions of the TLC plate times 100.

For identification of clavicipitic acid, the chromatogram was developed with $CHCl_3$–MeOH–HOAc (75:20:5) and the clavicipitic acid was eluted from the TLC plate with 95% EtOH containing 1% $NH_4OH$ and respotted. Reference unlabeled clavicipitic acid was spotted on top of the sample. The TLC plate was then developed in 2 dimensions, first in BuOH–HOAc–$H_2O$ (4:1:1) and then in MeOAc–*iso*PrOH–$NH_4OH$ (9:7:4). A radioautogram was obtained and the TLC plate was then sprayed with Van Urk's reagent (5 g *p*-dimethylaminobenzaldehyde, 1 drop 0·9% $FeCl_3$, in 100 ml conc-HCl).

In the radioactive incorporation expt., the labeled compound was added to 5 ml of a 6-day-old culture of *Claviceps* sp. SD 58. Cultures were harvested after 15 days and the alkaloids were extracted as described previously [8]. For quantitative determination of total alkaloids the acidic extract was used [11]. The $Et_2O$ extract was evaporated and the residue was dissolved in EtOH. For separation of alkaloids, a TLC plate was developed in EtOAc–$Me_2CO$–DMF (5:1:1). Elymoclavine and agroclavine spots were located on the TLC plate with UV, and the Si gel containing the compounds was transferred to 25 ml conical flasks. 3 ml of MeOH–HOAc–$H_2O$ (4:1:2) was added to the flask and vigorously shaken for 10 min. Three ml of Van Urk's reagent was added and shaking was continued for 10 min. The mixture was centrifuged for 15 min at 10000 *g*. The *A* of the supernatant was read at 550 nm [12]. Reference agroclavine was spotted on TLC and treated exactly the same as the samples in order to obtain a standard curve.

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