REGULATION OF ENZYMES INVOLVED IN THE BIOSYNTHESIS OF THE SESQUITERPENE ANTIBIOTIC PENTALENOLACTONE IN *STREPTOMYCES ARENAE*

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The production of the sesquiterpenoid antibiotic pentalenolactone in the producer strain *Streptomyces arenae* TÜ 469 is controlled by the activity of the enzyme farnesylpyrophosphate cyclase. In contrast to the activity of this enzyme, the specific activities of all other enzymes of the mevalonoid pathway tested so far, proved to be not rate-limiting. Several metabolites of the pentalenolactone pathway were tested for inhibitory effects on the activity of the HMG-CoA reductase and farnesylpyrophosphate cyclase. The activity of the cyclase was inhibited by low concentrations of pentalenolactone and its derivatives, thus suggesting an end product inhibition of the starting enzyme of the pentalenolactone pathway. The activity of HMG-CoA reductase was not inhibited by pentalenene or any pentalenolactone-derivatives. According to these results, an end-product inhibition of the first enzyme which is specific for pentalenolactone synthesis seems to be a mechanism involved in the regulation of pentalenolactone biosynthesis.

The antibiotic pentalenolactone is one of the rare sesquiterpenoids produced by procaryotes. The mevalonoid origin of pentalenolactone (PL) was shown by CANE *et al.*[1] in incorporation experiments, where specifically labeled PL was produced by *Streptomyces* UC 5319 from ^{13}C and ^{2}H-labeled glucose in the culture medium. After the synthesis of the sesquiterpene hydrocarbon pentalenene by OHFUNE *et al.*[2], SETO *et al.*[3] isolated the compound from the cells of a PL-producing *Streptomyces* strain and thus demonstrated that pentalenene was a PL-precursor.

CANE and TILLMAN[4] measured the activity of an enzyme which catalyzes the cyclization of *trans-trans*-farnesylpyrophosphate to pentalenene and showed that the cyclization is catalyzed by a single enzyme *via* an enzyme-bound intermediate humulene[5]. The hypothetical pathway of PL-biosynthesis was revised recently by SETO *et al.*[6] after the isolation and structure elucidation of epipentalenolactone F (Fig. 1).

Streptomyces arenae TÜ 469 produces PL (arenaemycin)[7] when cultivated in defined media. Research directed towards increasing the amount of PL produced by *S. arenae* gave evidence that PL-production by this strain was highly regulated. Our investigations concentrated on studying the activity of the enzymes of the isoprenoid pathway to find the rate-limiting steps of PL-biosynthesis. Various metabolites of the bacterial isoprenoid pathway were therefore tested for their inhibitory or regulatory effects on the activity of PL biosynthetic enzymes.

Materials and Methods

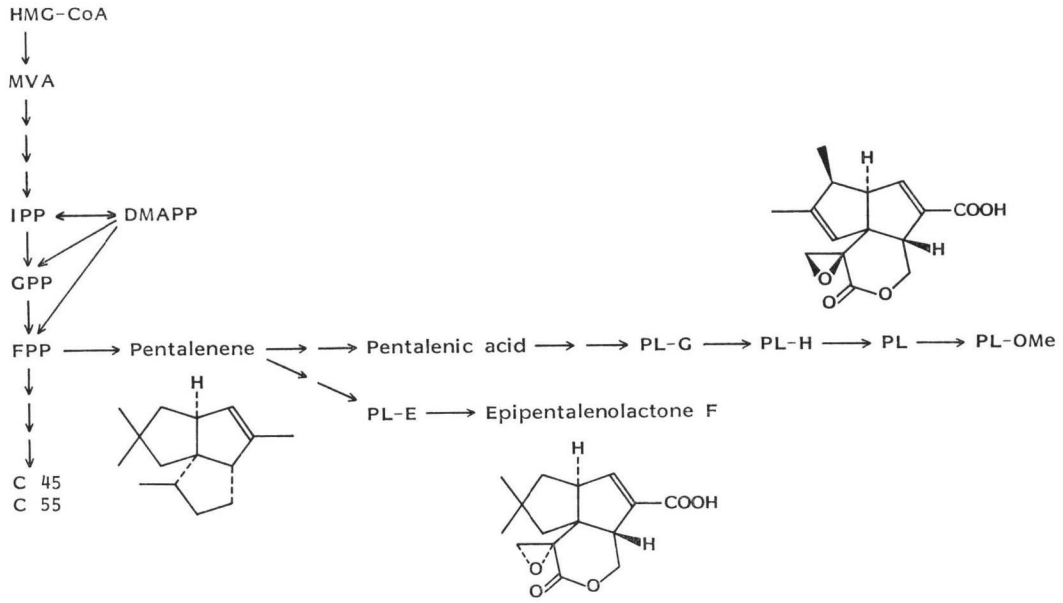
Abbreviations

PL, pentalenolactone; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenylpyrophosphate; DMAPP, dimethylallylpyrophosphate; GPP, geranylpyrophosphate; FPP, farnesyl-

Fig. 1. Principle of isoprenoid pathway in pentalenolactone-producing Streptomycetes.

Hypothetical pathway of PL-biosynthesis by SETO *et al.*[6]. The further synthesis of a PL-*O*-methyl ether is reported in the present investigation.

MVA, mevalonic acid; C 45, triterpenoids (hopanoids, menaquinone residues); C 55, undecaprenyl phosphate sugar carrier.



pyrophosphate.

Chemicals and Reagents

[3-^{14}C]HMG-CoA was purchased from New England Nuclear (Boston, Mass.), [1-^{14}C]isopentenylpyrophosphate from Amersham-Buchler (Braunschweig, FRG), [1-^{14}C]farnesylpyrophosphate was synthesized enzymatically from [1-^{14}C]isopentenylpyrophosphate and geranylpyrophosphate according to POPJAK[8]. IPP, DMAPP, GPP and FPP were prepared as described by CORNFORTH and POPJAK[9]. (±)Pentalenene was synthesized from humulene according to OHFUNE *et al.*[2]. Pentalenolactone and its derivatives were prepared from the fermentation broth of *S. arenae* as described before[10].

Enzyme Assays

Assay of the HMG-CoA Reductase: The assay was performed according to the method of SHAPIRO *et al.*[11]. The enzyme activity in all cases was linear within 20 minutes incubation time and up to 0.25 mg protein under the above mentioned conditions. The enzyme assays were done in triplicate.

Assay of Isopentenylpyrophosphate Isomerase: The assay of IPP isomerase was performed as described by PEREZ *et al.*[12]. The enzyme assays were done in triplicate and the activity was calculated as described[12].

Assay of Dimethylallyl- and Geranyltransferase: The assay of prenyl transferases was performed as described by TAKAHASHI and OGURA[13]. The results were corrected for the IPP isomerase activity.

Assay of Farnesylpyrophosphate Cyclase Activity: The test was performed in a modified procedure according to CANE and TILLMAN[4]. 25 ml of cell-free supernatant of ultrasonic disrupted cells in 100 mM Tris-HCl pH 7.5, containing 0.4 mM dithioerythritol, 1 mM EDTA and 5% glycerol, were degassed by sparging with nitrogen for 1 minute. The reaction was started by addition of 5 μmol $MgCl_2$ and 0.2 μmol [1-^{14}C]-*trans-trans*-farnesylpyrophosphate (5.9×10^8 dpm/mmol). After 60 minutes at 30°C, the reaction was quenched by addition of 25 ml acetone. After addition of 0.4 mg of synthetic (±)pentalenene, the mixture was extracted with hexane. The hexane phase was dried,

concentrated and applied to preparative thin-layer chromatography with hexane - chloroform, 9: 1. The pentalenene containing area was scraped out and counted in toluene-scintillator. The results were corrected for the counts of boiled protein samples.

Determination of Pentalenolactone: Pentalenolactone was determined in an enzymatic test system with glyceraldehyde-3-phosphate dehydrogenase as described earlier[10].

Media and Culture Conditions

S. arenae TÜ 469 was grown on a peptone, yeast extract, malt extract and glucose medium as complex nutrition medium providing good growth. All experiments were done with cells grown in 100 ml medium in 1-liter Erlenmeyer flasks at 30°C with shaking. *S. arenae* produced PL when transferred from complex medium to a minimal medium containing mannitol 4%, asparagine 0.25%, $(NH_4)_2SO_4$ 0.2%, NaCl 0.1%, $K_2HPO_4 \cdot 3H_2O$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.4%, $FeSO_4 \cdot 7H_2O$ 0.01% and $ZnSO_4 \cdot 7H_2O$ 0.001% at pH 6.2 and 30°C. For PL-production, 1 ml of a 48-hour culture of *S. arenae* was transferred to the minimal medium. For the determination of enzyme activities, cells were harvested from $1 \sim 6 \times 100$ ml cultures in minimal medium (depending on growth phase and enzyme activity) and washed with destilled water, 1 M KCl and 0.9% NaCl. The washed cells were suspended in the buffer according to the respective enzyme assay and were broken by ultrasonic disruption in a Branson sonifier 12 S for 60 seconds in four 15-second pulses at 0°C. Cell debris was removed by 30-minute centrifugation at $20,000 \times g$. The supernatants of such centrifugations normally were used in the enzyme assays described. No pentalenolactone could be found in these crude extracts.

Protein Determination: Protein determination was by the procedures of BRADFORD[14] and LOWRY *et al.*[15].

Results

The characteristic growth and PL-production of *S. arenae* cells is shown in Fig. 2. The production of PL started shortly after the beginning of the growth phase. At the end of the growth phase, the concentration of PL decreased rapidly. At least part of this decrease can be attributed to a rapid degradation of PL. This could be shown by feeding experiments with ^3H-labeled PL which gave a

Fig. 2. Growth and PL-production rate of *S. arenae* after inoculation of minimal medium with cells grown in complex medium.

■ Mycelium dry weight, ● specific production rate for PL.

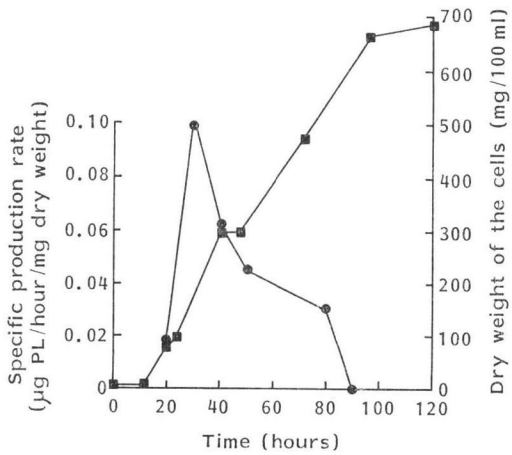


Fig. 3. Specific activity of HMG-CoA reductase in cell-free extracts of *S. arenae* during the cultivation for PL-production.

The means of the experimentally derived values were shown.

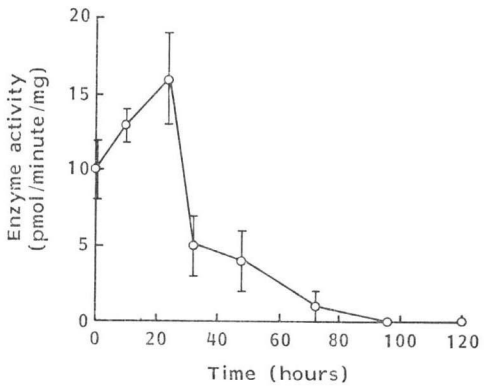


Fig. 4. Specific activity of isopentenylpyrophosphate isomerase, dimethylallyl transferase and geranyl transferase in cell-free extracts of *S. arenae* during the cultivation in the synthetic medium.

Enzyme activities were tested as described in Materials and Methods.

▲ Isopentenylpyrophosphate isomerase, ○ dimethylallyl transferase, ■ geranyl transferase.

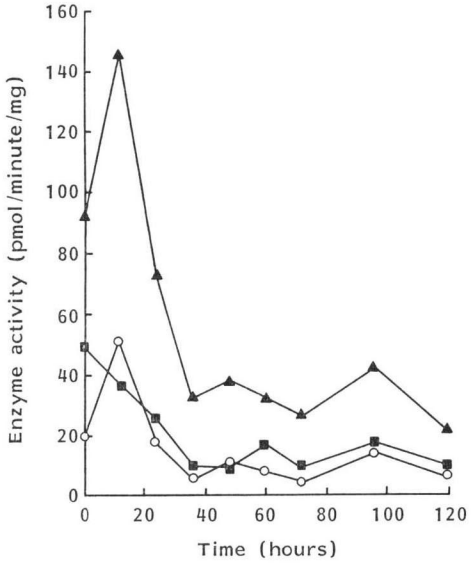
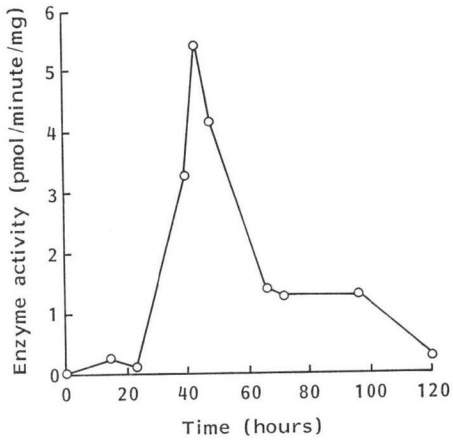


Fig. 5. Specific activity of the farnesylpyrophosphate cyclase of cell-free extracts of *S. arenae* during the cultivation in the synthetic medium.



variety of degradation products. The specific production rate had a maximum after 30~40 hours of cultivation.

The enzymes of the isoprenoid pathway were tested to look for steps which could be responsible for the regulation of PL-biosynthesis.

The specific activities of HMG-CoA reductase, IPP isomerase, DMA transferase, geranyl transferase and FPP cyclase were tested during cultivation. The HMG-CoA reductase activity in crude extracts of *S. arenae* cells was found to be unchanged after centrifugation at 100,000×*g* and by the use of detergents. Thus, it can be presumed that the enzyme is soluble in *S. arenae*. When cells were transferred from complex medium into chemically defined medium, there was a slight increase in the specific activity of HMG-CoA reductase (Fig. 3). This was followed by a rapid decrease to values approaching the limit of determination after 80~96 hours of cultivation, the time of maximum PL-production.

When the specific activities of IPP isomerase, DMA transferase and geranyl transferase were tested during the cultivation period (Fig. 4), their time-courses proved to be very similar to the time-course of HMG-CoA reductase activity. The activities of the transferases and IPP isomerase were markedly higher, but no correlation between enzyme activity and PL-production could be found.

The activity of FPP cyclase showed quite different characteristics when followed during cultivation (Fig. 5). No activity could be detected in cells grown in complex media or during the first 36 hours of cultivation in chemically defined medium. After a sharp maximum of specific activity at 40 hours, it decreased to an activity of 1 pmol/minute/mg. This behavior correlates to the time-course of the specific production rate of PL shown in Fig. 2. Pentalenene was the only product formed from FPP in crude extract which was extractable by hexane. To test the hypothesis of end product inhibition of HMG-CoA reductase and FPP cyclase activity, crude extracts with high specific activities were tested in the presence of various intermediates and products of the pentalenolactone pathway.

Table 1. Inhibition of the farnesylpyrophosphate cyclase by PL (chlorohydrine) and the supposed PL-*O*-methyl ether.

In each assay 25 ml of crude extract of a 40-hour culture were tested, showing a specific activity of 8.6 pmol/minute/mg. The inhibitors were added to the assay mixture together with the substrate [^{14}C]farnesylpyrophosphate.

Inhibitor	Inhibitor concentration (μM)	Inhibition (%)
PL (chlorohydrine)	100	50
	25	19
PL-*O*-methyl ether	30	69
	25	66
	15	50
	5	37

The HMG-CoA reductase assay was started by addition of [^{14}C]HMG-CoA, 30 minutes after preincubation of the crude extract with the substances tested. Because of the high instability of FPP cyclase in crude extracts[4], the enzyme assay was started immediately after addition of the test substances, by addition of the substrate [^{14}C]FPP.

The HMG-CoA reductase activity showed no reaction towards any of the PL compounds tested in a concentration of 30 μM. Furthermore the activity was not affected by 200 μM (±)pentalenene, 2.5 mM humulene, a crude lipid extract of *S. arenae* cells or 1 mM mevalonic acid.

In contrast to the HMG-CoA reductase, the FPP cyclase activity was highly sensitive to PL and PL-derivatives.

FPP-cyclase proved to be most sensitive towards a PL-derivative which according to IR and NMR data* is presumed to have the structure of a pentalenolactone-*O*-methyl ether. After feeding experiments with ^{3}H-labeled PL, this compound could be shown to represent one of the degradation products of PL. The sensitivity of FPP cyclase towards PL and PL-*O*-methyl ether is shown in Table 1. The relative inhibitory activity of PL and the PL-*O*-methyl ether was determined by the ratio of the concentration necessary to get a 50% inhibition of FPP cyclase. All other PL-derivatives tested so far have shown inhibitory effects on farnesylpyrophosphate cyclase, but no product inhibition of the enzyme has yet been found after addition of (±)pentalenene or humulene, although humulene caused inhibition of PL-production *in vivo*.

Discussion

HMG-CoA reductase in eucaryotic organisms is a highly regulated enzyme. In bacteria, the activity of the enzyme has only been measured in a *Pseudomonas* species grown on mevalonic acid as the sole carbon source[16]. The *Pseudomonas* HMG-CoA reductase is soluble and accepts NADH as a coenzyme. In *S. arenae* TÜ 469, the HMG-CoA reductase was soluble, but had no distinct coenzyme specificity towards NADPH and NADH. The activity was not inducible by mevalonic acid like in the *Pseudomonas* strain. No sign of product inhibition of the HMG-CoA reductase in *S. arenae* could be found, even when a crude lipid extract was incubated with the enzyme activity. Thus, regulation of the HMG-CoA reductase activity could possibly occur at the level of repression mechanisms.

The activity of FPP cyclase was regulated by end product inhibition by pentalenolactone and derivatives. This seems reasonable since FPP cyclase catalyzes the first step leading specifically to the secondary product pentalenolactone, whereas the rest of isoprenoid pathway is part of the primary metabolism. Possibly an end product inhibition of the HMG-CoA reductase does not occur because the product of the enzyme is the substrate for the synthesis of several different metabolites.

Humulene and pentalenene had no effect on the activity of FPP cyclase nor HMG-CoA reductase. Therefore, the detectable inhibition of PL-production by humulene *in vivo* is presumed to be a competi-

* Oil, FT-IR (KBr) 1769, 1702, 1666 cm^{-1}; ^{1}H NMR (400 MHz, in $CDCl_3$) 1.04 (3H, d, J=7 Hz), 1.69 (3H, s), 2.74 (1H, m), 3.18 (1H, d, J=10 Hz), 3.18 (1H, d, J=5 Hz), 3.27 (1H, s), 3.45 (3H, s), 3.88 (2H, d, J=9 Hz), 4.47 (1H, dd, J=10.7, 1 Hz), 5.22 (1H, dd, J=10.7, 5 Hz), 5.42 (1H, br s), 6.83 (1H, s).

tive inhibition of the enzymes which catalyze the oxidation of pentalenene to the different pentalenolactones.

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References

1) CANE, D. E.; T. ROSSI, A. M. TILLMAN & J. P. PACHLATKO: Stereochemical studies of isoprenoid biosynthesis of pentalenolactone from [*U*-^{13}C]glucose and [6-^{2}H]glucose. J. Am. Chem. Soc. 103: 1838~1843, 1981
2) OHFUNE, Y.; H. SHIRAHAMA & T. MATSUMOTO: Formolysis of protoilludyl cation equivalents. A new mode of rearrangement of bicyclo[4.2.0]oct-2-yl cation. Tetrahedron Lett. 1976: 2869~2872, 1976
3) SETO, H. & H. YONEHARA: Studies on the biosynthesis of pentalenolactone. III. Isolation of a biosynthetic intermediate hydrocarbon pentalenene. J. Antibiotics 33: 92~93, 1980
4) CANE, D. E. & A. M. TILLMAN: Pentalenene biosynthesis and the enzymatic cyclization of farnesyl pyrophosphate. J. Am. Chem. Soc. 105: 122~124, 1983
5) CANE, D. E.; C. ABELL & A. M. TILLMAN: Pentalenene biosynthesis and the enzymatic cyclization of farnesyl pyrophosphate: Proof that the cyclization is catalyzed by a single enzyme. Bioorg. Chem. 12: 312~328, 1984
6) SETO, H.; H. NOGUCHI, U. SANKAWA & Y. IITAKA: Studies on the biosynthesis of pentalenolactone. VI. The X-ray crystal structure investigation of pentalenolactone G and structural revision of pentalenolactone F. J. Antibiotics 37: 816~817, 1984
7) KELLER-SCHIERLEIN, W.; J. LEMKE, R. NYFELER & H. ZÄHNER: Stoffwechselprodukte von Mikroorganismen. 105. Arenaemycin E, D und C. Arch. Mikrobiol. 84: 301~316, 1972
8) POPJAK, G.: Enzymes of sterol biosynthesis in liver and intermediates of sterol biosynthesis. Methods Enzymol. 15: 393~494, 1969
9) CORNFORTH, R. H. & G. POPJAK: Chemical synthesis of substrates of sterol biosynthesis. Methods Enzymol. 15: 359~390, 1969
10) MAURER, K. H.; F. PFEIFFER, H. ZEHENDER & D. MECKE: Characterization of two glyceraldehyde-3-phosphate dehydrogenase isoenzymes from the pentalenolactone producer *Streptomyces arenae*. J. Bacteriol. 153: 930~936, 1983
11) SHAPIRO, D. J.; J. L. NORDSTROM, J. J. MITSCHELEN, V. W. RODWELL & R. T. SCHIMKE: Micro assay for 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts. Biochim. Biophys. Acta 370: 369~377, 1974
12) PEREZ, L. M.; R. LOZADA & O. CORI: Biosynthesis of allylic isoprenoid pyrophosphates by an enzyme preparation from the flavedo of *Citrus paradisii*. Phytochemistry 22: 431~433, 1983
13) TAKAHASHI, I. & K. OGURA: Farnesyl pyrophosphate synthetase from *Bacillus subtilis*. J. Biochem. 89: 1581~1587, 1981
14) BRADFORD, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248~254, 1976
15) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193: 265~275, 1951
16) RODWELL, V. W. & W. R. BENSCH: *S*-3-Hydroxy-3-methylglutaryl-CoA reductase from *Pseudomonas* sp. Methods Enzymol. 71: 480~487, 1981