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Inhibition of penicillin biosynthetic enzymes by halogen derivatives of phenylacetic acid

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SUMMARY

The effect of phenylacetic acid (PAA) and several analogs on the activity of isopenicillin N synthase (IPNS) and acyl-CoA: 6-APA acyltransferase (AT) from *Penicillium chrysogenum* Wis 54-1255 has been tested. Whereas the substitution on the ring of a hydrogen atom by hydroxy-, methyl- or methoxy- groups did not cause any effect, the presence of halogens (Cl or Br) at positions 3 and/or 4 of PAA strongly inhibited these two enzymes. The replacement of hydrogen atoms by fluorine in certain positions also caused inhibition, but to a lesser extent.

INTRODUCTION

The specific pathway of penicillins in *Penicillium chrysogenum* involves the participation of three different enzymes which sequentially catalyze the synthesis of a linear tripeptide (δ-L-α-aminoadipyl-L-cysteinyl-D-valine) (ACV) [6], its cyclization to isopenicillin N (IPN) [2,8] and the replacement of the α-aminoadipic acid (α-AAA) moiety of this antibiotic by other penicillin side-chain precursors [11] (Fig. 1).

The most important antibiotic produced in vivo by this fungus is benzylpenicillin (penicillin G) which contains as side chain a phenylacetic acid (PAA) moiety. It has been reported [5] that although PAA (free acid) is not a direct penicillin biosynthetic intermediate (since it must be previously activated to its CoA thioester, PA-CoA), some enzymes of the pathway (isopenicillin N synthase, IPNS and acyltransferase, AT) can use as substrates some analogs of ACV or PAA which contain, as a part of the molecule, PAA or PAA-derivatives. Thus, IPNS converts some non-natural heteropeptides into either benzylpenicillin or *m*-carboxybenzylpenicillin [3,4,10]. Moreover, a different enzyme, AT, which utilizes PA-CoA as substrate, can also acylate the amino group of 6-APA with different PA-CoA variants (with substitutions on the aromatic ring) leading to the enzymatic production of several penicillins [1,12].

It has been also reported that the addition of PAA to the fermentation broths increased the intracellular pool of some benzylpenicillin biosynthetic intermediates, indicating that PAA could cause a general stimulation of the biosynthetic pathway [9]. These results have never been confirmed in vitro. Therefore, we have tried to study the effect of PAA and different derivatives (hydroxy-, methyl-, methoxy- and several halogen substituents) on the activities of some target enzymes (IPNS and AT) that catalyze reactions of the biosynthetic pathways of some important β-lactam antibiotics.

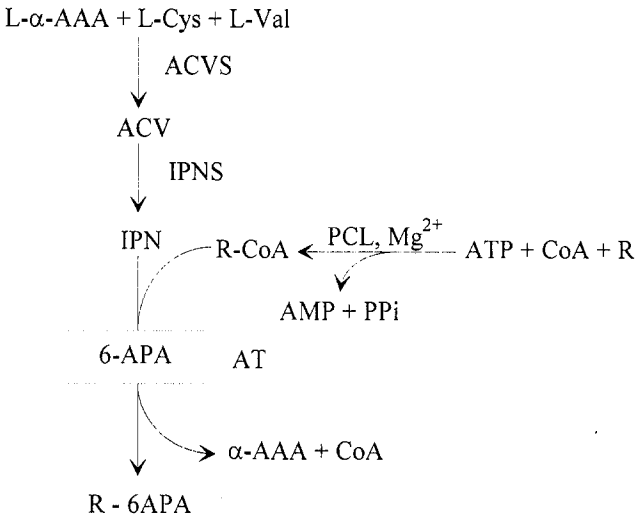

Fig. 1. Biosynthetic pathway of penicillins in *Penicillium chrysogenum*. ACVS, δ-(-L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase; IPNS, isopenicillin N synthase; 6-APA, 6-aminopenicillanic acid; AT, acyl-CoA: 6-APA acyltransferase. PCL, phenylacetyl-CoA ligase. R-6-APA corresponds to different penicillins (G, V, X or others).

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MATERIALS AND METHODS

Materials. PAA and derivatives were obtained from commercial sources: Br, F and di-Cl-derivatives of PAA were from Aldrich Química (Madrid, Spain); PAA was supplied by Fluka Chemika-Biochemika (Madrid, Spain) and mono-Cl-derivatives of PAA were from Lancaster (Strasbourg, France).

Microorganism. P. chrysogenum Wis 54-1255 (ATCC 28089) and *Micrococcus luteus* (ATCC 9341) were obtained from the American Type Culture Collection (Rockville, MA, USA). The strains were kept lyophilized.

Culture media and growth conditions. P. chrysogenum was maintained and cultured as previously reported [7]. Penicillin fermentations were carried out as reported in that publication.

Enzyme assays. Pure IPNS and AT were purified as reported [1,10]. The reaction mixtures contained:

a) IPNS assay: ACV 10 mM, 10 µl; DTT 20 mM, 10 µl; ascorbate 10 mM, 10 µl; $FeSO_4$ 2 mM, 10 µl and IPNS 60 µl (8 µg of pure protein).

b) AT assay: 6-APA 0.3 mM, 10 µl; DTT 20 mM, 10 µl; phenylacetyl-CoA (PA-CoA) 10 mM, 10 µl and AT 70 µl (10 µg of pure protein). PAA or its derivatives (20 mM) were added to the reaction mixtures and incubated at 25 °C for 30 min. The formation of the corresponding antibiotic (isopenicillin N or penicillin G) was measured by bioassay against *Micrococcus luteus* ATC 9341 as reported [10].

RESULTS AND DISCUSSION

When different PAA analogues were supplied to the IPNS or AT assay mixtures, neither PAA nor its hydroxy-, methyl- and methoxy- derivatives (*o*-, *m*- and *p*-) affected the synthesis in vitro of IPN or PG (data not shown). However, PAA variants containing halogen substituents on the aromatic ring inhibited both enzymes to different extents. Thus, chlorine (Cl) and bromine (Br) derivatives strongly inhibited IPNS and the highest inhibition, that inhibited activity completely, was obtained when the Cl or Br atoms were at position 3, 4 or 3 and 4 (dichloro derivatives) (Table 1). Other substitutions (2, or 2 and 6) also decreased the quantity of antibiotic produced but to a lesser degree. A similar effect was also caused by fluorine (F) derivatives of PAA and, as with the other PA-halogen derivatives, the highest inhibition was observed when F atom(s) substituted hydrogen atom(s) at position 3, 4 or 3 and 4, respectively.

AT activity was also affected by these compounds. However, this enzyme was not completely inhibited in any case (Table 1), the highest inhibition being caused by 2,6-di-Cl-PAA and by 3,4-di-Cl-PAA. These results indicate that the position of Cl atoms at 3 or 4 has a special significance.

Perhaps PAA-halogen derivatives do not affect the enzyme directly but some substrates (6-APA) or the reaction products (IPN or PG). To clarify this point we incubated

TABLE 1

Effect of halogen derivatives of PAA on IPNS and AT activities

Effector added	Inhibition of IPNS (%)	Inhibition of AT (%)
None	0	0
2Cl-PAA	32	24
3Cl-PAA	100 35*	58
4Cl-PAA	100 50*	40
2,4-di-Cl-PAA	100 28*	65
3,4-di-Cl-PAA	100 45*	76
2,6-di-Cl-PAA	40	85
2Br-PAA	42	42
3Br-PAA	100 30*	60
4Br-PAA	100 28*	60
2F-PAA	16	10
3F-PAA	40	14
4F-PAA	42	18
2,4-di-F-PAA	42	18
2,5-di-F-PAA	0	14
2,6-di-F-PAA	81	20
3,4-di-F-PAA	75	18
3,5-di-F-PAA	35	14

The concentration of effector added was 20 mM for IPNS and 30 mM for AT since lower quantities of inhibitor affect this latter enzyme very poorly. In some cases (*) the concentration of effector was reduced (10 mM). The data reported in this table are the average of three different determinations.

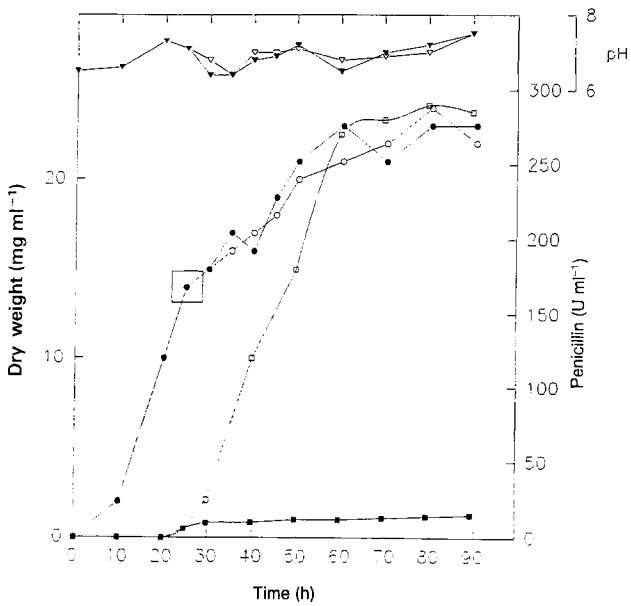

Fig. 2. Effect caused by 3,4-di-Cl-PAA (20 mM) on penicillin production when this compound was added (●, ▼, ■) or not (○, ▽, □) to the fermentation broths at the time indicated by the box (25 h). (■, □) Penicillin production; (●, ○) growth and (▼, ▽) pH variation.

IPN and PG with the effector. In no case were we able to observe any variation of the antibacterial activity of these antibiotics. Furthermore, when the PAA-halogen derivatives were added to the reaction mixtures previously incubated in the absence of effector (30 min), and incubated with it longer (30 additional min) the quantity of antibiotic generated was similar to that produced in the control reaction without effector (data not shown). These results suggest that inhibition was not an indirect effect caused by the reaction of the halogen derivatives with the antibiotics but by their interaction with the enzymes.

Moreover, this inhibitory effect does not seem to be reversible. Elimination of excess inhibitor by passing the reaction mixture through a Sephadex G-25 column (in non-diluting conditions) [13] did not lead to restoration of the original enzymatic activity, suggesting that either the inhibitor is strongly bound to the proteins or that some irreversible modification occurred in the structure of the enzymes that impaired their ability to catalyze the biosynthetic reactions.

When a 5- to 8-fold excess of substrate (ACV or PA-CoA) was added a decrease of inhibition was not observed; nor did addition of higher quantities of thiol-protecting molecules (DTT or GSH, 40 mM) restore enzymatic activity.

The mechanism underlying this effect is unclear. To confirm whether this inhibition is also caused in vivo, 20 mM 3,4-di-Cl-PAA was added to the fermentation broth just before penicillin production started. The growth of *P. chrysogenum* was not affected but the production of penicillin was decreased as expected (Fig. 2). These results indicate that this inhibition is not due to non-specific but to a specific effect, that directly affects the activity of some enzymes involved in penicillin biosynthesis.

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