DIRECT ENZYMATIC SYNTHESIS OF NATURAL PENICILLINS USING PHENYLACETYL-CoA: 6-APA PHENYLACETYL TRANSFERASE OF *PENICILLIUM CHRYSOGENUM*: MINIMAL AND MAXIMAL SIDE CHAIN LENGTH REQUIREMENTS

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(Received for publication June 26, 1986)

In vitro synthesis of different natural penicillins (hexanoyl, heptanoyl and octanoyl-penicillin) have been carried out by direct acylation of 6-aminopenicillanic acid (6-APA) with several fatty acid-CoA derivatives (hexanoyl-CoA, heptanoyl-CoA and octanoyl-CoA). The reactions were catalyzed by the enzyme Acyl-CoA: 6-aminopenicillanic acid acyltransferase from *Penicillium chrysogenum* AS-P-78. This enzyme only accepts as substrate, aliphatic side chain precursors whose carbon length is between 6 and 8 atoms. Although the enzymatic synthesis of octanoylpenicillin has been previously reported the *in vitro* synthesis of hexanoyl and heptanoyl penicillins is described here for the first time.

The biosynthesis of penicillins in *Penicillium chrysogenum* starts with the formation of a non bioactive tripeptide molecule (δ-L-α-aminoadipyl-L-cysteinyl-D-valine) (ACV)[1,2] which in a later step is cyclized to isopenicillin N[3,4]. This molecule which contains the residue of L-α-aminoadipic acid as a side-chain, notably increases its antibacterial activity when the α-aminoadipic acid is substituted by other side-chain precursors (phenylacetic, phenoxyacetic acid)[5]. The presence in the fermentation broths of several natural penicillins (F, DF, K)[5] in which the L-α-aminoadipic acid moiety had been replaced by a fatty acid (hexenoic, hexanoic and octanoic acid), implies that the biosynthesis of these molecules follows a pathway similar to that described for benzylpenicillin[6]. In the first step, the fatty acid must be activated to its CoA derivative by an acyl-CoA ligase[7], while in the second step the acyl-CoA must be transferred onto the 6-aminopenicillanic acid (6-APA) molecule by an acyl-CoA: 6-APA acyltransferase enzyme[8~12]. In this report we present evidence concerning the *in vitro* synthesis of different natural penicillins and about the maximal and minimal length requirements of substrates for the enzyme acyl-CoA: 6-APA acyltransferase.

Materials and Methods

Materials

Phenylacetyl-CoA, butyryl-CoA, valeryl-CoA, hexanoyl-CoA (lithium salt), heptanoyl-CoA, octanoyl-CoA, nonanoyl-CoA, decanoyl-CoA, undecanoyl-CoA, lauryl-CoA, tridecanoyl-CoA, myristoyl-CoA, pentadecanoyl-CoA, palmitoyl-CoA, stearoyl-CoA and oleoyl-CoA (free acid); heptanoic acid and a kit of fatty acid methyl esters were purchased from Sigma Chemical Co., St. Louis, Mo, U.S.A. Hexanoic and octanoic acid were from Merck (Germany). Benzylpenicillin (potassium salt), 6-APA, hexanoylpenicillin (penicillin dihydro F), heptanoylpenicillin, octanoylpenicillin (penicillin K) and *Escherichia coli* penicillin acylase were from Antibioticos S.A., León, Spain, and β-lactamase from *Bacillus cereus* was from Difco. All other products were of analytical grade.

Microorganisms

The strain of *Penicillium chrysogenum* AS-P-78 used in all experiments was from Antibioticos S.A. This fungus produces about 8,000 U/ml of benzylpenicillin when incubated in complex production medium[13]. *Micrococcus luteus* ATCC 9341 was used for the routine determination of the different penicillins by bioassay[14].

Strains were kept lyophilized or in liquid N_2 (gas phase).

Media and Culture Conditions

Inoculum of *P. chrysogenum* was developed in complex medium using 2.5 ml to inoculate 50 ml of complex medium as previously described[15]. For production of natural penicillins no phenylacetic acid was added to the medium.

Enzyme Preparation

Preparation of the mycelial extract and enzyme purification was carried out as previously described[9,12].

Enzyme Assay

In a total volume of 100 μl the reaction mixture contained the following: Buffer solution (Tris 0.05 M, NaCl 0.1 M and KH_2PO_4 0.02 M, pH 8.4), 25 μl; 10 mM phenylacetyl-CoA (or another side-chain precursor), 10 μl; 0.3 mM 6-APA, 10 μl; 15 mM dithiothreitol, 5 μl; enzyme, 50 μl (0.05 mg protein). Incubations were carried out at 20°C for 30 minutes and stopped by addition of 100 μl of pure methanol. The quantification of the reaction products was carried out by bioassay (against each standard penicillin) as previously described[9,14]. One unit of enzymatic activity was defined as the amount of enzyme required to synthesize 1 μg of benzyl, hexanoyl, heptanoyl or octanoylpenicillin at 20°C in 30 minutes.

Chemical Synthesis of Octanoyl, Heptanoyl and Hexanoylpenicillins

Octanoyl, heptanoyl and hexanoylpenicillins used as standards were synthesized from 6-APA and the corresponding acid chloride. 6-APA (54 g) was dissolved in 1.2 liters of 10% (w/v) aqueous $NaHCO_3$ and 55 g of octanoyl, heptanoyl or hexanoyl chloride, previously dissolved in 100 ml of dry acetone, were added dropwise to the 6-APA solution. The mixture was stirred for 20 minutes at 16°C after which the acetone was evaporated under reduced pressure. Ammonium sulfate (45% w/v) was added to each solution and a pale white solid quickly precipitated[16]. The product (penicillin DF, K or heptanoylpenicillin) was filtered, washed with dry acetone, dried in an oven (28°C overnight) and later identified by HPLC using a Perkin-Elmer Chromatograph Series 3B with a LC-75 Spectrophotometric Detector and a Sigma 10 B Chromatography Data Station. A μBondapack C-18 column of 30 cm (Water Associates Inc.) was used. The mobile phase for benzylpenicillin, hexanoyl and heptanoylpenicillin was 83% 0.1 M sodium acetate - acetic acid, pH 4.5, and 17% of acetonitrile. Retention times were; 12.1, 17.1 and 24.7 minutes for benzylpenicillin, hexanoylpenicillin and heptanoylpenicillin, respectively. The mobile phase used for octanoylpenicillin was 0.025 M KH_2PO_4 - methanol (50: 50). The retention time for this penicillin was 9.6 minutes. In these later conditions the retention time for heptanoylpenicillin was 4.3 minutes while hexanoylpenicillin was not retained.

Isolation and Purification of Natural Penicillins Present in the Fermentation Broth

Five day-old production broth[13] was centrifuged at 18,000$\times g$ for 10 minutes. The supernatant fluid was centrifuged again for 5 minutes at 15,000$\times g$ and ammonium sulfate was added to the clear supernatant fluid (final concentration, 45% w/v). Penicillins with non polar side chains present in the solution immediately began to precipitate[16]. The precipitate was separated the supernatant fluid (which contained 6-APA and polar penicillins) by filtration through a No. 4 Pyrex glass-fiber filter. Once resuspended in 1/30 of the original volume the precipitate was acidified to pH 2.0 with 2 N H_2SO_4 and quickly extracted with butyl acetate as previously described[17]. The amount of aliphatic penicillins present in the aqueous phase was less than 1%. The rich butyl acetate fraction was treated with ammonium 2-ethylhexanoate and after drying "*in vacuo*", a powder was obtained. This was washed with dry acetone and dried in an oven (28°C overnight). The purity of the penicillins obtained was

Fig. 1. Gas chromatograms obtained from: (A) standards of hexanoic, heptanoic and octanoic acid; (B) fatty acids released from the natural penicillins isolated from *Penicillium chrysogenum* fermentation broth, and (C) untreated natural penicillins.

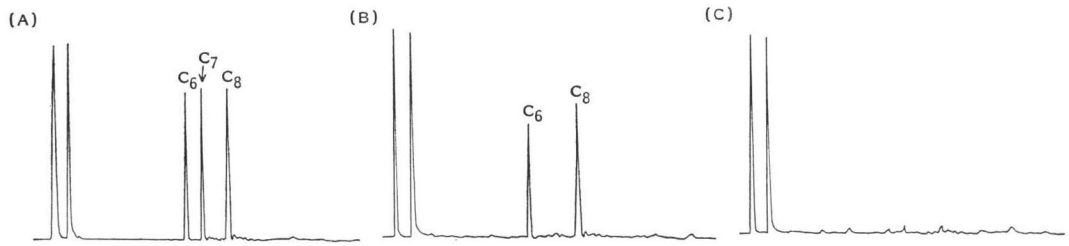


Table 1. Formation of natural penicillins by incubation of 6-APA and different side chain precursors in the presence of Acyl-CoA: 6-APA acyltransferase of *Penicillium chrysogenum* AS-P-78.

Side chain precursor*	Concentration in reaction mixture (mM)	Antibiotic (μg/ml)	Sensitivity to	
			Penicillin-acylase	β-Lactamase
Butyryl-CoA (C_4: 0)	1 ; 10	ND		
Valeryl-CoA (C_5: 0)	1 ; 10	ND		
Hexanoyl-CoA (C_6: 0)	1 ; 10	0.2; 0.4	−	+
Heptanoyl-CoA (C_7: 0)	1 ; 10	0.5; 0.8	−	+
Octanoyl-CoA (C_8: 0)	1 ; 10	1.0; 1.2	−	+
Noneanyl-CoA (C_9: 0)	1 ; 10	Trace (0.1)		
Decanoyl-CoA (C_{10}: 0)	1 ; 10	Trace (0.1)		
Undecanoyl-CoA (C_{11}: 0)	1 ; 10	ND		
Lauryl-CoA (C_{12}: 0)	1 ; 10	ND		
Tridecanoyl-CoA (C_{13}: 0)	1 ; 10	ND		
Myristoyl-CoA (C_{14}: 0)	1 ; 10	ND		
Pentadecanoyl-CoA (C_{15}: 0)	1 ; 10	ND		
Palmitoyl-CoA (C_{16}: 0)	1 ; 10	ND		
Heptadecanoyl-CoA (C_{17}: 0)	1 ; 10	ND		
Stearoyl-CoA (C_{18}: 0)	1 ; 10	ND		
Oleoyl-CoA (C_{18}: 1)	1 ; 10	ND		
Phenylacetyl-CoA	1 ; 10	1.0; 1.3	+	+

ND: Not detected.

* When 6-APA was not added to the reaction mixture no formation of antibiotic took place.

greater than 95% (w/w). If potassium 2-ethylhexanoate was employed, the yield of the process was lower than 5%.

Identification of the Side Chains

The white solid obtained as above (1 g) was hydrolyzed with 5 N HCl (5 ml) at 80°C for 10 hours (N_2 atmosphere). The resulting solution was vigorously extracted with diethyl ether (15 ml), the organic phase was separated, dried under reduced pressure and 10 mg of the residual oil was analyzed by gas chromatography after forming the methyl esters with diazomethane[18] using a 5830 Hewlett-Packard Chromatograph with a DEGS (diethyleneglycol succinate) column. By this methodology the fatty acids present in the natural penicillins were identified (Fig. 1). Samples without hydrolysis and pure fatty acid methyl esters were used as standards.

Results and Discussion

When the enzyme phenylacetyl-CoA: 6-APA phenylacetyltransferase of *P. chrysogenum* was incubated in the presence of 6-APA and different possible side-chain precursors (Table 1), the forma-

Fig. 2. HPLC chromatograms of: (A) Benzyl, hexanoyl, heptanoyl and (B) octanoylpenicillin used as standards and chromatograms of the natural penicillins (C, D) purified from *Penicillium chrysogenum* complex broth when the fungus was grown in the absence of phenylacetic acid.

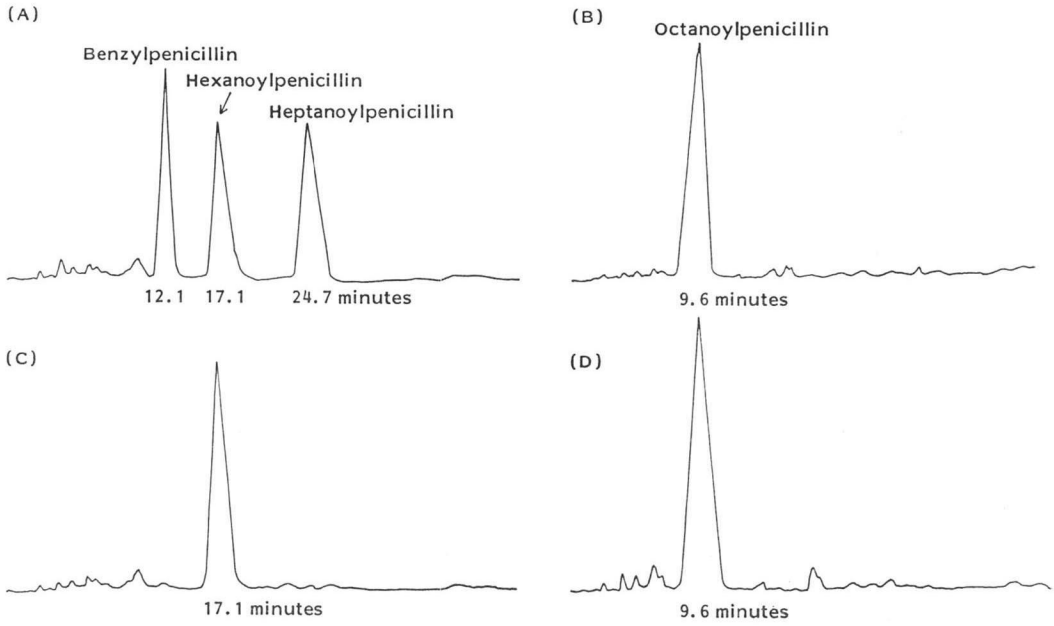
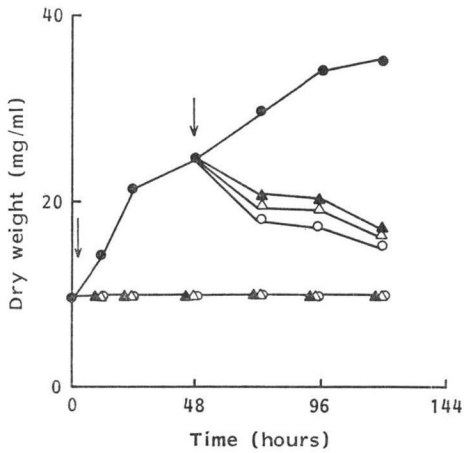


Fig. 3. Determination of *Penicillium chrysogenum* AS-P-78 growth when the fungus was grown in complex medium in the absence (●) or presence of hexanoic (▲), heptanoic (△) or octanoic (○) acid.

Arrows indicate the time at which fatty acids were added.



tion of antibiotic substances active against *M. luteus* was only shown when hexanoyl, heptanoyl, octanoyl and phenylacetyl-CoA were used as substrates. With other aliphatic molecules (butyryl, valeryl, nonanoyl, decanoyl, undecanoyl, lauryl, tridecanoyl, myristonyl, pentadecanoyl, palmitoyl, stearoyl, heptadecanoyl and oleoyl-CoA) the enzyme did not form penicillins even when a 10-fold increased concentration of precursors (10 mM) was added to the reaction mixture. In these last conditions only traces of penicillins (<0.1 μg) were detected when nonanoyl-CoA and decanoyl-CoA were employed. Moreover, the quantity of penicillin produced increased as a function of the length of the aliphatic side chain precursor. The maximal yield was obtained with octanoyl-CoA and the minimal with hexanoyl-CoA (Table 1). These results suggest that the enzyme acyl-CoA: 6-APA acyltransferase accepts as substrates fatty acids previously activated to their CoA derivatives as long as the length of the aliphatic side-chain ranges between 6 and 8 carbon atoms. Fatty acids shorter (lower than 6) or longer (higher than 8) are not bound to the enzyme (or with very low efficiency as in the case of nonanoyl or decanoyl-CoA). This is

supported by the fact that when these unuseful molecules are pooled together with phenylacetyl-CoA or with octanoyl-CoA (even at concentrations of 10 mM) the amount of benzylpenicillin and penicillin K produced were similar in all cases (data not shown).

The presence of some of these natural penicillins (DF and K) in the fermentation broth of *P. chrysogenum* AS-P-78, when the microorganism was cultured with no phenylacetic acid (see Materials and Methods), are in good agreement with these "*in vitro*" results (Fig. 2).

The lack of penicillin F from the *P. chrysogenum* AS-P-78 fermentation broth suggest either that this antibiotic is not produced, in the above fermentation conditions, by this mutant or that the quantity of penicillin F is very low if compared with hexanoyl or octanoylpenicillins. Fig. 1 shows the presence of octanoic and hexanoic acids when the natural penicillins isolated from the fermentation broth were hydrolyzed in order to release the side chain precursors. The absence of heptanoylpenicillin in the broth and consequently the lack of heptanoic acid in the chromatogram could be due to the fact that this molecule is rare in nature and though naturally occurring lipids sometimes contain fatty acids with an even number of carbon atoms, fatty acids with odd number of carbon atoms are only found in significant amounts in the lipids of some marine organisms[19,20]. The absence of fatty acids with an even number of carbon atoms in the nutrients used for penicillin fermentation (soya oil or corn oil) is probably the reason why heptanoyl-CoA is not generated and therefore heptanoylpenicillin cannot be produced. When octanoic, heptanoic or hexanoic acid were added at zero time to the complex medium as potential side chain precursors, the results are unexpectedly completely negative, since these molecules were extremely toxic for the fungus even at low concentrations (0.3%). Furthermore, the addition of any of these fatty acids to 48 hour-grown cultures caused a rapid lysis of the mycelia (Fig. 3). Similar inhibitory effect have been described for yeast[21] and spoilage microorganisms[22].

The isolation of mutants of *P. chrysogenum* able to grow in the presence of high concentrations of these fatty acids or these others in which the acyltransferase has a broader substrate specificity could be very important in the future since it opens the possibility for obtaining, using soluble or immobilized acyl-CoA: 6-APA acyltransferase, new aliphatic penicillins. The industrial relevance of these findings lies in the possibility that simple chemical modifications of the side chain precursors could be accepted by the acyltransferase, generating, as in the case of isopenicillin N synthetase[23], different penicillins with broader antibacterial spectra.

Acknowledgments

We are gratefully indebted to Mr. CESAR MIRAVALLES (Refinesa) for his help in the gas chromatography analysis and to Mr. E. GIL for his excellent technical assistance. We also thank Antibióticos S.A. León, Spain, for support.

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