REVIEW ARTICLE

Cyclization of α-Aminoadipic Acid into the δ-Lactam 6-Oxo-piperidine-2-carboxylic Acid by *Penicillium chrysogenum*

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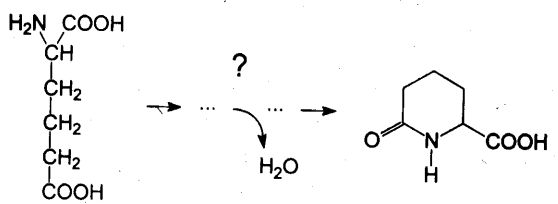
(Received for publication December 22, 1997)

I. Introduction

When penicillin is produced by fed-batch cultivations of the filamentous fungus *Penicillium chrysogenum* there is a significant excretion into the extracellular medium of byproducts and intermediates related to the penicillin biosynthetic pathway[1]. Whereas the mechanisms behind formation of most of the byproducts are known, the route leading to the byproduct 6-oxo-piperidine-2-carboxylic acid (OPC), the cyclized δ-lactam of α-aminoadipic acid (α-AAA), is unknown (Fig. 1). The open literature contains only a few reports on OPC formation during β-lactam cultivations[1~9], although its formation has a significant impact on the overall process costs for the industrial production of β-lactams. Table 1 summarizes the reported values on OPC formation relative to the formation of penicillin. Apparently, the extent of OPC formation depends both on the applied strain and on the cultivation conditions. KURZATKOWSKI *et al.*[7] reported relative values on OPC formation as high as 60% during penicillin G cultivations. It is, however, likely that industrial strains of *P. chrysogenum*

Fig. 1. Formation of OPC by cyclization of α-AAA.



α-Aminoadipic acid (α-AAA) 6-Oxo-piperidine-2-carboxylic acid (OPC)

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Table 1. Reported values on OPC formation during penicillin cultivations.

Reference	OPC formation[a]	Comments
JØRGENSEN *et al.*[1]	6~11%	Production of penicillin V in lab-scale fed-batch cultivation
BRUNDIDGE[2]	28%	Industrial penicillin V cultivation and shakeflasks
HERSBACH *et al.*[5]	15%	Industrial penicillin G cultivation
KURZATKOWSKI *et al.*[7]	36~60%	Production of penicillin G in shakeflasks

[a] Relative to the formation of penicillin (on a molar basis).

produce relatively smaller amounts of OPC due to an indirect selection for low OPC producing strains when screening for high penicillin producing strains. In fact, the high yielding strain applied by JØRGENSEN *et al.*[1] known to produce approx. 25 g/liter of penicillin V during 200 hours of fed-batch cultivation, shows a relative OPC formation in the range of 6~11%.

The biosynthesis of penicillin implies no direct net utilization of α-AAA since the α-AAA moiety is cleaved off and replaced by the penicillin sidechain precursor in the final enzymatic step in the pathway. Formation of OPC by cyclization of α-AAA in an enzymatic reaction associated with penicillin synthesis represents an undesirable loss of carbon and Gibbs free energy and causes a requirement for a net synthesis of α-AAA by the microorganism. Table 2 illustrates the influence of OPC formation on the maximum theoretical yield of penicillin on glucose, $Y_{sp,max}$ (mole/mole) at non-growth conditions, *i.e.* the specific growth rate $\mu=0$. According to JØRGENSEN *et al.*[11] even a moderate OPC formation of 15% on a molar basis reduces $Y_{sp,max}$ significantly (from 0.43 to 0.39) and at a ratio of 1 : 1 between penicillin and OPC formation $Y_{sp,max}$ has decreased by almost 50%. The impact of OPC formation on $Y_{sp,max}$ is not limited to non-growth conditions, OPC formation will also at $\mu>0$ cause a significant decrease of $Y_{sp,max}$.

The formation of OPC is assumed to be related to the biosynthetic pathway of β-lactam antibiotics since it is formed in a fixed stoichiometric ratio to penicillin during fed-batch cultivations[1]. However, the route of its formation is unknown and in this paper several hypotheses on the mechanism behind OPC formation in *P. chrysogenum* are presented and discussed on the basis of both new experimental results and literature data.

II. Possible Mechanisms for OPC Formation

Despite the apparant influence (Table 2) of OPC formation on $Y_{sp,max}$ (and thereby on the overall process costs), the exact mechanism behind OPC formation is not known. OPC is most likely formed in a single biosynthetic reaction, but could possibly also be produced by more than one mechanism. Several mechanisms responsible for the OPC formation may be proposed:

- Spontaneous, non-enzymatic ring closure of α-AAA.
- Formation by the glutamine synthetase in the absence of ammonia.
- Formation by the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase, probably in the absence of at least one of the two other precursor amino acids L-cysteine and L-valine.
- Formation by the acyl-CoA : isopenicillin N acyltransferase (AT) when the α-AAA moiety of isopenicillin N (IPN) is either cleaved off or exchanged by the penicillin sidechain precursor to form 6-amino-penicillanic acid and penicillin, respectively.
- *Via* the γ-glutamyl cyclotransferase activity of the

Table 2. Maximum theoretical yield of penicillin on glucose at non-growth conditions reported in literature.

Reference	OPC formation[a]	$Y_{sp,max}$[b]
COONEY & ACEVEDO[10]	0	0.56
	1.00	0.33
HERSBACH *et al.*[5]	0	0.44
	1.00	0.31
JØRGENSEN *et al.*[11]	0	0.43
	0.15	0.39
	0.60	0.31
	1.00	0.26

[a] Relative to the formation of penicillin (on a molar basis).

[b] Maximum theoretical yield of penicillin on glucose [moles penicillin/mole glucose].

γ-glutamyl cycle.

- Other mechanisms than mentioned above.

A. Spontaneous, Non-Enzymatic Ring Closure

The ring closure of α-AAA into the six-membered piperidone form OPC is analogous to the ring closure of glutamic acid into the corresponding five-membered pyrrolidonecarboxylic acid (synonymous to pyroglutamic acid and 5-oxoproline), the latter being extensively studied by WILSON & CANNAN[12] and GREENSTEIN & WINITZ[13]. In contrast, aspartic acid and α-aminopimelic acid do not form the corresponding four- and seven-membered ring systems[13], but cyclic forms of the α-keto acids analogous of arginine, citrulline, homoarginine and homocitrulline do exist[14]. If OPC formation proceeded by spontaneous, non-enzymatic ring closure of α-AAA, OPC would most likely be detected during growth of all microorganisms where α-AAA serves as an intermediate in the biosynthesis of lysine, *i.e.* in fungi. However, the spontaneous, non-enzymatic ring closure of α-AAA in aqueous solutions is far too slow at 25°C[13] to be responsible for the OPC formation observed during penicillin cultivations. By refluxing α-AAA in aqueous solution at 100°C equilibrium between α-AAA and OPC is established within 2 hours consisting of 73% OPC and 27% α-AAA[15].

B. Glutamine Synthetase

Evidence for the activity of glutamine synthetase from pea and from sheep brain towards α-AAA exists in literature[16~18]. Highly purified sheep brain glutamine synthetase was shown to act on both optical isomers of α-AAA thereby forming L- and D-homoglutamine. Furthermore, in the absence of ammonia and hydroxylamine the enzyme catalyzed the ring closure of α-AAA into OPC. Glutamine synthetase (GS) is one of the two enzymes constituting the so-called GS-GOGAT pathway (the other enzyme being glutamate synthase, abbreviated GOGAT from its previous trivial name: glutamine amide-2-oxoglutarate aminotransferase) which is an ATP dependent high affinity system for assimilation of ammonia. However, the GS-GOGAT pathway is repressed by ammonia[19,20], and at most cultivation conditions ammonia is not growth limiting, though penicillin biosynthesis in *P. chrysogenum* is repressed by ammonia[21], and nitrogen assimilation is mainly by the NADP-linked glutamate dehydrogenase as illustrated for the growth of *Acremonium chrysogenum*[22]. Since OPC formation is also observed when ammonia is in excess, it is unlikely that glutamine synthetase is the enzyme catalyzing the ring closure of α-AAA during penicillin cultivations.

C. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine Synthetase

The initial enzymatic step in the biosynthetic pathways of all β-lactam antibiotics is the condensation of the three precursor amino acids L-α-AAA, L-cysteine and L-valine into the tripeptide ACV which is catalyzed by the multifunctional enzyme ACV synthetase. The first function of the ACV synthetase is supposed to be the activation of the three precursor amino acids by ATP hereby forming aminoacyladenylates. These aminoacyladenylates are bound non-covalently to the enzyme before they are transfered to three distinct acceptor sites and coupled to thiol-groups of the enzyme[23]. It is therefore likely that α-AAA in some cases, perhaps in the absence of one or two of the other precursor amino acids, is released from the enzyme in the activated form thus being more susceptible to cyclization into OPC. In this case, OPC formation should be a common phenomenon observed during the synthesis of all β-lactam antibiotics.

Recently, THEILGAARD *et al.*[24] reported the purification and characterization of the ACV synthetase from *P. chrysogenum*. This purified enzyme has been used to investigate a potential link between OPC formation and ACV synthetase activity. At optimal *in vitro* conditions[24] enzyme assays were performed both with all three precursor amino acids present and with either one or two of the precursor amino acids L-cysteine and L-valine absent. When all three precursor amino acids were present ACV formation was observed. However, none of the assays showed any detectable level of OPC formation. ACV synthetase is therefore most likely not the enzyme responsible for OPC formation.

D. Acyl-CoA : isopenicillin N Acyltransferase

The tripeptide ACV is further converted into IPN in the second enzymatic step common to all β-lactam synthesizing microorganisms, and represents the completion of the bicyclic penam ring structure. IPN is a branchpoint in microorganisms producing different kinds of β-lactam antibiotics. In cephem producing microorganisms IPN is epimerized into penicillin N (penN) by a specific epimerase and further processed into different cephalosporins and cephamycins, whereas *P. chrysogenum* and *Aspergillus nidulans* produce a series of penicillins through the exchange of the α-AAA moiety of IPN with the corresponding CoA-thioester activated sidechain precursors. This sidechain exchange is carried

out by the enzyme AT and can proceed by either a one step mechanism or by a two step mechanism[25]. The two step mechanism differs from the one step mechanism with respect to the release of the intermediate 6-amino-penicillanic acid from the enzyme. When the supply of the activated sidechain precursor is low the two step mechanism most likely prevails and in the absence of activated sidechain precursor accumulation of 6-amino-penicillanic acid is observed[3,26]. The removal of the α-AAA moiety from IPN could potentially lead to formation of OPC in both the one step mechanism and in the two step mechanism. If this holds true, OPC formation would be limited to microorganisms possessing AT activity, *i.e.* penicillin producing strains of *P. chrysogenum* and *A. nidulans*.

BRUNDIDGE *et al.*[2] noted that OPC was formed in an almost constant 1 : 1 ratio to 6-amino-penicillanic acid and the carboxylated form 8-hydroxy-penillic acid in cultures of *P. chrysogenum* grown in the absence of sidechain precursor. This observation strongly suggests that OPC formation is linked to the IPN amidohydrolase activity of the AT which is responsible for removing the α-AAA moiety from IPN in the two step mechanism. However, HENRIKSEN *et al.*[3] reported a ratio of 1 : 2.8 between the specific formation rates of OPC and the sum of 6-amino-penicillanic acid and 8-hydroxy-penillic acid in steady state continuous cultures without sidechain precursor added to the feeding medium. Moreover, TROWN *et al.*[9] did also observe formation of OPC during growth of a cephalosporin C producing strain of *Cephalosporium*. GUTIERREZ *et al.*[27] found no homologous DNA sequence to the *pen*DE gene of *P. chrysogenum* encoding the AT in a number of strains of *C. acremonium*. Since OPC formation is not limited to penicillin producing microorganisms but also observed during the biosynthesis of cephalosporin, the AT can be ruled out as being responsible for OPC formation.

E. γ-Glutamyl Cyclotransferase

The tripeptide ACV formed in the initial step of the biosynthetic pathways of all β-lactam antibiotics is structurally very similar to glutathione (GSH), the tripeptide γ-(L-glutamyl)-L-cysteinyl-glycine. Both contain the free thiol-group of the cysteine residue, but also one unusual peptide bond between the δ-carbon atom of α-AAA and the γ-carbon atom of glutamate and the cysteine residue in respectively ACV and GSH. As a consequence of the structural similarity, it has been suggested that ACV and GSH were synthesized by the same set of enzymes[28], and speculations have been made whether at one time GSH was a universal penem-like precursor of antibiotics in a variety of life forms, since GSH potentially could be converted into the β-lactam glutacillin which is structurally very similar to IPN[29]. GSH is present in most living cells in rather high concentrations and serves a large number of metabolic functions[30,31]. In higher eukaryotes, GSH is a part of the γ-glutamyl cycle which among other things serves to transport extracellular amino acids across the plasma membrane. It is though questionable whether the entire γ-glutamyl cycle exists in microorganisms[32]. There has been some controversy concerning the existence of the entire γ-glutamyl cycle in yeast and its importance with respect to transport of extracellular amino acids. MOOZ & WIGGLESWORTH[33] and OSUJI[34,35] claimed that the γ-glutamyl cycle takes an active part in the transport of amino acids in yeast, whereas ROBINS & DAVIES[36] reported that no correlation exists between amino acids uptake and GSH turnover in yeast. Later, JASPERS *et al.*[32] observed that only part of the enzyme activities in the cycle could be detected in *Saccharomyces cerevisiae*, *i.e.* activity of γ-glutamyl transpeptidase and cysteinyl-glycine dipeptidase was detected whereas activity of γ-glutamyl cyclotransferase and 5-oxoprolinase remained undectected. The question whether the entire γ-glutamyl cycle is present in *P. chrysogenum* therefore remains unanswered, though *P. chrysogenum* is known to secrete GSH into the extracellular medium[3,4,37].

The enzyme γ-glutamyl cyclotransferase forms a potential link between the γ-glutamyl cycle and OPC formation. In the active cycle for transporting extracellular amino acids across the plasma membrane, the enzyme is responsible for the release of the transported amino acids and the cyclization of the γ-glutamyl moiety of GSH into 5-oxoproline. If, somehow, ACV is able to enter the γ-glutamyl cycle in a similar way as GSH, OPC could potentially be formed by cyclization of α-AAA in the enzymatic step catalyzed by γ-glutamyl cyclotransferase. The formed 5-oxoproline subsequently undergoes a decyclization into glutamate performed by the enzyme 5-oxoprolinase at the expense of ATP. This enzyme has been shown to accept OPC as substrate and carry out a similar decyclization into α-AAA though at the expence of four times as much ATP as for the decyclization of the natural substrate 5-oxoproline[38,39]. The potential decyclization of OPC by 5-oxoprolinase could explain the reversion of L-lysine inhibition of penicillin biosynthesis observed by KURZATKOWSKI *et al.*[8] when adding OPC to the extracellular medium of growing cultures of *P. chrysogenum*.

If ACV enters the γ-glutamyl cycle and causes formation of OPC in the step catalyzed by γ-glutamyl cyclotransferase, a correlation between the intracellular pool of ACV and OPC formation is likely to be observed. Such a correlation was indeed observed by HENRIKSEN *et al.*[4] in a series of steady state continuous cultivations performed at different levels of dissolved oxygen concentration. Thus both the intracellular pool of ACV and the specific formation rate of OPC were constant at high dissolved oxygen concentration, and both increased at low dissolved oxygen concentrations.

F. Other Mechanisms

All the above mentioned hypotheses for OPC formation have in common that OPC is formed exclusively as the L-isomer by cyclization of L-α-AAA. However, BRUNDIDGE *et al.*[2] observed the formation of an almost racemic mixture of OPC with a slight excess of the L-isomer during growth of *P. chrysogenum*. This implies that a racemization of either α-AAA prior to cyclization or of the formed OPC takes place. Normally, amino acids, including α-AAA, do not readily racemize at physiological conditions[40], and the spontaneous, non-enzymatic cyclization itself does not involve any racemization[15,41]. Racemization of α-AAA or OPC therefore has to be catalyzed enzymatically. The D-isomer of α-AAA does not play any known physiological role in *P. chrysogenum* or in any other β-lactam producing microorganism, except that the α-AAA moiety of cephalosporins and cephamycins is the D-isomer. The D-α-AAA moiety arises from the enzymatic conversion of IPN to penN by the IPN epimerase, an enzyme which carries out an almost complete racemization of the α-AAA moiety of IPN/penN. The IPN epimerase is encoded by the gene *cef*D, which in cephamycin producing bacteria is located in the single cluster containing the genes encoding all the enzymes in the pathway, whereas it is still unknown whether the gene is linked to any of the two clusters containing the cephalosporin biosynthetic genes in *Cephalosporium acremonium*[42]. According to CANTWELL *et al.*[43], *P. chrysogenum* expresses no IPN epimerase activity and a probe of the *cef*D gene encoding the enzyme in *Streptomyces lipmanii* does not hybridize to the DNA of *P. chrysogenum*. However, observations by ALVI *et al.*[44] indicate the presence of some IPN epimerase activity in *P. chrysogenum*. Recombinant strains of *P. chrysogenum* expressing deacetoxycephalosporin C synthase (expandase) activity were found to produce deacetoxycephalosporin C, and since the expandase shows no affinity towards IPN[45,46], epimerization of IPN into penN has to take place. As pointed out by ALVI *et al.*[44], the epimerase activity observed in *P. chrysogenum* does not necessarily have to be related to the IPN epimerase encoded by the gene *cef*D in cephalosporin and cephamycin producing microorganisms, but could arise from an unspecific amino acid racemase. If such an unspecific racemase exists, it could potentially also act on α-AAA thereby forming a racemic mixture of L- and D-α-AAA. The IPN epimerase is, however, known to be unaffected by α-AAA[47]. Once the racemization of the α-AAA moiety of IPN and penN or perhaps of the free α-AAA has been performed cyclization into OPC can proceed by a mechanism catalyzed by an unknown enzyme distinct from the AT.

III. OPC Formation by Strains of *P. chrysogenum* Impaired in Penicillin Biosynthesis

Formation of OPC was furthermore studied in *P. chrysogenum* Wis 54-1255 and in two derived mutant strains npe6 and npe10. The strain npe6 carries a loss-of-function point mutation in the gene *pen*DE encoding the AT, thus expressing the gene but synthesizing a non-functional AT with respect to all its enzymatic activities[48], whereas npe10 has lost the entire cluster of genes encoding the enzymes in the penicillin biosynthetic pathway[49]. The three strains serve as an excellent tool to check whether OPC formation is linked directly to the penicillin biosynthetic pathway or whether OPC formation more specifically is correlated to the activity of the AT. Formation of OPC by the three strains was monitored in batch cultures on a chemically defined medium. *P. chrysogenum* Wis 54-1255 and npe6 produced small and comparable amounts of OPC, whereas OPC was found in significantly lower but detectable amounts in cultures of npe10 (Table 3). The very small amounts of OPC in cultures of npe10 may arise by another mechanism than in *P. chrysogenum* Wis 54-1255 and

Table 3. Specific formation rate of OPC in batch cultures of *P. chrysogenum* Wis 54-1255, npe6 and npe10.

Strain	OPC formation [μmoles/g DW/h]
Wis 54-1255	0.35
npe6	0.17
npe10	0.02

npe6, *e.g.* by spontaneous non-enzymatic cyclization of α-AAA. In that case, formation of OPC is most likely correlated to the activity of an enzyme distinct from the AT acting on IPN (and possibly also penN). If, however, the OPC found in cultures of npe10 is formed by the same mechanism as in *P. chrysogenum* Wis 54-1255 and npe6, formation of OPC would not be linked directly to the penicillin biosynthetic pathway but must arise from *e.g.* the activity of an unknown enzyme acting on α-AAA.

IV. Conclusion

Using purified ACV synthetase from *P. chrysogenum* it was found that this enzyme is not involved in OPC formation. The formation of OPC was furthermore investigated in *P. chrysogenum* Wis 54-1255 and the derived mutant strains npe6 and npe10, of which npe6 carries a loss-of-function mutation in the *pen*DE gene encoding the AT and npe10 lacks the entire penicillin gene cluster. By virtue of the findings by BRUNDIDGE *et al.*[2] of a racemic mixture of OPC in cultures of *P. chrysogenum* and the comparable OPC formation by *P. chrysogenum* Wis 54-1255 and npe6, the most plausible explanation for the OPC formation in β-lactam producing microorganisms is by an enzyme distinct from the AT acting on IPN and penN. Due to the findings of small but detectable amounts of OPC in cultures of npe10, it can, however, not be ruled out that OPC formation is only indirectly linked to the penicillin biosynthetic pathway in *P. chrysogenum*.

V. Acknowledgments

The authors would like to thank W. T. A. M. DE LAAT and D. J. HILLENGA (Royal Gist-Brocades N. V., Delft, The Netherlands) for their valuable contributions to the discussion on OPC formation, and Prof. J. F. MARTÍN (Universidad de León, León, Spain) for the kind donation of *P. chrysogenum* Wis 54-1255 and the derived mutant strains npe6 and npe10.

VI. Appendix: Experimental Details

Strains

P. chrysogenum Wis 54-1255 (ATCC 28089) and the two derived mutant strains npe6[48] and npe10[49] were kindly donated by Prof. J.F. MARTÍN, Universidad de León, León, Spain.

Cultivation Medium

The chemically defined medium for the batch cultivations contained: 25 g/liter sucrose, 1.6 g/liter KH_2PO_4, 7.0 g/liter $(NH_4)_2SO_4$, 0.5 g/liter KCl, 0.04 g/liter $FeSO_4 \cdot 7H_2O$, 0.10 g/liter $MgSO_4 \cdot 7H_2O$, 0.05 g/liter $CaCl_2 \cdot 2H_2O$, 0.5 ml/liter Pluronic® F68 (Fluka, Buchs, Switzerland, prod. no. 81112), and 5 ml/liter trace metal solution. The trace metal solution contained: 1.0 g/liter $CuSO_4 \cdot 5H_2O$, 4.0 g/liter $ZnSO_4 \cdot 7H_2O$, and 4.0 g/liter $MnSO_4 \cdot H_2O$.

Cultivation Conditions

The applied strains of *P. chrysogenum* were all grown in a high-performance Chemap bioreactor with a working volume of approximately 6.5 liters[50]. The temperature, aeration rate and head space pressure were kept constant at 25.0°C, 1 vvm, and 1.5 bar, respectively, and pH was kept at 6.50 by addition of 4 M NaOH and 1 M H_2SO_4. The agitation rate was kept constant at a level sufficiently high to maintain a dissolved oxygen concentration above 100% (air saturation at 1 bar). Batch cultures of the strains Wis 54-1255, npe6 and npe10 were inoculated with spores from mycelium grown on agar plates containing lactose, corn steep liquor and mineral salts to an initial concentration of 1.0×10^8 spores per kg cultivation medium.

Sampling

Cell free samples were taken automatically by means of an *in-situ* membrane module[51], and collected for later analysis in a fraction collector positioned in a refrigerator. Biomass samples were taken manually.

Assay for OPC Formation by ACV Synthetase

ACV synthetase purified from *P. chrysogenum*[24] with a specific activity of 41.4 nkat/g protein was assayed for potential OPC formation. A standard assay based on L-[^{14}C(U)]-valine with subsequent detection of LLD-[^{14}C(U)]-ACV was initially performed as described in THEILGAARD *et al.*[24] to check for full activity of the applied fraction of the purified enzyme. Subsequently, assays were carried out containing the following components in a total volume of 50 μl: 0.1 M MOPS (pH 7.5), 20% (v/v) glycerol, 1 mM EDTA, 5 mM ATP, 20 mM $MgCl_2$, 2 mM L-α-AAA, 1 mM L-cysteine, 192 μM L-valine and 5 μl purified enzyme (352 pkat/ml). Series of assays were performed with all three precursor amino acids present and with either one or two of the amino acids L-cysteine and L-valine left out. All assay mixtures were incubated at 26°C for 30 minutes and terminated by

addition of 15 μl 20% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation (20,000 g for 20 minutes) prior to analysis by HPLC for potential OPC formation.

Measurement of OPC

OPC was quantified by HPLC according to HENRIKSEN *et al.*[52].

VII. References

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