"*IN VITRO*" SYNTHESIS OF DIFFERENT NATURALLY-OCCURRING, SEMISYNTHETIC AND SYNTHETIC PENICILLINS USING A NEW AND EFFECTIVE ENZYMATIC COUPLED SYSTEM

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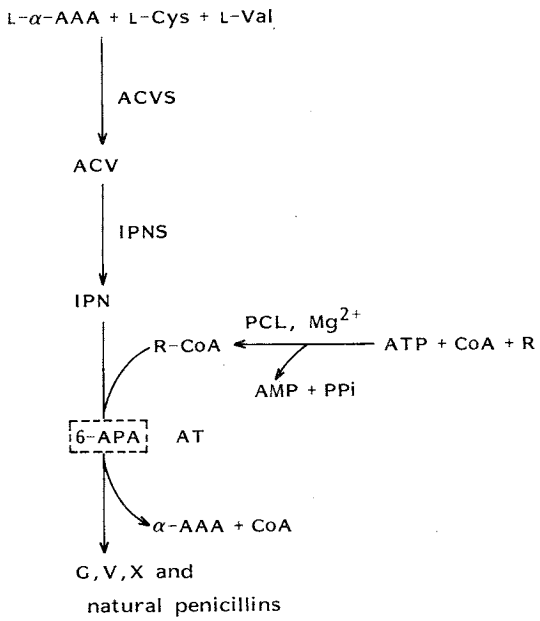
Forty-seven different penicillins, including some of great clinical importance, have been synthesized "*in vitro*" by coupling the newly described enzyme phenylacetyl-CoA ligase (PCL) from *Pseudomonas putida* and acyl-CoA: 6-aminopenicillanic acid (6-APA) acyltransferase (AT) from *Penicillium chrysogenum*. Incubations were carried out at 30°C in 50 mM HCl-Tris buffer pH 8.0. The reaction mixtures contained 6-APA, CoA, ATP, dithiothreitol, Mg^{2+} and the corresponding penicillin side-chain precursor. This is the first description of the enzymatic synthesis of all the natural penicillins known, many of the semisynthetic until now reported, and some penicillins that could only be currently obtained by chemical synthesis. The efficiency of this prokaryotic-eukaryotic enzymatic-coupled system and its application to the synthesis of different β-lactam antibiotics are discussed.

The biosynthetic pathways of different β-lactam antibiotics have been addressed over many years[1]. However, until recently the main enzymatic steps and the nature of some of their intermediates were almost unknown[2]. Currently, the application of modern biotechnology to industrial microbes has allowed the isolation, characterization and purification of some of the enzymes involved in such pathways[3] and has also led to the study of the physical organization of some of the genes coding for these important biosynthetic enzymes[4]. A good example of this kind of research is that performed with the early antibiotics, benzylpenicillin and cephalosporin C. Thus, in recent years, several laboratories in different countries have been carrying out interesting work on the enzymatic synthesis of these β-lactam antibiotics using purified proteins of different microbial origin[5~8].

In the particular case of benzylpenicillin, the most important limiting aspect in obtaining complete enzymatic synthesis of this antibiotic from its biosynthetic intermediates (6-aminopenicillanic (6-APA) and phenylacetic acid) PAA)) is the step catalyzed by phenylacetyl-CoA ligase (PCL), Fig. 1). This protein although described some years ago[9] was never purified and therefore has been characterized.

We solved this problem by using a strain of *Pseudomonas* (*Pseudomonas putida* U) that efficiently degrades PAA through a new catabolic pathway which required the participation of a PCL activity. This enzyme was purified to homogeneity and characterized. Its optimal physico-chemical assay conditions (30°C and pH 8.0) were very similar to those reported for other proteins involved in penicillin biosynthesis (isopenicillin N synthetase (IPNS) and acyl-CoA: 6-APA acyltransferase (AT), Fig. 1), thereby suggesting that they could be coupled "*in vitro*"[10]. Some time later, we successfully demonstrated the formation of benzylpenicillin by incubating IPNS, AT (from *Penicillium chrysogenum*) and PCL (from *P. putida*) with some penicillin biosynthetic intermediates (ACV/6-APA, PAA and CoA)[11]. Accordingly, it was "a priori" feasible that, if the substrate specificity of these enzymes was broad enough, use of this coupled system (PCL-AT) might lead to the synthesis "*in vitro*" of many different penicillins. In this report we have tested the enzymatic synthesis of several penicillins using different side-chain precursors, 6-APA and

Fig. 1. Biosynthetic pathway of benzylpenicillin and natural penicillins.



α-AAA: α-Aminoadipic acid, ACV: δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, IPN: isopenicillin N, 6-APA: 6-aminopenicillanic acid, ACVS: ACV synthetase, IPNS: IPN synthetase, AT: acyl-CoA: 6-APA acyltransferase, PCL: phenylacetyl-CoA ligase, G: benzylpenicillin, V: phenoxymethylpenicillin, X: *p*-hydroxybenzylpenicillin, R: penicillin side chain precursor.

Scheme 1. Sequence of coupled reactions leading to the production of different penicillins by incubating PCL (I), AT (II) and different side chain precursor (X).

$$\text{(I):}\quad X + ATP + CoA \xrightarrow[Mg^{2+}]{PCL} X\text{-CoA} + AMP + PPi$$

$$\text{(II):}\quad X\text{-CoA} + 6\text{-APA} \xrightarrow[DTT]{AT} X\text{-6-APA} + CoA$$

$$\text{(I+II):}\quad X + ATP + 6\text{-APA} \xrightarrow[Mg^{2+},\ DTT]{PCL/AT} X\text{-6-APA} + AMP + PPi$$

the enzymes PCL and AT.

Materials and Methods

Materials

All the products tested as penicillin side-chain precursors were obtained from Lancaster Synthesis Ltd. (UK) or from Aldrich-Chemie (Germany).

Microorganisms

P. putida U was kindly supplied by Dr. J. M. ALONSO from our Department. The strain was originally obtained from Prof. R. A. COOPER (Department of Biochemistry, University of Leicester, UK). This bacteria was used for obtaining pure PCL as previously described[10].

P. chrysogenum Wis 54-1255 (ATCC 28089) was maintained and cultured as previously reported. This strain was used for obtaining pure AT[7].

Micrococcus luteus ATCC 9341 was routinely employed for the determination of penicillins by bioassay[6].

Coupled Assay of PCL and AT

The reaction mixture contained: $MgCl_2$ 0.2 M, 12.5 μl; ATP, 0.1 M, 50 μl; CoA, 20 mM, 30 μl: potassium phenylacetate (or the corresponding side chain precursor) 0.2 M , 30 μl; 6-APA 0.3 mM, 30 μl (at this concentration 6-APA does not inhibit the growth of *M. luteus*); dithiothreitol (DTT), 20 mM, 10 μl; PCL (enzyme I) 100 μl (2 μg of protein), AT (enzyme II) 100 μl (8 μg of protein). All the substrate except $MgCl_2$ were dissolved in 50 mM Tris and adjusted to pH 8.0. Incubations were carried out at 30°C for 1 hour (or the required time) and halted by the addition of a similar volume of methanol (362.5 μl). The antibiotics generated were measured by bioassay against *M. luteus* ATCC 9341. Control reactions were carried out under the same conditions without PAA or CoA. In the cases in which antibiotic was produced, we concluded that the side chain precursor tested was a substrate of enzyme I and also, when activated CoA, of enzyme II. When no bioactive molecules were generated, to establish whether the substrates were recognized by PCL, formation of CoA-thioesters was followed by different procedures: HPLC[10]; spectrofluorometrically[12]; measurement of the color generated in the presence of hydroxylamine[10], or by evaluation of the pyrophosphate released during the reactions[13] (see Scheme 1). When necessary, some reactions were incubated for longer times (2~3 hours) to discover whether, at low rates, these substrates are recognized or not by the enzymes. The combination of such results allowed us to conclude which compounds are substrates of PCL (I) or PCL and AT (I and II), respectively.

Results and Discussion

To study the synthesis "*in vitro*" of different penicillins by coupling PCL from *P. putida* and AT from

P. chrysogenum these two enzymes were incubated with several molecules (see tables) that can be used as penicillin side chain precursors. Five groups of substrates were selected: (i) includes aliphatic acids (linear or branched, saturated or unsaturated) (see Table 1); (ii) are structural analogues of PAA with substitution on the aromatic ring (Table 2); (iii) includes PAA-analogues with substitution at the α-position (see (A), Table 4); (iv) are PAA-analogues containing a longer acyl-chain joined to the aromatic ring (see (B), Table 4), and (v) includes compounds in which the PAA ring is replaced by a different one (see (C), Table 4). Evidence of penicillin formation was based on the bioactivity towards *M. luteus*. However, for some substrates this might have given a poor indication of coupling if the produced penicillin had only a weak activity against this microorganism.

The study carried out with aliphatic molecules (Table 1) showed that most of these compounds were activated to CoA-derivatives by PCL (I), whereas only some of them — those whose carbon length ranges between C-6 and C-8 carbon atoms — were efficiently recognized by both enzymes and therefore transformed into antibiotics. Of interest is the low rate (or the absence) of transformation observed when compounds

Table 1. Study of the incorporation into penicillins of different apliphatic acids when incubated with PCL (enzyme I) and AT (enzyme II) in the presence of ATP, CoA, Mg^{2+}, DTT and 6-APA.

Side chain precursor tested	Activation to its CoA derivative (enzyme I)	Production of penicillin (I+II)
Acetic acid	+	ND
Propionic acid	+	ND
Butyric acid	+	+ (3 hours)
Pentanoic acid	+	+ (2 hours)
trans-2-Pentenoic acid	+	ND
Pent-4-enoic acid	+	ND
4-Pentynoic acid	+	ND
3,3-Dimethylbutyric acid	+	+
2-Methylpent-4-enoic acid	+	++
Hexanoic acid	+	++*
Hexadienoic acid (adipic acid)	+	+ (2 hours)
trans-2-Hexenoic acid	+	+ (3 hours)
2,4-Hexedienoic acid	+	ND
trans-3-Hexenoic acid	+	++*
2-Methylhexanoic acid	+	+
Heptanoic acid	+	++
Heptadioic acid (pimelic acid)	+	+ (2 hours)
trans-2-Heptenoic acid	+	+ (3 hours)
trans-3-Heptenoic acid	+	++
2-Ethylhexanoic acid	ND	ND
Octanoic acid	+	++*
trans-2-Octenoic acid	+	+ (3 hours)
trans-3-Octenoic acid	+	++
2-Octynoic acid	ND	ND
Nonanoic acid	+	+ (2 hours)

The number and letter in brackets indicate the time of incubation and the common name of the penicillin produced.

All the antibiotics included in this table and in the following ones were sensitive to the β-lactamase (*Bacillus cereus*) attack. Penicillin production was measured as inhibition zone against *Micrococcus luteus* (+: 8~15 mm, ++: 15~30 mm and +++: 30~45 mm).

ND: Not detected.

* Indicates natural penicillins (DF, F and K, respectively).

Table 2. Study of the incorporation into penicillins of different PAA derivatives with substitution on the ring when incubated with PCL (enzyme I) and AT (enzyme II) in the presence of ATP, CoA, Mg^{2+}, DTT and 6-APA.

Side chain precursor tested	Activation to its CoA derivatives (enzyme I)	Production of penicillin (I+II)	Side chain precursor tested	Activation to its CoA derivatives (enzyme I)	Production of penicillin (I+II)
PAA	+	+++ (G)	3-Nitro-PAA	ND	ND
2-Hydroxy-PAA	+	+	4-Nitro-PAA	ND	ND
3-Hydroxy-PAA	+	++	3-Amino-PAA	+	++
4-Hydroxy-PAA	+	++	4-Amino-PAA	+	++
2-Methyl-PAA	+	ND	2-Fluoro-PAA	+	+++
3-Methyl-PAA	+	++	3-Fluoro-PAA	+	+++
4-Methyl-PAA	+	++	4-Fluoro-PAA	+	+++
2-Methoxy-PAA	+	ND	2,4-Difluoro-PAA	+	+++
3-Methoxy-PAA	+	+	2,5-Difluoro-PAA	+	+++
4-Methoxy-PAA	+	++	2,6-Difluoro-PAA	+	+++
2-Nitro-PAA	ND	ND	3,4-Difluoro-PAA	+	+++

ND: Not detected.

which contains a double or triple bond between C-2 and C-3 were employed. These results suggest that free mobility at C-2 in the substrate molecule is an important property required by AT to achieve a good penicillin conversion. Similar data have been previously reported by us using AT and chemically synthesized acyl-CoA variants[14,15]. A low synthesis of penicillins was also obtained when either shorter or longer fatty acid, (butyric, valeric, nonanoic acids) or substrates containing an extra polar function in their molecule (adipic acid and pimelic acid) were assayed (see Table 1).

Table 3. Inhibition of PCL by Cl and Br phenylacetyl derivatives.

Inhibitor (20 mM)	Residual activity (%)
None	100
2-Cl-PAA	65
3-Cl-PAA	40
4-Cl-PAA	45
2,4-diCl-PAA	22
3,4-diCl-PAA	10
2,6-diCl-PAA	46
2-Br-PAA	70
3-Br-PAA	41
4-Br-PAA	40

Incubations were carried out as reported[10].

When the second group of substrates were analyzed, penicillins were obtained when *o*-, *m*- or *p*-hydroxy-; *m*- or *p*-methyl-; *m*- or *p*-methoxy-; *m*- or *p*-amino- and *o*-, *m*- or *p*-fluoro derivatives of PAA were tested (see Table 2); whereas the presence of a NO_2 group on the aromatic ring handicapped these molecules for use as substrates (failure of enzyme I). Other halogen-derivatives tested (Cl- or Br-PAA) were not transformed into penicillins since these are potent inhibitors of PCL (see Table 3).

The use of compounds containing substitutions at the α-position in the acetyl-moiety of PAA (see (A), Table 4), disclosed that, whereas they were mostly activated to CoA thioesters by the enzyme I, only some of them were transformed into antibiotics. These results quite agree with those previously reported about the substrate specificity of AT[7,14,15] suggesting, again, the importance of free mobility at C-2 on the substrate molecule. Furthermore, the presence of an NH_2 at C-2 (α-aminophenylacetyl-CoA and *p*-hydroxy, α-aminophenylacetyl-CoA) also handicapped these molecules to be transformed, by AT, into ampicillin and amoxycillin, respectively (see Table 4). However, this effect cannot be explained only in terms of the negative influence of this group on the free rotation of C-1 ~ C-2 linkage since other substitutions (hydroxy-,

Table 4. Study of the incorporation into penicillin of different PAA analogues with substitution in the α-carbon atom (A), with longer acyl-chain (B) and containing a different ring (C) when incubated with PCL (enzyme I) and AT (enzyme II) in the presence of ATP, CoA, Mg^{2+}, DTT and 6-APA.

	Side chain precursor tested	Activation to its CoA derivatives (enzyme I)	Production of penicillin (I+II)
(A)	α-Methyl-PAA	+	+ (3 hours)
	α-Ethyl-PAA	+	++
	α-Amino-PAA	+	ND
	p-Hydroxy-α-amino-PAA	+	ND
	α-Phenylglyoxylic acid	+	ND
	α-Hydroxy-PAA	+	+
(B)	3-Phenylpropionic acid	ND	ND
	o-Hydroxyphenylpropionic acid	ND	ND
	m-Hydroxyphenylpropionic acid	ND	ND
	p-Hydroxyphenylpropionic acid	ND	ND
	trans-Cinnamic acid	ND	ND
	4-Phenylbutyric acid	ND	ND
	POA	+	++ (V)
	2-Phenoxybutyric acid	ND	ND
	4-Phenoxybutyric acid	ND	ND
	Thiophenoxyacetic acid	ND	ND
(C)	Ciclopentyl acetic acid	+	+ (3 hours)
	Ciclopentenoic acetic acid	+	+ (3 hours)
	Ciclohexylacetic acid	+	+ (3 hours)
	2-Thiophene acetic acid	+	+++
	3-Thiophene acetic acid	+	+++
	DL-α-Amino-2-thiophene acetic acid	+	ND
	3-Thiophene malonic acid	+	++ (ticarcillin)
	2-Pyridyl acetic acid	+	++ (2 hours)
	3-Pyridyl acetic acid	+	++ (2 hours)
	4-Pyridyl acetic acid	+	++ (2 hours)

ND: Not detected.

carboxy-, methyl- and ethyl-radicals see tables) still allows the synthesis of penicillins. It seems more probable that, in this case, the presence of a positive charge at α-position generates electrostatic interactions which cause a difficulty of binding the substrate at the AT active site. Furthermore, the replacement of the methylene group at the α-position of PAA by a keto group (phenylglyoxyl-CoA) was not tolerated by AT (Table 4) suggesting the importance of an acetyl moiety in the substrate molecule[7].

When molecular analogues of PAA containing a rather long acyl chain, were tested, only phenoxyacetic acid (POA) was efficiently incorporated as a penicillin side-chain (penicillin V). The lack of conversion observed with the rest of compounds is caused by the narrow specificity showed by PCL with these substrates (only recognizes POA, see (B), Table 4). Moreover, the replacement of the oxygen atom (POA) by the sulfur atom (thiophenoxyacetic acid) is sufficient a variation to handicap this compound for use as a substrate by both enzymes (I and II), suggesting that the larger volume of the sulfur atom causes steric hindrance. Furthermore, the fact that α-ethyl-PAA (but not α-ethylhexanoic acid) was recognized by PCL and AT (see Tables 1 and 4) could also be explained in similar terms. Thus, the lower length of the linkages between groups of atoms (caused by the presence of double bonds on the PAA-aromatic ring) could help achieve the appropriate molecular size required for both enzymes (PCL and AT) to bind any substrate at their active sites. The results obtained with 2-methylpent-4-enoic acid, 2-methylhexanoic acid and

α-methyl-PAA (see Tables 1 and 4) agree with this hypothesis.

To complete this study, some compounds in which the aromatic ring of PAA had been replaced by others were tested (see Table 4). All of them were activated to CoA-thioesters and, except DL-α-aminothiopheneacetyl-CoA, were mostly accepted as substrate by AT. However, these compounds required longer times of incubation (2~3 hours) to be converted into penicillins, suggesting the importance of: (a) the size and (b) the existence of a rigid and planar structure on the substrate molecule to facilitate its utilization by AT. Thus, when compounds containing aromatic rings with such characteristics were employed (2 and 3-thiopheneacetic acids or 3-thiophenemalonic acid), an efficient synthesis of penicillins was achieved.

The results obtained with 3-thiophenemalonyl-CoA (α-carboxy-3-thiopheneacetyl-CoA) was of great interest since it is the first description of the enzymatic synthesis of the broad spectrum antibiotic ticarcillin. Furthermore, the fact that α-carboxy-3-thiopheneacetyl-CoA was used by AT, indicates that the presence of a negative charge at α-position is, to some degree, tolerated by this enzyme, and contrasts with the results obtained when an NH_2 group was present at the same position (see Table 4).

The foregoing data show, for the first time, that the PCL-AT enzymatic couple system described herein can be efficiently used for the synthesis of many different penicillins (naturally-occurring, semisynthetic and those ones that can only be obtained by chemical synthesis). These findings allow us to speculate that the manipulation of the genes that code for these two enzymes (PCL and AT) could lead to "mutated" proteins (maybe with broader substrate specificity) and therefore provide new or modified β-lactam antibiotics. Furthermore, the cloning of PCL-gene from *P. putida* in high-producing strains of *P. chrysogenum* could have important industrial applications since the expression of this protein in such microbes might: (a) improve the final titres of benzylpenicillin and (b) increase the number of antibiotics generated "*in vivo*" by this fungus if as expected this enzyme does in fact represent a true biosynthetic limitation. In summary, the above results pave the way of obtaining, through highly efficient enzymatic reactions, of new or modified β-lactam molecules.

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