On the Mechanism of the Formation of Indole Alkaloids in *Penicillium roqueforti*†

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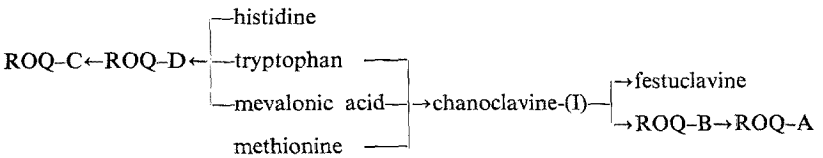
Studies on the biosynthesis of indole alkaloids in *Penicillium roqueforti* were carried out with the growing mycelia of a selected strain of the same mold by the use of DL-tryptophan-3-^{14}C, DL-mevalonic acid-2-^{14}C-lactone or L-methionine-methyl-^{14}C as a precursor of ergoline alkaloids and DL-tryptophan-3-^{14}C, DL-tryptophan-carboxy-^{14}C, DL-mevalonic acid-2-^{14}C-lactone or L-histidine-ring-2-^{14}C as a precursor of non-ergoline alkaloids. The results of these experiments seem to suggest that in the *Penicillium* mold employed there exist the following biosynthetic routes:



Previously, we[1~3] isolated festuclavine together with three new indole alkaloids, roquefortine A (ROQ-A), B (ROQ-B) and C (ROQ-C), from the cultures of *Penicillium roqueforti*. After that, we[4] additionally isolated a new indole alkaloid, roquefortine D (ROQ-D), in a small quantity from the said cultures.

Of these five indole alkaloids, festuclavine, ROQ-A and -B are ergoline derivatives, while ROQ-C and -D are non-ergoline compounds, and accordingly the biosynthetic interrelation among these indole alkaloids is very interesting.

In this paper, we describe the mechanism of the formation of indole alkaloids in *P. roqueforti*.

MATERIALS AND METHODS

Fungus and cultivation. A strain of *Penicillium roqueforti*, which was selected in the previous experiments,[5] was used throughout this experiment. The strain was cultivated at 26°C in 300 ml Erlenmeyer flasks each containing 40 ml of a modification of MGS-medium[5,6] composed of 30 g of mannitol, 10 g of glucose, 1 g of KH_2PO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, 10 g of succinic acid, NH_4OH (to pH 5.6) and tap water (1 liter).

Labelled chemicals. DL-Mevalonic acid-2-^{14}C-lactone (as the DBED salt), DL-tryptophan-3-^{14}C, DL-tryptophan-carboxy-^{14}C, L-methionine-methyl-^{14}C and L-histidine-ring-2-^{14}C were purchased from Radiochemical Centre Amersham.

Biosynthesis of alkaloids. When the alkaloids began to be produced in the culture (about on the 8th day), 5 μCi of each labelled precursor mentioned above was aseptically added to the culture, and the same cultivation was continued for 2~4 additional days.

Extraction of alkaloids. The alkaloids were extracted from the cultures with ethyl acetate after being made alkaline with ammonia (pH *ca.* 10). The alkaloidal extract was dissolved in 2% aqueous solution of tartaric acid and reextracted with chloroform after being made alkaline. The alkaloidal solution thus obtained was concentrated to dryness, and the residue was dissolved in a definite amount of methanol as a sample for alkaloid analysis.

Alkaloid analysis. The sample was quantitatively subjected to thin-layer chromatography on silica gel (WAKOGEL B-5F), using one or another of the following solvent systems: i) chloroform–ethanol (20: 3, v/v), ii) chloroform–acetone (2: 3, v/v) and iii) chloroform–diethylamine (10: 1, v/v). The rradioactivities

† Production of Alkaloids and Related Substances by Fungi. Part XV.

of the separated alkaloids were determined with a chromatogram–radioscanner *Aloka* JTC-201 (*Aloka* Co., Ltd.). The amount of each alkaloid was determined with a chromatogram–scanner OZUMOR-82 (*Asuka* Manufacturing Co., Ltd.), after treating the chromatogram with a modified Ehlrich's reagent[6] or Pauly reagent.[7]

RESULTS AND DISCUSSION

Table I shows the incorporation of the radioactive precursors into ergoline derivatives. The added DL-tryptophan-3-^{14}C, DL-mevalonic acid-2-^{14}C lactone and L-methionine-methyl-^{14}C were well incorporated into ROQ-A and -B as well as into festuclavine and chanoclavine-(I).* These results were expected from the structural similarity of ROQ-A and -B to festuclavine. The specific activities of the resulting radioactive ROQ-A were lower than those of resulting radioactive chanoclavine-(I) in all cases of supply of radioactive precursors. Further, the specific activities of resulting radioactive ROQ-B were also lower than those of the resulting radioactive chanoclavine-(I) but higher than that of the resulting radioactive ROQ-A in all cases of supply of precursors. Moreover, the specific activities of the resulting radioactive festuclavine were very close to those of the resulting radioactive ROQ-B in all cases of supply of precursors. These data support the hypothesis that in the tested fungus there exist the following biosynthetic route which is inferred from the chemical structure of these alkaloids:

tryptophan + mevalonic acid + methionine →

chanoclavine-(I) → ROQ-B → ROQ-A
chanoclavine-(I) → festuclavine

In this biosynthetic route, the transformation from chanoclavine-(I) [tricyclic ergoline] to tetracyclic ergoline such as ROQ-B is characteristic of the tested fungus in comparison to the biosynthetic route of rugulovasines[5] produced by *P. concavo-rugulosum*. Certainly, other known tetracyclic ergolines, such as agroclavine and elymoclavine, should be con-

* Newly identified by means of co-chromatography with authentic chanoclavine-(I) in this experiment.

TABLE I. INCORPORATION OF RADIOACTIVE TRYPTOPHAN, MEVALONIC ACID AND METHIONINE INTO ROQUEFORTINE A, ROQUEFORTINE B, FESTUCLAVINE AND CHANOCLAVINE-(I) IN THE CULTURES OF *Penicillium roqueforti*

Experimental procedures are described in the text.

Substrate 1.1×10^7 dpm/flask	Days of reaction	Roquefortine A		Roquefortine B		Festuclavine		Chanoclavine-(I)	
		Radioactivity incorporated (dpm/flask)	Specific activity (μCi/μmol)	Radioactivity incorporated (dpm/flask)	Specific activity (μCi/μmol)	Radioactivity incorporated (dpm/flask)	Specific activity (μCi/μmol)	Radioactivity incorporated (dpm/flask)	Specific activity (μCi/μmol)
DL-Tryptophan-3-^{14}C	2	2.8×10^4	0.019	6.7×10^3	0.039	2.3×10^4	0.042	3.0×10^3	0.069
	4	1.1×10^4	0.008	2.1×10^3	0.024	7.9×10^3	0.022	2.2×10^3	0.051
DL-Mevalonic acid-2-^{14}C-lactone	2	4.4×10^4	0.012	9.2×10^3	0.021	1.8×10^4	0.020	6.1×10^3	0.089
	4	3.8×10^4	0.010	5.0×10^3	0.019	8.0×10^3	0.012	1.2×10^3	0.028
L-Methionine-methyl-^{14}C	2	1.6×10^4	0.013	5.6×10^3	0.032	1.3×10^4	0.028	$<10^3$	**
	4	7.2×10^3	0.006	1.8×10^3	0.021	3.2×10^3	0.012	$<10^3$	**

** Impossible to give the value.

TABLE II. INCORPORATION OF RADIOACTIVE TRYPTOPHAN, MEVALONIC ACID AND HISTIDINE INTO ROQUEFORTINE C AND ROQUEFORTINE D IN THE CULTURES OF *Penicillium roqueforti*

Experimental procedures are described in the text.

Substrate 1.1×10^7 dpm/flask	Days of reaction	Roquefortine C		Roquefortine D	
		Radioactivity incorporated (dpm/flask)	Specific activity (μCi/μmol)	Radioactivity incorporated (dpm/flask)	Specific activity (μCi/μmol)
DL-Tryptophan-3-^{14}C	2	1.7×10^5	0.054	1.9×10^4	0.084
	4	3.0×10^5	0.069	2.1×10^4	0.075
DL-Tryptophan-carboxy-^{14}C	2	1.0×10^5	0.039	1.2×10^4	0.053
DL-Mevalonic acid-2-^{14}C-lactone	2	7.0×10^4	0.027	9.9×10^3	0.035
	4	7.5×10^4	0.023	9.1×10^3	0.032
L-Histidine-ring-2-^{14}C	2	4.3×10^5	0.061	4.2×10^4	0.093
	4	5.1×10^5	0.061	4.0×10^4	0.077

tained in a small quantity in the cultures of the tested fungus as in those[8] of *Aspergillus fumigatus*.

Table II shows the incorporation of the tracers into non-ergoline alkaloids, ROQ-C and -D. That is to say, the added DL-tryptophan-3-^{14}C, DL-tryptophan-carboxy-^{14}C, DL-mevalonic acid-2-^{14}C lactone and L-histidine-ring-2-^{14}C were well incorporated into ROQ-C and -D. These results were naturally expected from the structures of the two alkaloids. Especially, the incorporation of DL-tryptophan-carboxy-^{14}C indicated that the carboxy group of tryptophan constitutes the amide group directly with the amino group of histidine. The specific activities of the resulting radioactive ROQ-D were certainly higher than those of the resulting radioactive ROQ-C in any of the radioactive precursors used. These data suggest that ROQ-D is the precursor of ROQ-C. Tryptophanyl-histidinyl lactam is possibly an intermediate of the biosynthesis of ROQ-D on the assumption that the tested fungus own such a biosynthetic route as that of echinuline[9] in *Aspergillus glaucus*. Indeed as a metabolite of microorganism, a tryptophanyl-diketopiperazine having no C_5-unit derived from mevalonate was isolated by Kakimura *et al.*[10]

All these findings seem to indicate that in the *Penicillium* mold employed, the alkaloids are biosynthesized by way of the schema depicted in Fig. 1. The remarkable relationship between the cultural temperature and the alkaloid production in *P. roqueforti*, which was found in the previous experiments,[3] could be explained in this way: In the cultivation at low temperature (22°C), dimethylallyl group (C_5-unit), via mevalonic acid, is incorporated rapidly into the peculiar position C-4 of tryptophan, giving rise to the formation of an inter-

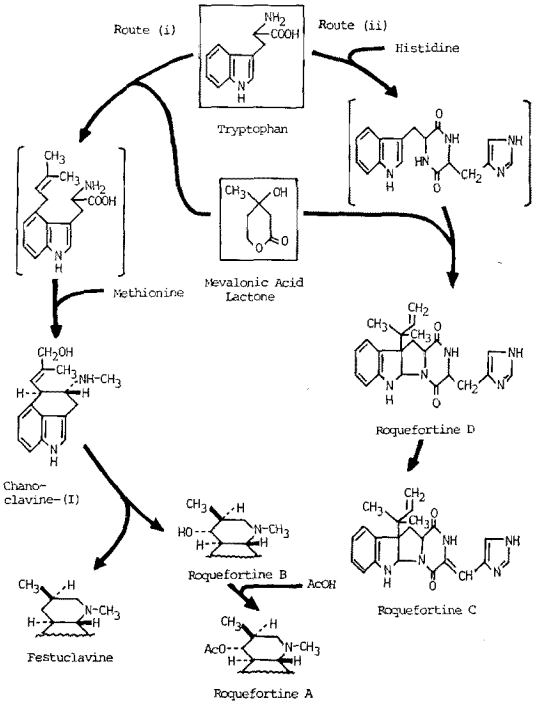


FIG. 1. Mechanism of the Formation of Indole Alkaloids in *Penicillium roqueforti*.

mediate such as 4-dimethylallyltryptophan[11] shown in Fig. 1, and from such an intermediate, chanoclavine-(I) and the other ergoline derivatives are biosynthesized via the ergoline biosynthetic route similar to that found[5] in *P. concavo-rungulosum* [route (i) in Fig. 1]. On the other hand, in the cultivation at higher temperature (30°C) tryptophan reacts, first of all, with histidine to form an intermediate such as tryptophanyl-histidinyl-lactam shown in the figure. The said C_5-unit is unable to be incorporated into the position C-4 of indole ring of such an intermediate, but instead it can be incorporated into the position C-3 of the indole ring, giving rise to the formation of ROQ-D and -C [route (ii) in Fig. 1].

Incidentally, the above reasoning also explains the fact[2] that Roquefort-type cheeses ripening under low temperature contained little or no roquefortine C (ROQ-C).

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