Phytochemistry, 1978, Vol. 17, pp 1705-1709 © Pergamon Press Ltd. Printed in England 0031 9422/78/1001-1705 $02.00/0

ACTIVITIES *IN VITRO* AND *IN VIVO* OF ENZYMES OF BENZODIAZEPINE ALKALOID BIOSYNTHESIS DURING DEVELOPMENT OF *PENICILLIUM CYCLOPIUM*

SABINE VOIGT, SALAH EL KOUSY, NICOLA SCHWELLE, LUTZ NOVER and MARTIN LUCKNER

Section of Pharmacy of the Martin Luther University and Institute of Plant Biochemistry of the Academy of Sciences of the GDR, 402 Halle/Saale, Weinbergweg, German Democratic Republic

(*Received* 27 *February* 1978)

Key Word Index—*Penicillium cyclopium*; Aspergillaceae; Ascomycetes; cyclopeptine synthetase; cyclopeptine dehydrogenase; dehydrocyclopeptine epoxidase; benzodiazepine alkaloids; biosynthesis; regulation.

Abstract—In the hyphae of *Penicillium cyclopium* the *in vitro* measurable activities of 3 enzymes of alkaloid biosynthesis are induced endogenously during development and increase in a coordinated manner. These are cyclopeptine dehydrogenase, dehydrocyclopeptine epoxidase and anthranilate adenylyltransferase (which is probably part of the cyclopeptine synthesizing enzyme complex). In contrast, in the conidiospores, the 3 enzymes are constitutive proteins. Conidiation of *P. cyclopium* is thus one of the rare cases where enzymes of secondary metabolism are formed in rapidly dividing cells. In the conidiospores as well as in the hyphae under certain conditions the *in vivo* rates of alkaloid synthesis and the *in vitro* activities of the measured enzymes do not increase in parallel. In the hyphae, synthesis of a protein which apparently is not an enzyme of alkaloid metabolism limits the rate of *in vivo* cyclopenin–cyclopenol production.

INTRODUCTION

Formation of the benzodiazepine alkaloids, cyclopenin and cyclopenol, is one of the characteristic idiophase events in the development of emerged cultures of *Penicillium cyclopium* [1]. Up to now 3 of the enzymes involved in the biosynthesis of these compounds (cf. Fig. 1) have been measurable *in vitro*: cyclopeptine dehydrogenase (CD) [2, 3], dehydrocyclopeptine epoxidase (DE) [4] and cyclopenin *m*-hydroxylase [5]. Feeding experiments with labelled compounds *in vivo* have indicated that the formation of cyclopeptine is catalysed by an enzyme complex (cyclopeptine synthetase) with tightly bound intermediates [6]. From the activities of this hypothetical complex, anthranilate adenylyltransferases (AA) is measurable in cell-free preparations. In the following paper, experiments are discussed in which the *in vitro* activities of AA, CD and DE are determined in hyphae and conidiospores, and are compared with the *in vivo* rates of cylopenin–cyclopenol biosynthesis.

RESULTS AND DISCUSSION

In accordance with the trophophase–idiophase concept of differentiation in *P. cyclopium* [1], the appearance of AA, CD and DE activities in the hyphae of batch cultures show a delay in comparison with the increase of hyphal dry wt (Fig. 2a). This demonstrates that the hyphae first grow and later on specialize with subsequent formation of the enzymes of alkaloid metabolism. The temporal separation of tropho- and idiophase depends very much on the method of cultivation. It is relatively large in batch cultures (Fig. 2a) and decreases if, by reduction of the nutrient regime, the expression of the idiophase is accelerated (Fig. 2b). Best separation was found when cultures were grown first under submerged conditions, in which the alkaloid metabolism as well as the other idiophase events do not occur, and then were transferred to emerged conditions (Fig. 2c).

During the development of the hyphae, the activities of AA, CD and DE increase more or less coordinately (cf. Figs. 2a and b). This agrees with former *in vivo* experiments which have shown that at the beginning of the idiophase cyclopeptine, dehydrocyclopeptine, cyclopenin and cyclopenol (cf. Fig. 1) appear simultaneously in the cultures and in spite of a large increase in the absolute rates of their synthesis, are released in the same relative proportions into the culture medium [6]. The increase in CD and DE activities is blocked by inhibitors of gene expression, e.g. cycloheximide (cf. Fig. 2) and 5-fluorouracil (100 μg/ml; not shown in Fig. 2) Hence it obviously depends on continuous synthesis of RNA and protein [7].

In batch cultures at the beginning of the idiophase, the *in vitro* measurable enzyme activities and the rates of cyclopenin–cyclopenol production increase almost simultaneously. Thus, at this stage of development, the amount of alkaloids formed by the hyphae can be taken as a measure of the enzyme activities present, as proposed by Nover and Luckner [1]. Later on, however, there is no further parallelism because due to starvation, the rates of alkaloid production rapidly decrease, whereas the activities of AA, CD and DE are more or less stable. This is in accordance with *in vivo* experiments where in old batch cultures (13 days *post inoculum*, p.i.) labelled phenylalanine was incorporated into cyclopenin and cyclopenol at even higher rates than in younger cultures (5 days p.i.) [8].

After discontinuous or continuous exchange of the nutrient solution, i.e. under conditions of a steady high level of nutrients, the rates of alkaloid formation are higher than under batch conditions (cf. legend to Fig. 2). Furthermore, by this kind of cultivation, the rates of alkaloid production markedly increase after the *in*

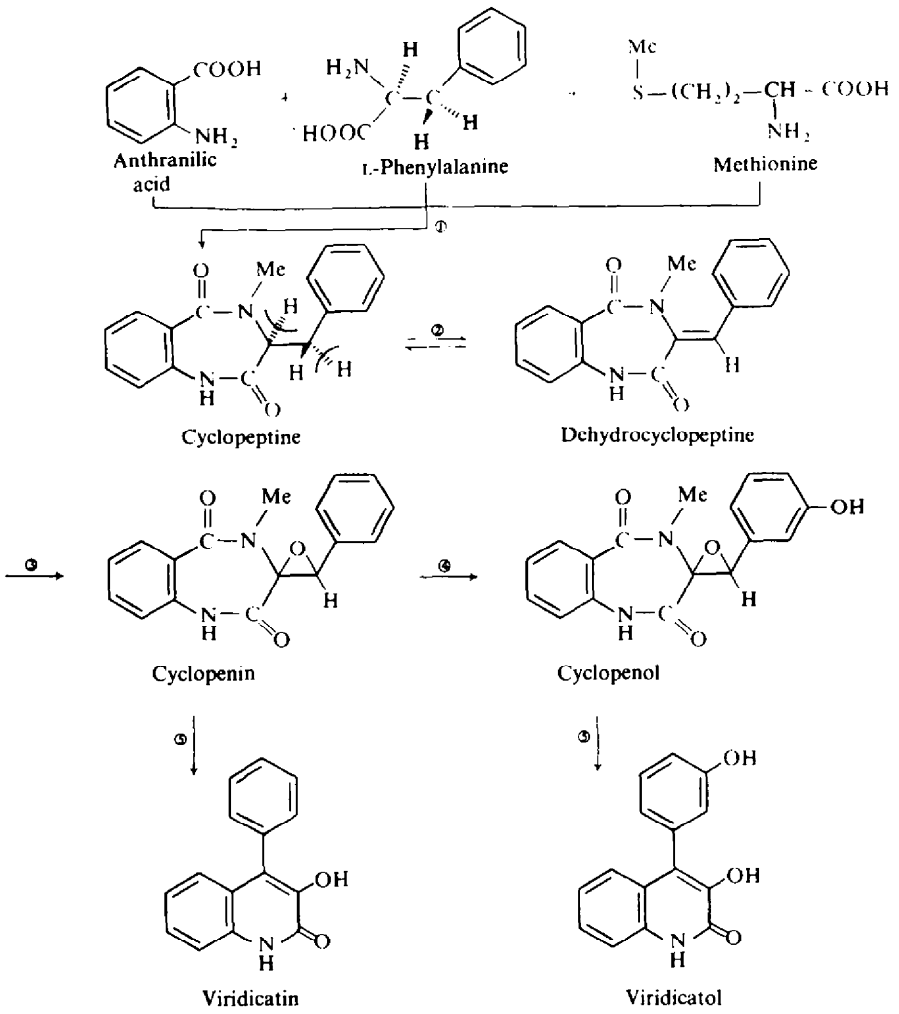

Fig. 1. Alkaloid biosynthesis in *P. cyclopium*: (1) cyclopeptine synthetase complex, hypothetical —assumed to include the enzyme activating anthranilic acid (AA); (2) cyclopeptine dehydrogenase (CD); (3) dehydrocyclopeptine epoxidase (DE); (4) cyclopenin *m*-hydroxylase; (5) cyclopenase.

vitro measurable enzyme activities have reached maximum values. This increase amounts to *ca* 3 times by discontinuous exchange and to *ca* 10 times by continuous exchange of the nutrient solution.

Experiments with cycloheximide and 5-fluorouracil, indicate that the *in vivo* rates of alkaloid biosynthesis are limited by the formation of a protein. Fig. 2 demonstrates that even at the period during which AA, CD and DE activities have reached maximum values, the further increase of the *in vivo* alkaloid formation is immediately stopped after addition of cycloheximide. Similar results were obtained after the addition of 5-fluorouracil (100 μg/ml; not shown in Fig. 2). Hence the *in vivo* rates of alkaloid formation are restricted by synthesis of a protein. The nature of this protein is still unknown. However, on the base of the arguments given above indicating a coordinated expression of all enzymes of cyclopenin–cyclopenol biosynthesis, it may be speculated that this protein limits the formation of precursors, cosubstrates, etc. or the transport of these substances to the site of alkaloid biosynthesis.

In contrast to the hyphae, AA, CD and DE are constitutive proteins of the conidiospores of *P. cyclopium*. The activities of the enzymes are detectable in homogenates of even very young spores and remain constant during conidiospore maturation (Fig. 3). Because each spore-detaching cell produces a conidiospore every 90–120 min, a fast synthesis goes on during conidiation. Conidia detachment in *P. cyclopium* is thus one of the rare examples where formation of enzymes of secondary metabolism proceeds in rapidly dividing cells. Conidiation in *P. cyclopium* shows all the features of a quantal cell cylce (cf. [9, 10]). The phialidae, the spore bearing cells, produce a clone of daughter cells, which are determined to differ in their mature state in many characteristics from their mother cell. Properties found in the spores but not in the phialidae and the other hyphal cells are, for instance, the thick rigid cell wall incrusted

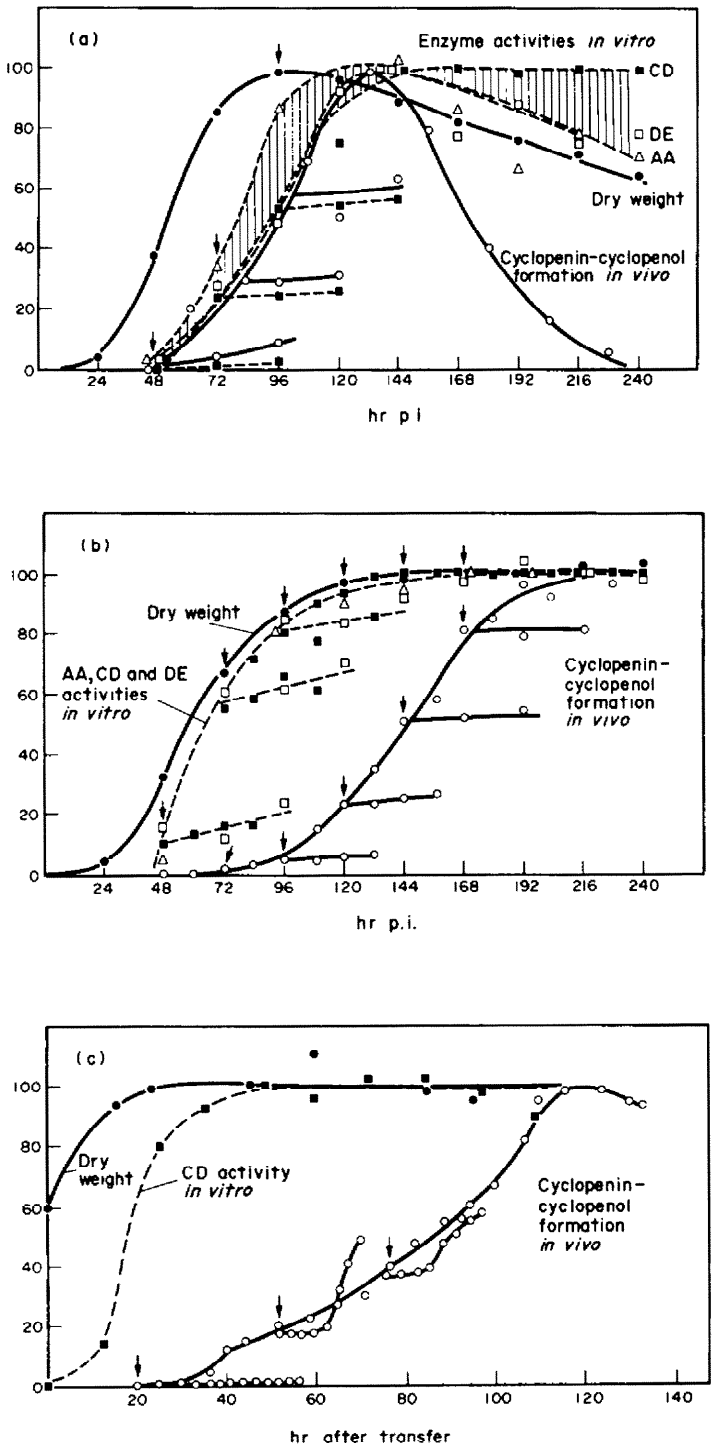

Fig. 2. *In vitro* activities of AA, CD and DE and rates of alkaloid biosynthesis in hyphae of *P. cyclopium* using different methods of cultivation: (a) batch cultures, (b) discontinuous exchange of the nutrient soln; (c) continuous exchange of the nutrient soln.

At the time indicated by arrows, cycloheximide (100 μg/ml), was added to the culture medium. The drug was removed by (b) after 24 hr, by (c) after 15 hr. In the cycloheximide treated cultures were determined in (a): CD activits and cyclopenin–cyclopenol formation, in (b): CD and DE activities and cyclopenin–cyclopenol formation, in (d): cyclopenin–cyclopenol formation. Hyphae and conidiospores were separated according to [1]. Ordinate: All values are given in units/cm^2 culture area. ●——● Dry wt: 100 = 3.6 (a), 3.5 (b) and 0.5 mg (c); △——△ AA activity: 100 = 9 (a), 5.6 pkat (b); ■——■ CD activity: 100 = 50 (a), 40 (b) and 130 pkat (c); □——□ DE activity: 100 = 0.10 (a) and 0.42 pkat (b); ○——○ Cyclopenin–cyclopenol formation: 100 = 3 (a), 9 (b) and 20 (c) pmol/sec.

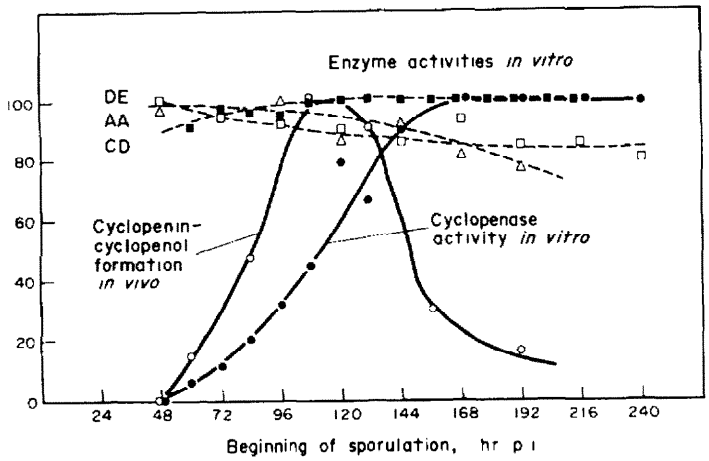

Fig. 3. *In vitro* activities of AA, CD, DE and cyclopenase, and rates of alkaloid biosynthesis during ripening of conidiospores of *P. cyclopium*.

Cultures were grown by discontinuous exchange of the nutrient soln (cf. Fig. 2b). At the time indicated by symbols, culture discs were frozen with dry ice and the conidiospores in the frozen state were brushed off. The rate of alkaloid formation was calculated from the increasing alkaloid content of the spores.

Ordinate: All values are given/mg dry wt. △——△ AA activity (100 = 1.15 pkat); ■ - - ■ CD activity (100 = 16 pkat); □ - - □ DE activity (100 = 0.007 pkat); ●·····● Cyclopenase activity (100 = 250 pkat); ○ - - ○ Rates of cyclopenin–cyclopenol formation (100 = 0.5 pmol/sec).

with the green and black spore melanins [11], and cyclopenase, the last enzyme of alkaloid metabolism (cf. Fig. 1), which becomes active during spore maturation (Fig. 3. [12]).

Furthermore Fig. 3 shows that the rates of cyclopenin–cyclopenol formation increase during the early period of spore maturation in spite of constant AA, CD and DE activities. This phenomenon exactly parallels the discrepancy between the *in vitro* activities of these enzymes in the hyphae and the *in vivo* rates of alkaloid biosynthesis mentioned above. During aging of spores, the rates of alkaloid formation rapidly drop due to starvation and probably also to the general deceleration of metabolism at the beginning of spore dormancy.

EXPERIMENTAL

P. cyclopium strain SM 72 [1] was cultivated by the following methods: (a) *Batch cultures.* The mould was grown emerged in Petri dishes (14 cm dia) with 100 ml nutrient soln NL I containing as the main nutrients 5% Glc, 0.12% NH_4 and 0.025% PO_4^{3-} [1]. (b) *Discontinuous exchange of the nutrient soln.* Beginning 48 hr p.i. the filtrate of emerged cultures was replaced every 12 hr by a nutrient soln (NL II) containing 20% of the C and N amounts of NL I and 2% of the phosphate, respectively [1]. (c) *Continuous exchange of the nutrient soln.* The mould was precultivated 48 hr in NL I under submerged conditions on a rotary shaker. The globular colonies so formed were spread in a monolayer on glass plates covered with filter paper and a dialysis membrane. The culture was covered with a glass plate to limit evapn, kept in a stream of sterile air and supplied continuously via the filter paper with nutrient soln, using NL I during the first 12 hr of emerged cultivation and later on diluted NL I containing only 15% of the original nutrient concn [13].

The dry wt of hyphae, the alkaloid content of conidiospores and the rates of cyclopenin–cyclopenol excretion by the hyphae were determined according to ref. [1]. Activities of cyclopeptine dehydrogenase, dehydrocyclopeptine epoxidase and cyclopenase were measured using the methods of [7], [4] and [12], respectively.

Anthranilate adenylyltransferase activity. Hyphae of total cultures were disintegrated by grinding with 2 parts of sand. The mixture was suspended in 2 parts of 0.25 M Tris–HCl pH 7.5 containing 10 mM mercaptoethanol. After centrifugation (15000 *g*/30 min) the supernatant was treated with $(NH_4)_2SO_4$ 40–60% satn. The ppt was dissolved in 0.04 parts of Tris–HCl pH 7.5. Conidiospores were brushed off from cultures into H_2O and collected by centrifugation. Spores (1 part) were ground with 2 parts of dry ice and mixed with 1.5 parts of H_2O. Me_2CO (13.5 parts) (−20°) were added to the conidiospore suspension for cell disintegration. After 5 min the conidia were sucked off and the residue was dried in a stream of air for 1 hr. It was suspended in 1 part of 0.25 M Tris–HCl pH 7.5 using a glass homogenizer. The test for AA activity contained in μmol: 25 anthranilic acid, 7 ATP Na salt, 20 Mg $(MeCO_2)_2$, 12.5 Tris–HCl pH 7.5, 0.5 mercaptoethanol and 0.05 ml enzyme prepn in 0.24 ml. The test was incubated 60 min at 35°. Then conidia were removed by centrifugation at 0° (2500 *g*/10 min). After addition of 20 μl 4 M KOH and 50 μl 10 M NH_2OH, the soln was left for 20 min at 35°. 10 M HCl (20 μl) and 0.5 ml $FeCl_3$-reagent were added and the mixture was centrifuged (2500 *g*/10 min). After 1 hr its *A* was determined at 530 nm against a blank which contained 0.29 ml H_2O instead of the test and the NH_2OH solns. From the measured value the *A*s of samples were substracted in which (a) KOH was added before the enzyme soln (colour of test constituents and non-enzymatic reactions with NH_2OH) and in which (b) anthranilic acid was absent (enzymatic side reactions with NH_2OH).

NH_2OH. To a methanolic soln of NH_2OH–HCl, MeOH KOH was added to pH 7.5. After centrifugation the supernatant was concd *in vacuo* until a soln of *ca* 25 M NH_2OH was formed.

$FeCl_3$-*reagent*. Equal amounts of 10% $FeCl_3$ soln, 12% TCA and 3N HCl were mixed.

REFERENCES

1. Nover, L. and Luckner, M. (1974) *Biochem. Physiol. Pflanzen* **166**, 293.

2. Aboutabl, El S. A. and Luckner, M. (1975) *Phytochemistry* **14**, 2573.
3. Aboutabl, El S. A., El Azzouny, A., Winter, K. and Luckner, M. (1976) *Phytochemistry* **15**, 1925.
4. Voigt, S. and Luckner, M. (1977) *Phytochemistry* **16**, 1651.
5. Richter, I. and Luckner, M. (1976) *Phytochemistry* **15**, 67.
6. Framm, J., Nover, L., El Azzouny, A., Richter, H., Winter, K., Werner, S. and Luckner, M. (1973) *European J. Biochem.* **37**, 78.
7. El Kousy, S., Pfeiffer, E., Ininger, G., Roos, W., Nover, L. and Luckner, M. (1975) *Biochem. Physiol. Pflanzen* **168**, 79.
8. Luckner, M. and Nover, L. (1971) *Abh. Dtsch. Akad. Wiss.*, 525.
9. Holtzer, H., Weintraub, H. and Biehl, J. (1973) in *Biochemistry of Cell Differentiation* (Monroy, A. and Tsanev, R. eds.) pp. 41–53. Academic Press, London.
10. Holtzer, H. and Rubinstein, N. (1977) in *Cell Differentiation in Microorganisms, Plants and Animals* (Nover, L. and Mothes, K., eds.) p. 424. VEB Gustav Fischer, Jena and Elsevier, Amsterdam.
11. Ha-Huy-Kê and Luckner, M. (1979) *Z. Allg. Mikrobiologie* **19**, in press.
12. Wilson, S., Schmidt, I., Roos, W., Fürst, W. and Luckner, M. (1974) *Z. Allg. Mikrobiologie* **14**, 515.
13. Nover, L. and Müller, W. (1975) *FEBS Letters* **50**, 17.