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ENZYMATIC SYNTHESIS OF CYCLOPEPTINE INTERMEDIATES IN *PENICILLIUM CYCLOPIUM*

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Key Word Index—*Penicillium cyclopium*; Aspergillaceae; fungi; enzymatics; cyclopeptine synthetase system; anthranilate adenylyltransferase; phenylalanine adenylyltransferase; alkaloids; benzodiazpines; cyclopeptine.

Abstract—Cyclopeptine, a benzodiazepine alkaloid of Penicillium cyclopium, is formed from anthranilic acid, L-phenylalanine and the methyl group of L-methionine by cyclopeptine synthetase. The following partial activities of this enzyme system were determined *in vitro*: anthranilic acid and L-phenylalanine adenylyltransferase activity, binding of anthranilic acid and L-phenylalanine as thioesters to proteins, formation of thioester-bound *N*-methyl-L-phenylalanine and *N*-methyl-L-phenylalanylanthranilic acid. The obtained results indicate that cyclopeptine is formed via enzyme-bound intermediates by the thiotemplate mechanism of peptide biosynthesis.

INTRODUCTION

Surface cultures of *Penicillium cyclopium* strain SM 72 form the benzodiazepine alkaloids cyclopeptine, dehydrocyclopeptine, cyclopenin and cyclopenol (for a summary cf. ref. [1]). Feeding experiments with isotope-labelled precursors indicated the existence of an enzyme system synthesizing cyclopeptine (1) [2]. The cyclopeptine synthetase system probably activates the precursors L-phenylalanine (Phe) and anthranilic acid (An), methylates Phe and forms cyclopeptine via enzyme-bound intermediates by the thiotemplate mechanism [3–5] (Fig. 1). Recently protein extracts from cyclopeptine-synthesizing cultures of *P. cyclopium* were obtained which catalysed the activation of An by the ATP-dependent formation of AnAMP probably as part of cyclopeptine biosynthesis [6]. This paper reports on further partial activities of the cyclopeptine synthetase system.

RESULTS

Anthranilic acid adenylyltransferase activity (Fig. 1, reaction 1)

The formation of AnAMP was determined by the PP exchange test as well as by the photometric measurement of $(AnNHOH)_3Fe$. It proceeded for more than 60 min and depended on the concentration of An, ATP and Mg^{2+} (Fig. 2). In routine tests 100 mM An (*ca* 0.75 K_m), 30 mM ATP (*ca* 8 K_m) and 80 mM Mg^{2+} (*ca* 6.3 K_m) were used.

The K_m for An (140 mM) calculated from the rates of AnAMP formation was high if compared to that of the substrates of other adenylyltransferases (cf. [7–9]). It may be caused by accumulation of AnAMP and PP in the test mixture, since relatively large amounts (~ 1–3 mM) AnAMP were necessary for the determination as $(AnNHOH)_3Fe$. PP and AMP inhibit the formation of AnAMP (Fig. 2). A similar inhibition was also shown with

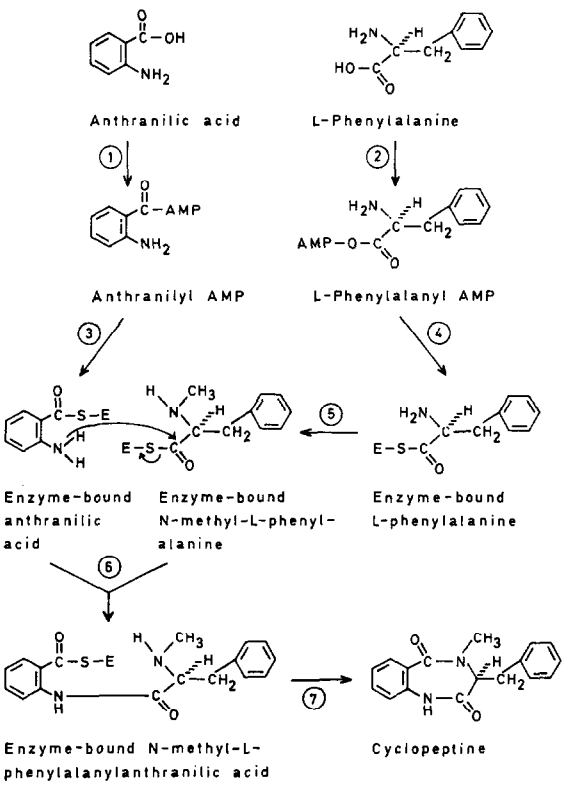


Fig. 1. Partial activities of the cyclopeptine synthetase system. (1) Anthranilic acid adenylyltransferase; (2) L-phenylalanine adenylyltransferase; (3) covalent binding of anthranilic acid; (4) covalent binding of L-phenylalanine; (5) methylation of enzyme-bound L-phenylalanine by *S*-adenosyl-L-methionine; (6) translocation of *N*-methyl-L-phenylalanine to enzyme-bound anthranilic acid; (7) release of cyclopeptine by cyclization.

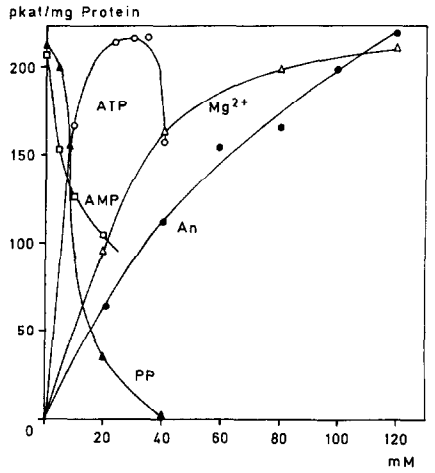


Fig. 2. Activity of An adenylyltransferase in relation to concentrations of substrates and effectors.

other enzymes forming substrate–AMP derivatives, e.g. aminoacyl tRNA synthetases [10] and bacterial peptide synthetases [11–13]. High concentrations of ATP decreased the measurable amount of AnAMP due to the increase of An-independent reactions of ATP with the constituents of the test mixture. Addition of ADP had no influence on AnAMP biosynthesis, but substitution of ATP by equimolar amounts of GTP or UTP reduced the enzyme activity to 15% and < 1%, respectively.

The An adenylyltransferase was activated by NH_4^+ (0.2 M NH_4^+ increased the enzyme activity by > 40%), a phenomenon also described for other enzyme systems incorporating amino acids [14–16]. It was inhibited by *p*-chloromercuribenzoate (2 mM caused 80% inhibition) indicating the presence of SH-groups necessary for enzyme activity. Addition of Na^+, K^+ and Ca^{2+} (20 mM) as well as of CoA (1–5 mM) did not alter the activity.

The benzodiazepines cyclopeptine, cyclopenin and cyclopenol as well as the quinoline derivatives viridicatin and viridicatol formed in later steps of the alkaloid metabolism of *P. cyclopium* [1] showed no influence on An adenylyltransferase activity. This is in contrast to inhibition by these compounds of cyclopeptine dehydrogenase, dehydrocyclopeptine epoxidase and cyclopenin *m*-hydroxylase, enzymes also involved in benzodiazepine alkaloid biosynthesis [17–19].

The An adenylyltransferase activity depended strongly on the pH of the reaction mixture (pH 6.7, 100%, pH 6.0 and pH 7.2, 50%) and on the concentration of the Tris–HCl buffer used (20 mM 50%, 90 mM 100%). However, the addition of NaCl up to concentrations of 3 M did not influence the activity. An adenylyltransferase activity was stable for 20 min at 35°, but was reduced to 70% by treating for 20 min at 50°.

L-Phenylalanine adenylyltransferase activity (*Fig.* 1, *reaction* 2)

The activation of Phe was determined with the PP exchange test or more reliably by the formation of PheNHOH. The PheNHOH may be determined photometrically as the $(PheNHOH)_3Fe$ complex or more precisely by use of *Phe (L-[U-^{14}C]phenylalanine) and separation of the *PheNHOH (L-[U-^{14}C]phenylalanyl hydroxamic acid) formed from excess *Phe by TLC. The pattern of *PheNHOH formation and the optimum values of temperature, pH and buffer concentrations resembled those in the synthesis of AnAMP. Maximum Phe adenylyltransferase activity was measured with 70 mM Phe, 28 mM ATP, 50 mM Mg^{2+} and 2.5 mM NH_2OH (if added to the enzymatic test) or 1.5 M NH_2OH (if present during the post-incubation period). Phe adenylyltransferase activity was inhibited by the MeOH, which was present in the NH_2OH preparations used (inhibition by 50 µl MeOH/test 22%, by 100 µl/test 87%). It was increased by NH_4^+ (100 mM 50%). The Phe adenylyltransferase activity measurable was small (0.1–0.2 pkat/mg protein) if NH_2OH was added directly to the incubation mixture (cf. [20]). It reached values of *ca* 100 pkat/mg protein if PheNHOH was formed during a post-incubation period as described for AnNHOH.

Covalent binding of anthranilic acid and L-*phenylalanine* (*Fig.* 1, *reactions* 3 *and* 4)

Table 1 shows that in the presence of ATP and Mg^{2+} *An ([^{14}COOH]anthranilic acid) and *Phe were bound (acid-stable) to the protein fraction investigated. Phe gave maximum binding at pH 7.0 (50% values at pH 5.0 and 8.3) in concentrations from 5–25 mM. The optimum temperature was 35°. Binding increased linearly to 2 mg protein/test. Bivalent cations stimulated it considerably. Best results were obtained with 13 mM Mg^{2+}. Substitution of Mg^{2+} by equal amounts of Mn^{2+} or Fe^{2+} reduced the binding of *Phe to 70% and 30%, respectively. As with alamethicine synthetase [21] the Phe-binding capacity of the protein fraction used was nearly saturated within 5 min and did not change considerably within the next 60–90 min.

Two types of protein-bound Phe seem to exist in *ca* equal amounts. In typical experiments a total of *ca* 18 pmol Phe/mg was bound to the proteins of the test mixture. By treatment with TCA 10 pmol Phe/mg protein were released whereas 8 pmol Phe/mg protein remained bound. The Phe bound TCA-labile may correspond to PheAMP which is liberated during the denaturation of proteins by TCA. The Phe bound acid-stable seems to be

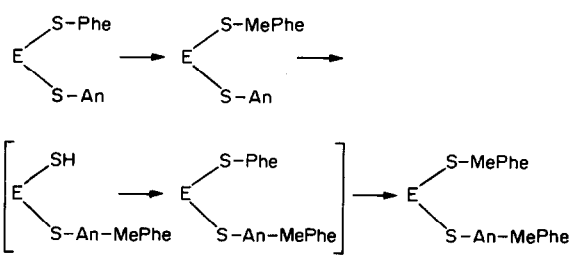
Table 1. Binding of Phe, An and the Me-group of SAM to purified protein fractions from *P. cyclopium*

Radioactive-labelled substrate	Non-radioactive substrate added	Radioactive-labelled substrate bound acid-stable [pmol/mg protein]
Phe	—	8
Phe	SAM	7
Phe	An	19
Phe	SAM + An	13
An	—	24
An	Phe	16
An	SAM + Phe	16
SAM	—	0.7
SAM	Phe	7
SAM	Phe + An	16

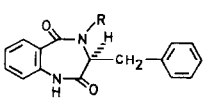
thioester-bound Phe as indicated by the inhibition of its formation by iodoacetamide (1.25 mM iodoacetamide reduce Phe binding to less than 25%) and by the instability of the TCA-stable bonds to dilute alkali (cf. [22]). After alkaline hydrolysis free *Phe and *An were identified by TLC.

Formation of enzyme-bound N-*methyl*-L-*phenylalanine and* N-*methyl*-L-*phenylalanylanthranilic acid* (*Fig.* 1, *reactions* 5 *and* 6)

Binding of *An or *Phe to the proteins was not influenced by amino acids, like L-tyrosine or L-valine. Incubation of *Phe together with An, however, increased the enzyme-bound amount of Phe considerably (Table 1). In contrast *An was bound less in the presence of Phe. Binding of the methyl group of *SAM (*S*-adenosyl-L-[Me-^{14}C]-methionine) to the proteins is very small in the absence of Phe but equal to Phe in the presence of Phe or a mixture of Phe and An. These results are in agreement with the idea that (a) there are different binding sites for Phe and An in the cyclopeptine synthetase system, which probably do not occur in equal amounts (cf. the larger binding of An in comparison to Phe), (b) that the bound Phe is methylated and (c) that MePhe is transferred to the bound An, forming protein bound *N*-methyl-L-phenylalanylanthranilic acid (3). The Phe-binding site is set free by this transfer so that in the presence of An, Phe may again be bound and methylated:

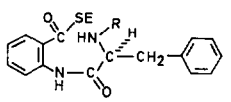


Incubation of *Phe together with An even in the absence of SAM increases Phe binding considerably (Table 1). Hence unmethylated Phe may also be transferred to An, forming enzyme-bound L-phenylalanylanthranilic acid (4) so that the Phe binding site is again set free. However, demethylcyclopeptine (2), which may be formed from L-phenylalanylanthranilic acid by cyclization, could never be detected in cultures of *P. cyclopium* and radioactive-labelled demethylcyclopeptine administered to cultures of *P. cyclopium* synthesizing benzodiazepine derivatives was not incorporated into cyclopenin and cyclopenol [2]. Hence the formation of protein-bound L-phenylalanylanthranilic acid may be an *in vitro* artefact similar to those formed in the biosynthesis of enniatin B [23].



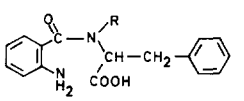
1 Cyclopeptine (R=CH_3)

2 Demethylcyclopeptine (R=H)



3 Enzyme-bound N-methyl-L-phenylalanylanthranilic acid (R=CH_3)

4 Enzyme-bound L-phenylalanylanthranilic acid (R=H)



5 Anthranilyl-N-methylphenylalanine (R=CH_3)

6 Anthranilylphenylalanine (R=H)

Cyclization of the enzyme-bound N-*methyl*-L-*phenylalanylanthranilic acid to cyclopeptine* (*Fig.* 1, *reaction* 7)

As yet the formation of cyclopeptine (1) could not be demonstrated in the experiments described. But the results discussed above indicate that enzyme bound *N*-methyl-L-phenylalanylanthranilic acid cyclizes with release from the enzyme protein to this compound. In chemical experiments derivatives of anthranilyl-*N*-methylphenylalanine (5) cyclized easily to cyclopeptine [24]. There are, however, no indications that compounds of this type are formed by the enzyme preparations of *P. cyclopium*.

DISCUSSION

The results given above indicate that the cyclopeptine synthetase system catalyses the partial reactions 1–7 shown in Fig. 1 in correspondence with the thiotemplate mechanism of peptide biosynthesis. With the exception of reaction 7 these were measurable *in vitro*. Their optimum conditions resembled each other in several respects. The activating and binding activities were soluble (no sedimentation during centrifugation at 150 000 *g* for 90 min) and were precipitated with $(NH_4)_2SO_4$ (40–60% saturation) indicating that they are related to each other. The Phe binding activity (which included the Phe adenylyltransferase activity) was shown to be part of a high M_r protein by chromatography on Sepharose 6B. This protein, extracted after lyophilization of the hyphae, might also contain the other partial activities and represent the native cyclopeptine synthetase system. It could not be isolated after mechanical disruption, whereas An and Phe adenylyltransferase activities were measurable in the respective preparations. These results indicated that cyclopeptine synthetase is an unstable system, which like other peptide synthetases (cf. [21]) can be isolated only by the use of very careful methods. Proteinases seem to destroy it rapidly, since An and Phe adenyltransferase activities were found only in enzyme preparations containing protease inhibitors like PMSF or *o*-phenanthroline.

EXPERIMENTAL

Cultivation of P. cyclopium. Strain SM 72 [25] was grown in Petri dishes ϕ 14 cm on the surface of 100 ml nutrient soln NL I [26]. The cultures were harvested at the sixth day after inoculation.

Degradation of hyphae. For determination of An and Phe adenylyltransferase activities one part of the sporulating mycelium was ground with two parts of sand at 0° for 10 min. The mixture was suspended in two parts of 0.25 M Tris–HCl pH 7.5 containing 0.01 M EtSH and centrifuged (30 min, 15 000 *g*). The proteins precipitating with $(NH_4)_2SO_4$ (40–60% satn) were redissolved in 0.04 parts 0.05 M Tris–HCl pH 7.5 and passed through a column of Sephadex G 50.

For determination of An and Phe binding the mycelial mats were rinsed with H_2O, pressed between filter paper and permeabilized by lyophilization. Dried mycelium (17 g) was pow-

dered and suspended in 300 ml of a soln containing 0.2 M Tris–HCl, pH 7.5, 0.005% PMSF (phenylmethyl sulfonylfluoride) and 0.05% EtSH. After 1 hr the suspension was centrifuged (30 min, 20000 *g*) and the supernatant treated with $(NH_4)_2SO_4$ (40–60% satn). The proteins ppted were dissolved in 30 ml 0.05 M Tris–HCl, pH 7.5 containing 10% sucrose and passed through a column of Sephadex G 50.

Determination of An and Phe adenylyltransferase. (a) Pyrophosphate exchange. 50 μl enzyme soln, 25 μmol An, 7 μmol ATP, 20 μmol $Mg(OAc)_2$, 100 μmol KF, 12.5 μmol Tris–HCl pH 7.5, 10 μmol EtSH, 1 μmol phenanthroline, 1 μmol ^{32}PP (total vol. 400 μl) were incubated for 20 min at 35°. To the blank An or Phe were added immediately before stopping the incubation by cooling to 0° and addition of 250 μl of a 15% soln of $HClO_4$ containing 0.4 M $Na_4P_2O_7$. For the *A* of ATP 1 ml of a suspension of 6 g charcoal in 0.1 M NaOAc buffer, pH 4.5, was added and the mixture shaken for 90 min at 0°. The charcoal was removed by centrifugation, washed successively with 6 ml each of a mixture of 100 ml 0.05 M NaOAc buffer, pH 4.5 and 100 ml of a 0.1 M soln of $Na_4P_2O_7$, and of 100 ml 0.05 M NaOAc buffer, pH 4.5 and 100 ml H_2O by shaking for 3 min at 0° followed by centrifugation. The ATP bound was hydrolysed with 1 ml 2 M HCl by heating at 100° for 30 min. After centrifugation 0.1 ml of the supernatant was used for detm of radioactivity.

(b) Photometric determination of $(AnNHOH)_3Fe$ (cf. [6]). The reaction with NH_2OH was carried out in an alkaline post-incubation medium, because near pH 7 the optimum of An adenylyltransferase, *O*-(anthranoyl)-hydroxylamine was formed, which gave no colour with Fe^{3+} [27]. Optimum conditions were tested with An Me ester as AnAMP equivalent. The formation of AnNHOH increased to 1.5 M NH_2OH, but depended on the amount of MeOH present in the NH_2OH preparations used (a 10 M NH_2OH soln prepared after ref. [6] contained *ca* 5–10% MeOH. 20 μl MeOH/test decreased the measurable *A* by more than 50%). The alkaline pH necessary for AnNHOH formation was adjusted with *ca* 60 μmol NaOH/test. The addition of higher amounts (to 200 μmol NaOH/test) did not inhibit the reaction. The formation of AnNHOH proceeded within 20 min. It was stopped by addition of HCl. The Fe^{3+} complex was formed with $FeCl_3$-reagent (optimum $FeCl_3$ concn 10%) [6]. The complex was stable for more than 2 hr. With the relatively crude adenylyltransferase preparations used side reactions occurred. The *A* of impurities reacting with NaOH or NH_2OH and of substances other than An activated by enzyme preparations were therefore subtracted. For each test four variants were prepared: variant I (full test mixture), variant II (full test mixture, NaOH added already before the beginning of the incubation to avoid enzyme reactions), variant III (test mixture without An) and variant IV (NaOH added to the An-free test mixture before the beginning of the incubation). The difference of the *A* of variants I and II represented the *A* produced by AnAMP and enzymatic side reactions, corrected by the *A* produced by substances present in the mixture already at the beginning of the incubation. The differences of the *A* of variants III and IV gave the *A* produced by enzymatic side reactions. Subtraction of the value (III–IV) from (I–II) eliminated most enzymatic and non-enzymatic interfering processes.

(c) Photometric determination of $Phe(NHOH)_3Fe$: 100 μl enzyme soln, 20 μmol Phe, 7 μmol ATP, 20 μmol $Mg(OAc)_2$, 10 μmol Tris–HCl pH 7.5 (total vol. 290 μl) were incubated 60 min at 35°. After addition of 50 μl 10 M NH_2OH the mixture was left 10 min at 34°. The post-incubation was stopped by cooling to 0°; 500 μl $FeCl_3$-reagent [6] were added. The proteins ppted were removed by centrifugation (10 min, 2500 *g*) and the *A* of the supernatant was measured after 60 min at 500 nm. As blank the mixture without Phe was used. The reaction was calibrated with PheNHOH prepared from Phe Me ester with NH_2OH in MeOH (mp 195°, uncorr.).

(d) Chromatographic separation of *PheNHOH from excess *Phe: 0.03 μmol *Phe (= 5×10^6 ipm), 10 μmol ATP, 200 μl enzyme soln, 20 μmol $Mg(OAc)_2$, 55 μmol Tris–HCl pH 7.5, 2 μmol EtSH, 1 mol NH_2OH (total vol. 400 μl) were incubated 40 min at 35°. The samples were then held for 2 min at 70° to denature proteins and centrifuged (10 min, 3000 *g*). In the blanks the enzyme preparation was substituted by 200 μl 0.05 M Tris–HCl, pH 7.5. Phe and PheNHOH were separated by TLC on silica gel G, thickness 0.5 mm. 20 μl of the supernatant of the enzyme test and the blank were put on start bands 15 × 3 mm in triplicate. The plates were developed in EtOAc–pyridine–H_2O–HOAc (70:15:15:2). The area containing the *PheNHOH near the front was scraped off and the radioactivity determined. Synthetic PheNHOH (see above) was used for calibration.

Determination of TCA-stabile binding of Phe, An and SAM. Enzyme soln (500 μl), 5 μmol ATP, 10 μmol $Mg(OAc)_2$, 100 μmol Tris–HCl pH 7.0, 1 μCi *An, *Phe or *SAM and if necessary 1 μmol of Phe, An or SAM (total vol. 750 μl) were incubated for 30 min at 35°. As blank the mixture without ATP and $Mg(OAc)_2$ was used. Binding was stopped by addition of 50% TCA to a final concn of 7%. After 30 min at 0° the ppt was passed through glass microfibre paper (Whatman GF/A), washed × 5 with 30 ml 5% TCA, twice with 30 ml EtOH and once with 30 ml EtOH–Et_2O (1:3). The radioactivity of the ppt was measured by liquid scintillation counting.

Hydrolysis of thioester-bound Phe or An. The ppt obtained with TCA was pelleted by centrifugation (10 min, 5000 *g*) and washed by resuspension and centrifugation as given above. It was then incubated for 60 min at 40° with 200 μl 50 mM NaOH. After addition of 10 μl 1 M HCl and lyophilization the mixture was extracted with 400 μl 80% EtOH. The EtOH extract was chromatographed (silica gel, *n*-BuOH–HOAc–H_2O, 4:1:1) and EtOAc–pyridine–HOAc–H_2O (60:20:6:11); R_f: Phe 0.40, An 0.80. The radioactive substances present were determined after elution with EtOH.

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