ENZYMATIC SYNTHESIS OF PENICILLINS

M. J. ALONSO, F. BERMEJO[a], A. REGLERO, J. M. FERNÁNDEZ-CAÑÓN, G. GONZÁLEZ DE BUITRAGO[b] and J. M. LUENGO[c,†]

Departamento de Bioquímica y Biología Molecular, Universidad de León, España
[a]Departamento de Química Orgánica, Facultad de Químicas, Universidad de Salamanca, España
[b]Departamento de Investigación, Antibióticos S.A., Madrid, España
[c]Planta Piloto de Fermentación, Antibióticos S.A., León, España

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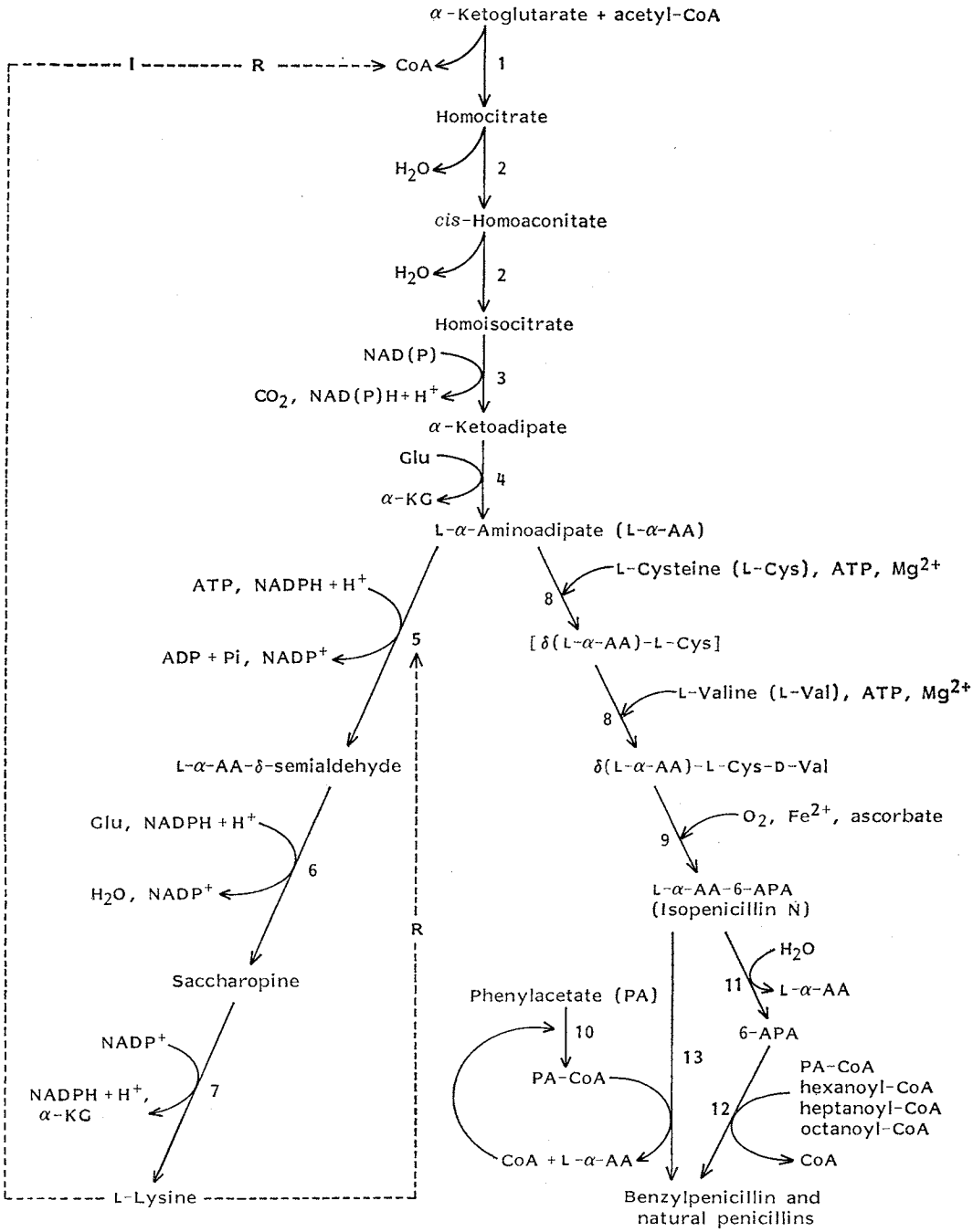
Different penicillins (phenylacetyl, 2-hydroxyphenylacetyl, 4-hydroxyphenylacetyl, phenoxyacetyl and 2-thiopheneacetylpenicillin) have been synthesized "*in vitro*" by direct *N*-acylation of 6-aminopenicillanic acid (6-APA) with the acyl group of several acyl-CoA derivatives. The enzyme that catalyzes these reactions, acyl-CoA: 6-APA acyltransferase of *Penicillium chrysogenum*, was purified to homogeneity (374-fold) and its amino acid composition is given. This protein accepts as substrates several aliphatic acids and different aromatic acids with the only requirement that an acetyl-CoA moiety must be present in the substrate molecule. Shortening or lengthening of the acyl moiety prevents the 6-APA-*N*-acylation reaction. The presence of an amino group in the α-position of the acetyl group does not allow this molecule to be used as substrate. However, different substitutions in the phenyl group (hydroxylation of the carbons 2 and 4) or its replacement by another aromatic ring (thiophene) were accepted with varying reactions rates in the acylation reaction when a 176-fold purified acyltransferase was employed. The homogeneity pure enzyme accepts as substrate thiophene acetyl-CoA but it did not 2-hydroxyphenyl and 4-hydroxyphenylacetyl-CoA. The presence of an oxygen atom between the aromatic and the acetyl moieties did not affect the catalysis.

The biosynthesis of penicillin in *Penicillium chrysogenum* (Fig. 1) 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 (IPN)[3,4]. Unlike other antibiotics (cephamycins, cephalosporins) the α-aminoadipic acid moiety present in the IPN molecule is exchanged with several side chain precursors (phenylacetic, phenoxyacetic acid) thereby generating different penicillins[5~9]. It has been reported that the enzyme which catalyzes this transfer, acyl-CoA: 6-Aminopenicillanic acid (6-APA) acyltransferase (so-called phenylacetyl CoA: 6-APA phenylacetyltransferase[8,9]) can use a number of CoA derivatives at different rates as substrates[6] and although it accepts to some degree single molecular changes in the structure of the side chain precursors, further modifications result in a lack of acylation[7]. However, the lack of a method that allows the ready evaluation of this enzyme considerably hinders the study of its substrate specificity. We have previously reported[8] a very easy biological assay (bioassay against *Micrococcus luteus* ATCC 9341) that permitted determination of optimal kinetic parameters of this enzyme, the minimal and maximal carbon length requirements for aliphatic side chain precursors[9], and the establishment of the bioactivity of the reaction products. Using this methodology we have been able to demonstrate that the enzyme acyl-CoA: 6-APA acyl-

† Present address: Departamento de Bioquímica y Biología Molecular, Universidad de León, España.

Fig. 1. Postulated branched pathway to L-lysine and benzylpenicillin biosynthesis in *Penicillium chrysogenum*.



I: Inhibition, R: repression, 1: homocitrate synthase, 2: homoaconitase (homoaconitate hydratase), 3: isocitrate dehydrogenase, 4: ketoadipate-glutamate-transaminase, 5: α-aminoadipate-δ-semialdehyde dehydrogenase, 6: saccharopine dehydrogenase (glutamate forming), 7: lysine-α-keto glutarate reductase (saccharopine dehydrogenase lysine forming), 8: L-α-aminoadipyl-L-cysteine-D-valine synthetase (ACV synthetase), 9: isopenicillin N synthase (ACV cyclase), 10: phenylacetyl-CoA ligase, 11: isopenicillin N amidolyase, 12: acyl-CoA: 6-APA acyltransferase, 13: acyl-CoA: isopenicillin N acyltransferase.

The enzymatic activities 11 and 12 could be associated "*in vivo*" in a single functional complex (acyl-CoA: Isopenicillin N acyltransferase, so called transacylase).

transferase has a fairly broad substrate specificity, being able to condense with 6-APA a wide variety of acetic acids containing aromatic substituents, thus generating different penicillins. The role of the acyl and aromatic moieties present in the substrates tested and their molecular implications in the 6-APA acylation reaction are described and discussed.

The broader substrate specificity of the acyl-CoA: 6-APA acyltransferase offers new possibilities for obtaining enzymatically penicillins with different antibacterial spectra and opens a new field in β-lactam antibiotic research.

Materials and Methods

Materials

Phenylacetylchloride, phenoxyacetylchloride, benzoylchloride, chlorobenzoyl, chloride (2-chloro, 3-chloro and 4-chloro), 3-phenylpropionylchloride, 2-thiopheneacetylchloride, phenylacetic acid, 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-phenylpropionic acid, 4-phenylbutyric acid, benzoic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid and 4-chlorobenzoic acid were obtained from Aldrich-Chemie (Germany).

D(−)-α-Aminophenylacetylchloride dihydrochloride hydrochloride was purchased from Deretil, S.A. (Spain). Phenylacetyl-CoA, hexanoyl-CoA, heptanoyl-CoA, octanoyl-CoA and D(−)-α-aminophenylacetic acid were from Sigma Chem. Co. (U.S.A.). Benzylpenicillin potassium salt (1,590 U/mg); phenoxyacetylpenicillin potassium salt (1,520 U/mg), 2-thiopheneacetylpenicillin potassium salt (1,540 U/mg), 2-hydroxybenzylpenicillin potassium salt (1,516 U/mg) and 4-hydroxybenzylpenicillin potassium salt (1,500 U/mg) were from Antibioticos, S.A.

Octyl-Sepharose CL-4B was from Pharmacia Fine Chemicals AB (Sweden).

Microorganisms

The strain of *P. chrysogenum* AS-P-78 used in the experimental work was from Antibioticos, S.A. This fungus produces about 8,000 U/ml of benzylpenicillin when incubated in a complex production medium[10].

M. luteus ATCC 9341 was used for the determination of the different penicillins by bioassay[11]. Strains were maintained in the lyophilized state.

Penicillin Synthesis

Aliphatic penicillins were synthesized from 6-APA and the corresponding acyl chloride as previously described[9].

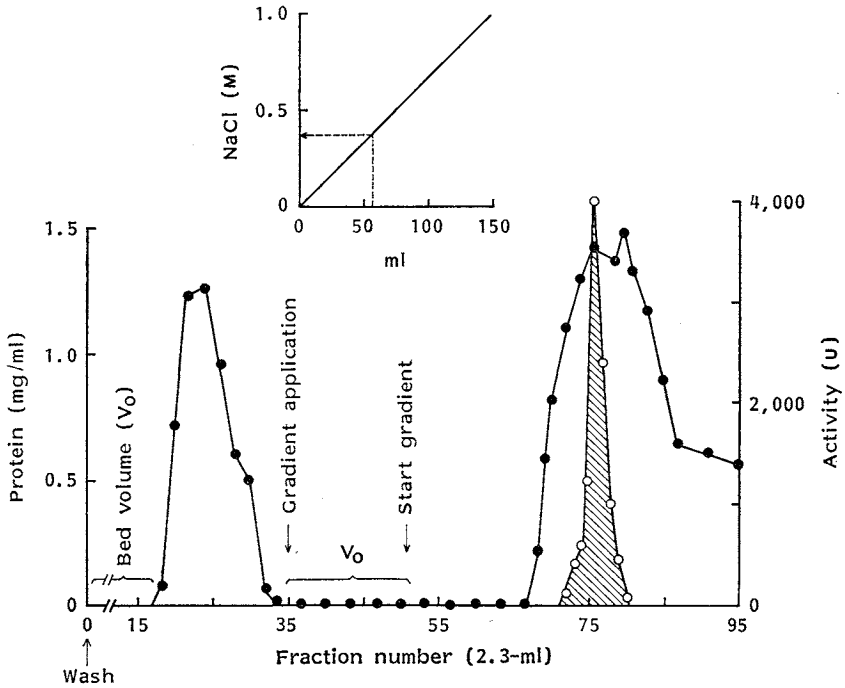
Chemical evaluation of the different antibiotics was carried out by the hydroxylamine procedure[12].

Experimental

Purification of Acyl-CoA: 6-APA Acyltransferase

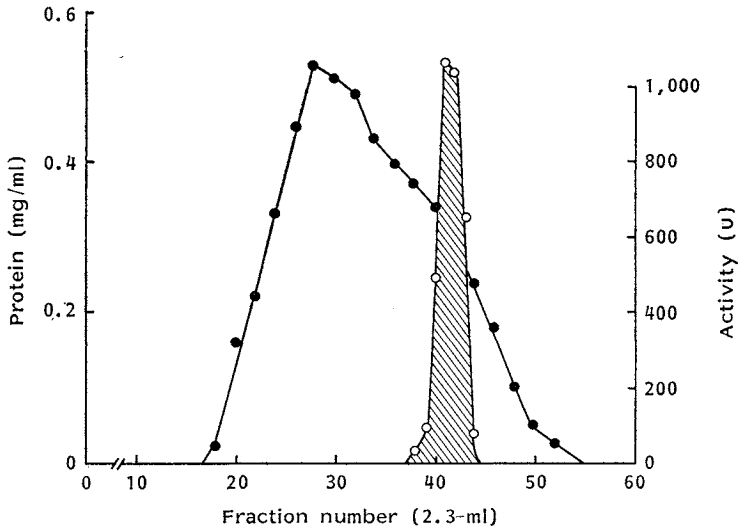
P. chrysogenum AS-P-78 was grown in two stages (seed and complex medium, as previously reported[10]). Mycelia were harvested from the fermentation broth at 36 hours, collected, centrifuged (12,000 × *g*, 30 minutes, 4°C) washed with sterile saline solution and processed as described[8]. In that previous report we obtained a 40-fold purified acyl-CoA: 6-APA acyltransferase; however, further purification was achieved by modification of the preceding procedure. The protein fraction that precipitated between 35~70% saturation of ammonium sulfate was dissolved in 5 ml of 0.015 M Tris, 0.02 M KH_2PO_4, pH 8.4 buffer (TP buffer) and the excess of ammonium sulfate was eliminated by passing the enzyme solution (2-ml aliquots) through a Sephadex G-25 PD-10 column (Pharmacia) equilibrated with TP buffer. Aliquots of 1 ml were collected and assayed for acyltransferase activity. Fractions 3 to 5 containing maximal activity were mixed and the total volume was ultracentrifuged (200,000 × *g*, 60 minutes, 2°C). The supernatant fluid was applied to a DEAE-cellulose column (2.3 × 22 cm) and elution was carried out with a NaCl gradient (0~1 M). Fractions of 2.3-ml were collected (flow rate 25 ml/hour). The acyl-CoA: 6-APA acyltransferase activity eluted between frac-

Fig. 2. Profile of elution from DEAE-cellulose column.



Fractions (2.3-ml) were collected at 25 ml/hour. Acyl-CoA: 6-APA acyltransferase (shaded area) eluted between the fractions 72 to 81. ● Protein, ○ acyltransferase activity.

Fig. 3. Gel filtration through a Sephadex G-200 column.



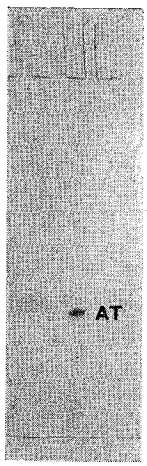
Fractions (2.3-ml) were collected at 25 ml/hour. Acyl-CoA: 6-APA acyltransferase activity (shaded area) eluted between the fractions 38 to 46. Maximal activity was found in fractions No. 41 and 42. ● Protein, ○ acyltransferase activity.

tions 72~81 with a peak in the fractions 76~77 (NaCl 0.35 M; bed volume 36.8 ml) (Fig. 2). The fractions containing activity were pooled, concentrated through an Amicon membrane (exclusion size 30,000) to 1 ml (32 mg protein) and applied to a Sephadex G-200 column (2.5×28 cm). Elution

Table 1. Purification of acyl-CoA: 6-APA acyltransferase.

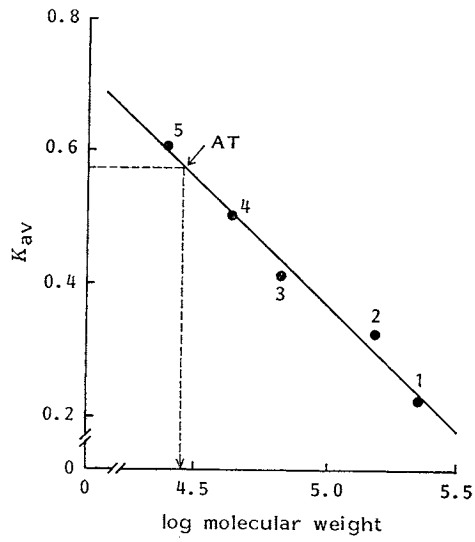
Treatment	Total protein (mg)	Enzyme activity (U)	Specific activity (U/mg protein)	Purification factor
Crude extract	3,944	27,642	7.00	1
Streptomycin sulfate precipitation	1,950	26,240	13.45	1.92
Ammonium sulfate precipitation (35~70%)	420	22,785	54.25	7.75
Sephadex G-25 eluate	276	16,229	58.8	8.4
Ultracentrifugation (200,000×g, 1 hour)	161	14,200	88.2	12.6
DEAE-cellulose	32	10,029	313.4	44.7
Sephadex G-200 eluate (fractions 41 and 43)	1.7	2,096	1,233	176.1
Octyl-Sepharose CL-4B (fractions 9~14)	0.5	1,310	2,621	374.4

Fig. 4. Electrophoretical mobility of purified acyltransferase (AT).



A sample (about 15 μg of protein) of the 374-fold purified acyltransferase (see Table 1) was run on 7% polyacrylamide gel electrophoresis. The enzyme appears as a single band with a Rf of 0.68.

Fig. 5. Determination of molecular weight of acyl-CoA: 6-APA acyltransferase from *Penicillium chrysogenum*.

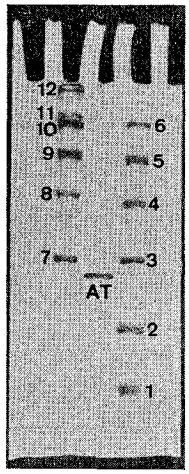


The molecular weight of the enzyme was calculated by using a calibrated Sephadex G-200 column with known proteins. 1: Catalase (232,000), 2: aldolase (158,000), 3: bovine serum albumin (66,000), 4: ovoalbumin (45,000), 5: chymotrypsinogen A (25,000), AT: acyltransferase.

These standard proteins were obtained from a Gel Calibration Kit (Pharmacia Fine Chem.) (Sweden). V_e: Elution volume, V_o: bed volume, V_t: total volume, K_{av}: partition coefficient, $K_{av}=(V_e-V_o)/(V_t-V_o)$.

was carried out with TP buffer at a low rate of 25 ml/hour (2.3-ml aliquots). Acyl-CoA: 6-APA acyltransferase activity was found in the fractions 38~46, showing a peak in the fractions 41 and 42 (Fig. 3). Fractions 41, 42 and 43 were mixed with the same volume of TP buffer containing 25% saturation of ammonium sulfate (TPS) and applied to an Octyl-Sepharose CL-4B column (6×1.5 cm). The column was washed with 100 ml of TPS buffer and eluted with the same buffer containing 30% (w/w) of ethylene glycol at a flow rate of 25 ml/hour (fractions of 1.1 ml). Acyltransferase activity eluted in the fractions 9~14 showing a peak in the fraction 11. By this procedure the enzyme was purified to homogeneity (374-fold) (Table 1). When necessary this enzyme solution was dialyzed against 1,000 volume of TP buffer for eliminating the ethylene glycol. The enzyme runs in 7% polyacrylamide gel electrophoresis[13] as a single band with a Rf of 0.68 (Fig. 4). According to the Sephadex G-200 column (Fig. 5) the acyltransferase is a small protein with a molecular weight of about 29±1.8 kdalton. The low molecular weight of this protein was confirmed

Fig. 6. SDS-10% polyacrylamide gel electrophoresis of purified acyltransferase and several molecular weight standard proteins.



1: α-Lactalbumin (MW 14,400), 2: soybean trypsin inhibitor (MW 20,100), 3 and 7: carbonic anhydrase (MW 29,000), 4 and 8: ovoalbumin (MW 45,000), 5 and 9: bovine serum albumin (MW 66,000), 6 and 10: phosphorylase B (MW 205,000), 11: β-galactosidase (MW 117,000), 12: myosin (MW 205,000), AT: acyltransferase. Proteins were stained with Coomassie Brilliant Blue R-250.

Table 2. Amino acid composition of acyltransferase.

Amino acid	Composition (residues/28 kdalton)
Asx (Asp+Asn)	24
Thr	16
Ser	27
Glx (Glu+Gln)	33
Gly	30
Ala	21
Val	13
Met	3
Ile	10
Leu	15
Tyr	7
Phe	8
His	4
Lys	10
Arg	8

by filtration through an Amicon membrane (exclusion size 30 kdalton). Part of the enzyme (10%) was found in the filtrate, indicating that the molecular weight of this protein is near to the critical retention size of the membrane. This result was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis[14] (Fig. 6). By this procedure we have estimated for this enzyme a MW of 28 kdalton.

Amino Acid Analysis

The homogeneity pure acyltransferase was hydrolyzed in thrice-distilled HCl (5.7 M) at 110°C for different times (24, 48, 72 and 96 hours) in evacuated sealed tubes. Hydrolysis were analyzed on a Durrum D-500 amino acid analyzer. Threonine and serine were extrapolated to time zero of hydrolysis. Amino acid composition is given in Table 2.

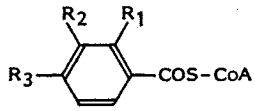
Stability of the Enzyme

The purified acyltransferase is very unstable, rapidly losing its activity at 0°C (more than 80% in 24 hours). This result, contrasts with the good stability of the enzyme in crude extract which can be stored at −20°C for months without significant loss of activity. Purified preparations are less stable under these conditions and are particularly sensitive to repeated freezing and thawing. Addition of sulfhydryl compounds such as dithiothreitol (DTT) or reduced glutathione (GSH) (1 mM) provide some protection. Inhibitors of proteolysis (phenylmethylsulfonylfluoride, leupeptin and pepstatin) did not improve stability. The most stable preparation was obtained with 20% (p/v) sucrose containing 1 mM DTT or with 50% ethylene glycol containing 1 mM DTT. In all the experiments in which substrate specificity was tested both 176-fold and homogeneity-pure acyltransferase were used.

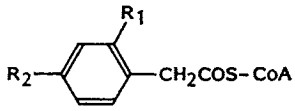
Enzyme Assay

At a total volume of 85 μl the reaction mixture contained the following: TCP buffer (0.05 M Tris, 0.1 M NaCl, 0.02 M KH_2PO_4) 50 μl, 10 mM phenylacetyl-CoA (or the corresponding acyl-CoA derivative) 10 μl, 0.3 mM 6-APA (or close structural molecule) 10 μl, 20 mM DTT 5 μl, enzyme 10 μl. Incubations were carried out at 20°C for 30 minutes (or the required time) and stopped by addition of 85 μl of pure methanol as previously described[8]. One U of enzymatic activity was defined as the amount of enzyme required to synthesize 1 μg of benzylpenicillin at 20°C over 30 minutes. Specific activity was expressed in U/mg protein.

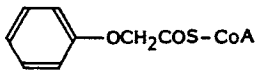
Fig. 7. Structure of the different acyl-CoA precursors tested as substrates of the acyl-CoA: 6-APA acyltransferase enzyme.



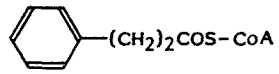
Benzoyl-CoA (**I**) $R_1 = R_2 = R_3 = H$
2-Chlorobenzoyl-CoA (**II**) $R_1 = Cl$ $R_2 = R_3 = H$
3-Chlorobenzoyl-CoA (**III**) $R_1 = R_3 = H$ $R_2 = Cl$
4-Chlorobenzoyl-CoA (**IV**) $R_1 = R_2 = H$ $R_3 = Cl$



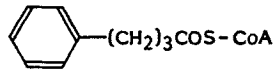
Phenylacetyl-CoA (**V**) $R_1 = R_2 = H$
2-Hydroxyphenylacetyl-CoA (**VI**) $R_1 = OH$ $R_2 = H$
4-Hydroxyphenylacetyl-CoA (**VII**) $R_1 = H$ $R_2 = OH$



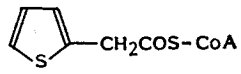
Phenoxyacetyl-CoA (**VIII**)



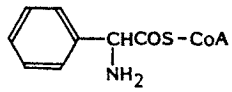
3-Phenylpropionyl-CoA (**IX**)



4-Phenylbutyryl-CoA (**X**)



2-Thiopheneacetyl-CoA (**XI**)



D(−)-α-Aminophenylacetyl-CoA (**XII**)

Synthesis of Acyl-CoA Derivatives

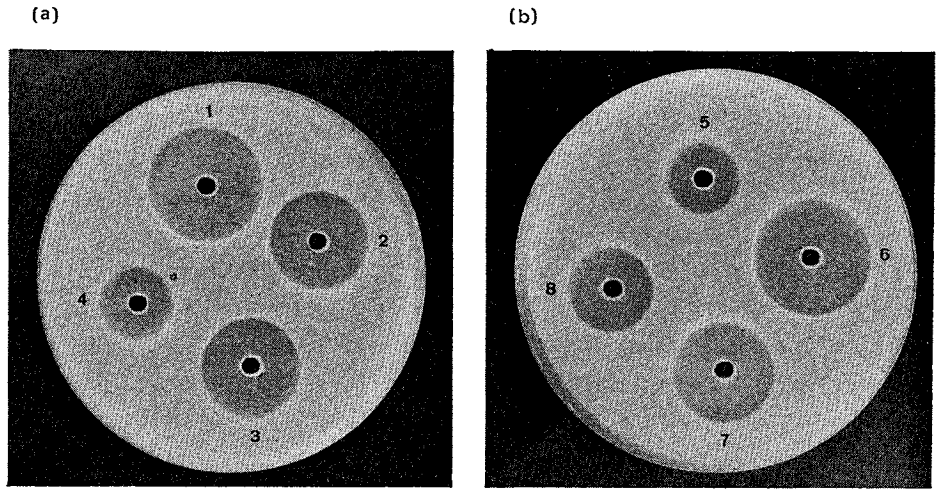
Benzoyl-CoA (I), chlorobenzoyl-CoA (2-chloro, 3-chloro and 4-chloro) (II, III and IV), phenylacetyl-CoA (V), 2-hydroxyphenylacetyl-CoA (VI), 4-hydroxyphenylacetyl-CoA (VII), phenoxyacetyl-CoA (VIII), 3-phenylpropionyl-CoA (IX), 4-phenylbutyryl-CoA (X), 2-thiopheneacetyl-CoA (XI) and D(−)-α-aminophenylacetyl-CoA (XII) (Fig. 7) were synthesized by standard procedures[15~17] using the acylchloride derivative and coenzyme A. A solution of 1.3 to 1.4×10^{-4} mmol of the acylchloride in 1.5 ml of ether or tetrahydrofurane was added dropwise to an ice-cooled vigorously-stirred solution of 77 mg (1×10^{-4} mmol) of coenzyme A in 1.5 ml of aqueous 0.2 M $KHCO_3$ (adjusted to pH 7.5).

When D(−)-α-aminophenylacetylchloride dihydrochloride was used, the crystalline compound was added at once to the ice-cooled CoA solution. The efficiency of conversion was evaluated using a quantitative assay of free-SH test[18,19] after separation of the organic and aqueous phases. The latter was lyophilized. The yields transformation (acyl-CoA formation) in the different reactions were: I 89%; II 72%; III 76%; IV 79%; V 85%; VI 65%; VII 69%; VIII 73%; IX 68%; X 70%; XI 83% and XII 60%.

Results and Discussion

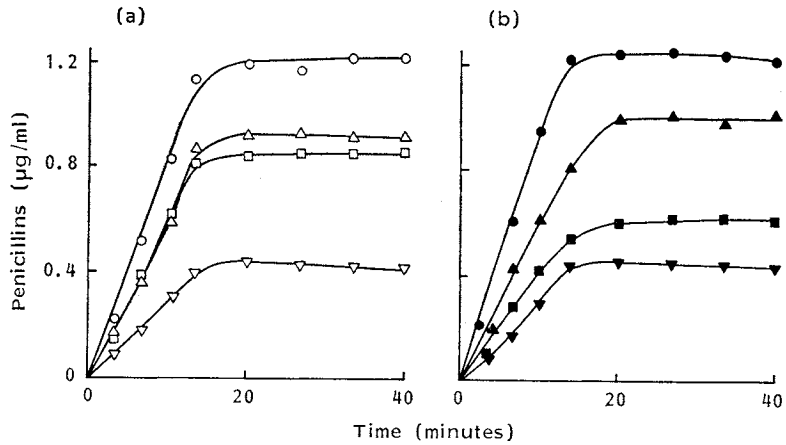
The incubation of acyl-CoA: 6-APA acyltransferase of *P. chrysogenum* (176-fold purified fraction) in the presence of 6-APA and different acyl-CoA derivatives (Fig. 7) generated molecules active against *M. luteus* ATCC 9341 when phenylacetyl, 2-hydroxyphenylacetyl, 4-hydroxyphenylacetyl, phenoxyacetyl and 2-thiopheneacetyl-CoA were used as side chain precursors (Fig. 8). This enzyme, like the 40-fold purified preparation[8,9] recognizes as substrates aliphatic side chain precursors whose carbon length ranges between 6 and 8 atoms (hexanoic, heptanoic and octanoic acids activated as CoA derivatives) (Fig. 8b); however, with benzoyl, 2-chloro, 3-chloro or 4-chlorobenzoyl-CoA, 3-phenyl-

Fig. 8. Bioassay against *Micrococcus luteus* of the product generated after incubating 6-APA, acyl-CoA: 6-APA acyltransferase and different acyl-CoA derivatives.



(a) 1: Phenylacetyl-CoA, 2: phenoxyacetyl-CoA, 3: 2-thiopheneacetyl-CoA, 4: 2-hydroxyphenylacetyl-CoA.
(b) 5: 4-Hydroxyphenylacetyl-CoA, 6: octanoyl-CoA, 7: heptanoyl-CoA, 8: hexanoyl-CoA.

Fig. 9. Time course formation of different penicillins.



(a) ○ Phenylacetylpenicillin, △ phenoxyacetylpenicillin, □ 2-thiopheneacetylpenicillin, ▽ 2-hydroxyphenylacetylpenicillin.
(b) ● Octanoylpenicillin, ▲ heptanoylpenicillin, ■ hexanoylpenicillin, ▼ 4-hydroxyphenylacetylpenicillin.

propionyl and 4-phenylbutyryl-CoA it did not. All the antibiotic molecules were destroyed by β-lactamase from *Bacillus cereus* (Difco). Control reactions without side chain precursors, 6-APA or enzyme were negative (data not shown). The kinetics of the synthesis of these penicillins evaluated by bioassay against *M. luteus*, followed at time-dependent course (Fig. 9). These results clearly indicate that chemical modification in the phenyl group (2-hydroxy, 4-hydroxy) or its substitution by another aromatic group (thiophene) can be accepted by this enzyme to a certain degree affecting the efficiency of the 6-APA-*N*-acylation reaction. However, the absence of bioactivity when benzoyl

and the three chlorobenzoyl-CoA derivatives were employed, strongly suggests that the acyl chain $CH_2COSCoA$ plays an important role in the formation of the enzyme-substrate complex. The fact that molecules with a longer acyl group (3-phenylpropionyl or 4-phenylbutyryl) are not used as substrates, implies that the acetyl-CoA moiety is of critical size in the substrate molecules. However, the substitution of a CH_2 group in 3-phenylpropionyl-CoA by an oxygen atom (phenoxyacetyl-CoA) implies a molecular variation that is sufficient for this compound to be used as substrate[6,7]. The use of other aromatic ring containing molecules such as indole derivatives (indole 3-carboxyl-CoA, indole 3-acetyl-CoA, 3-indole butyryl-CoA and 3-indole propionyl-CoA) did not generate bioactive products (data not shown).

A further aspect pointing to the need for a free moiety of acetyl-CoA is that the presence of an amino-group in the α-position of the acyl group (D(−)-α-aminophenylacetyl-CoA) also prevents 6-APA acylation. The presence of the NH_2 group in the side chain precursor molecule could cause steric impediments that hinder the access of phenylacetyl-CoA, blocking either its binding to the enzyme or its location in the active site. Since these inactive substrates (benzoyl-CoA, chlorobenzoyl-CoA, 3-phenylpropionyl-CoA, 4-phenylbutyryl-CoA and D(−)-α-aminophenylacetyl-CoA) when added (at a concentration of 5 mM) to the reaction mixtures containing 1 mM phenylacetyl-CoA, octanoyl-CoA, heptanoyl-CoA or hexanoyl-CoA, did not change the efficiency of 6-APA acylation (data not shown), it seem probable that these molecules are not bound to the enzyme.

When the other substrate (6-APA) of the acyltransferase reaction was replaced by similar molecules (7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid 7-ADCA) no acylation occurred. Furthermore if 6-APA and 7-ACA/7-ADCA were pooled together, the efficiency of the reaction did not decrease with any of the different acyl-CoA derivatives tested (phenylacetyl, phenoxyacetyl, hydroxylphenylacetyl or thiopheneacetyl-CoA). Such results imply that these molecules do not bind to the enzyme, indicating that this enzymatic system is specific for 6-APA and therefore for hydrophobic penicillin synthesis, but not for production of hydrophilic molecules such as cephalosporins. The absence of this enzyme activity in *Acremonium chrysogenum* cell free extracts[9] is in good agreement with the above results.

Moreover, the high affinity shown by this enzyme for 6-APA (*Km*: 1 μM)[8] contrasts with the *Km* values calculated for the side chain precursors (0.55 mM for phenylacetyl-CoA; 0.60 mM for octanoyl-CoA; 0.8 mM for heptanoyl-CoA; 1 mM for hexanoyl-CoA; 0.85 mM for 2-thiopheneacetyl-CoA; 2.6 mM for 2-hydroxyphenylacetyl-CoA and 2.1 mM for 4-hydroxyphenylacetyl-CoA). The lower specificity of these molecules could explain why *P. chrysogenum* is able to synthesize many different penicillins (G, V, DF, F, K and X) from a common nucleus (6-APA)[9].

When the homogeneity pure acyltransferase preparation was used, the results were similar to those previously described excepting when 2-hydroxy and 4-hydroxyphenylacetyl-CoA were employed as substrates. In these cases no bioactive products against *M. luteus* were generated. This fact could be explained by the instability of the purified enzyme (involving conformational changes in the acyltransferase molecule which affect its catalytic properties) or by the lost, during the last step of purification, of another protein (or subunit) with acyltransferase activity which is still present in the 176-fold purified preparation.

The results described in this report are important not only because they open the possibility of obtaining enzymatically a range of penicillins that cannot be produced by direct fermentation (adding the appropriate side chain precursor), but because they also permit an approach to the knowledge of

the biochemical and structural requirements of this important biosynthetic enzyme. These findings enlarge the scope of previously reported studies of other steps in penicillin biosynthesis[20~22].

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