Phenylalanine and Tyrosine Biosynthesis in Sporeforming Members of the Order Actinomycetales

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Dedicated to Professor H. Simon on the occasion of his 60th birthday

Actinomycetales, Biosynthesis of Phenylalanine and Tyrosine, Arogenate Dehydrogenase, Chorismate Mutase, Prephenate Dehydratase

The enzymes of the terminal steps of phenylalanine and tyrosine biosynthesis, chorismate mutase, prephenate dehydratase, arogenate dehydratase, prephenate dehydrogenase and arogenate dehydrogenase were studied in 13 sporeforming members of the order Actinomycetales. In these organisms tyrosine is synthesized exclusively *via* arogenate, phenylalanine, however, *via* phenylpyruvate. The regulation pattern of the corresponding enzymes was determined: No feedback inhibition of arogenate dehydrogenase by L-phenylalanine and L-tyrosine was observed. Chorismate mutase was found to be inhibited in all organisms by L-tyrosine and in most organisms by L-tryptophan. L-Phenylalanine was shown to inhibit prephenate dehydratase in the majority of bacteria tested and L-tyrosine activated this enzyme in most cases.

The elution profiles for the phenylalanine and tyrosine biosynthetic enzymes were studied in three members of the order Actinomycetales by anion exchange chromatography on DEAE-cellulose.

Introduction

The exact composition and definition of the order Actinomycetales are still open to question and modifications by the application of new taxonomic approaches. Single cells of these organisms show different morphology ranging from simple to complex, with two broad morphological groups, nocardiaform- and sporo-actinomycetes [1, 2]. Nocardiaform bacteria form hyphae which eventually fragment into coccoid or rod-like elements that give rise to new mycelia. The Sporoactinomycetes show a greater morphological complexity that includes the formation of spores in or on definite parts of the mycelium.

The application of new and reliable biochemical, chemical, genetical, numerical and molecular biological techniques has been responsible for rapidly changing views on how bacteria ought to be classified and identified [1].

The newer methods are now being applied to sporoactinomycetes with interesting results on the phylogenetic relationships of these organisms [3–5].

In a recent paper we reported on the biosynthesis of phenylalanine and tyrosine and their regulation patterns in a variety of Streptomycetes [6]. The biosynthesis from chorismate may proceed *via* prephenate or *via* arogenate (Fig. 1). The diversity in the use of certain enzymatic steps, in the regulatory properties of individual enzymes, and in the utilisation of either NAD^+ or $NADP^+$ (or both) as cofactors for the dehydrogenase(s) gives rise to a number of different biosynthetical and biochemical possibilities for a certain organism.

To broaden the basis of these studies, we investigated various bacteria of the family Actinoplanaceae and one representative of the families Streptomycetaceae, Micromonosporaceae and Nocardiaceae, respectively.

Reprint requests to Prof. Dr. Lingens.

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Materials and Methods

Organisms and culture conditions

All of the organisms investigated were obtained from O. Salcher, Bayer-AG, Wuppertal, Germany (Federal Republic), (Table I).

All organisms were grown in yeast-malt extract broth containing 0.4% yeast extract, 1% malt extract, 0.4% glucose and 0.2% $CaCO_3$. All strains were grown aerobically on a rotary shaker at 30 °C and harvested after five days.

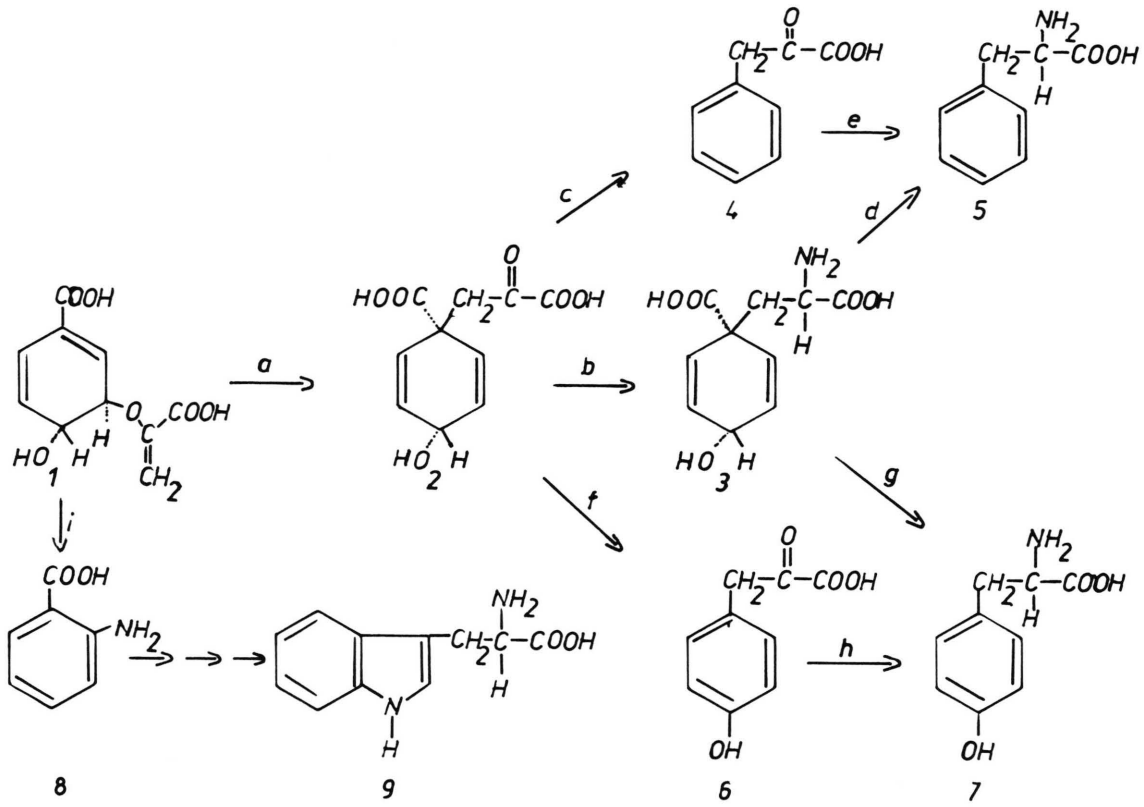

Fig. 1. Biochemical routes of tryptophan, phenylalanine and tyrosine biosynthesis. Trivial names of enzymes: chorismate mutase (a), prephenate aminotransferase (b), prephenate dehydratase (c), arogenate dehydratase (d), phenylalanine aminotransferase (e), prephenate dehydrogenase (f), arogenate dehydrogenase (g), tyrosine aminotransferase (h), anthranilate synthase (i), Compounds: chorismate (1), prephenate (2), arogenate (3), phenylpyruvate (4), phenylalanine (5), 4-hydroxyphenylpyruvate (6), tyrosine (7), anthranilate (8), tryptophan (9).

Table I. Sporeforming members of the order Actinomycetales.

Organism	DSM-Nr.	Family[a]	Term[b]
Actinoplanes missouriensis	43046	Actinoplanaceae	Actinoplanetes
Actinoplanes philippinensis	43019	Actinoplanaceae	Actinoplanetes
Amorphosporangium auranticolor	43031	Actinoplanaceae	Actinoplanetes
Ampullariella campanulata	43148	Actinoplanaceae	Actinoplanetes
Pilimelia terevasa	43040	Actinoplanaceae	Actinoplanetes
Dactylosporangium thailandensis	43158	Actinoplanaceae	Actinoplanetes
Micromonospora echinospora	43141	Micromonosporaceae	Actinoplanetes
Planobispora longispora	43041	Actinoplanaceae	Maduromycetes
Planomonospora venezuelensis	43178	Actinoplanaceae	Maduromycetes
Spirillospora albida	43034	Actinoplanaceae	Maduromycetes
Streptosporangium roseum	43021	Actinoplanaceae	Maduromycetes
Actinomadura citrea	43461	Nocardiaceae	Maduromycetes
Actinopycnidium caeruleum	43030	Streptomycetaceae	Streptomycetes

[a] According to Bergey's Manual of Determinative Bacteriology 8th ed. [7].
[b] As defined by [1] and by DNA-DNA- and DNA-rRNA-reassociation [4].

Preparation of cell-free extracts

Cells were suspended in 100 mM-potassium phosphate buffer, pH 8.0 (buffer A) and disrupted by sonification (Branson sonifier J-17A). Cell debris was removed by centrifugation at $20{,}000 \times g$ or if necessary at $150{,}000 \times g$ for 1 h. The resulting crude extract was dialyzed overnight against 1000 volumes of buffer A.

Analytical techniques

Protein concentration was determined according to Lowry *et al.* [8] using bovine serum albumine as standard.

Enzyme assays

All assays were performed at 30 °C. Specific activities are expressed as nmol of product formed per min per mg protein = mU/mg.

Chorismate mutase (chorismate pyruvate mutase EC 5.4.99.5): We used the method of Cotton and Gibson [9].

The reaction mixture (300 µl) contained 300–400 µg protein, 500 nmol chorismate and buffer A and was incubated for 10 min.

Prephenate dehydratase (prephenate hydro-lyase (decarboxylating) EC 4.2.1.51): The technique according to Cotton and Gibson [10] was used. The reaction mixture (500 µl) contained 300–400 µg protein, 500 nmol prephenate and buffer A and was incubated for 10 min.

Prephenate dehydrogenase (prephenate: NAD^+ oxidoreductase (decarboxylating) EC 1.3.1.12): This enzyme was assayed by the method of Cotton and Gibson [11]. The reaction mixture contained 1 µmol NAD^+ or $NADP^+$, 50 nmol prephenate, 100–500 µg protein and buffer A to a final volume of 500 µl.

Arogenate dehydratase: The enzymatic product phenylalanine was determined spectrofluorometrically (excitation wavelength 360 nm, emission wavelength 495 nm) after oxidation of non-reacted arogenate according to Shapiro *et al.* [12]. The reaction mixture was incubated for 30 min and contained: 500–1500 µg protein, 200 nmol arogenate and buffer A to a final volume of 600 µl.

Arogenate dehydrogenase: The continuous formation of NADH or NADPH was followed spectrophotometrically at 340 nm. The reaction mixture contained 100–500 µg protein, 1 µmol NAD^+ or $NADP^+$, 50 nmol arogenate and buffer A to a final volume of 500 µl. The zero-blank contained all ingredients except arogenate. The enzymatic reaction was started by addition of arogenate.

DEAE-cellulose chromatography

Crude extract containing up to 500 mg protein was applied at 4 °C to a 2.5×20 cm DE-52 (Whatman) ion exchange column previously equilibrated with 10 mM potassium phosphate buffer pH 7.0, containing 1 mM dithioerythritol (buffer B). The protein was washed into the column with buffer B and thereafter with buffer B containing 0.1 M NaCl and eluted with 500 ml of a linear gradient of NaCl (0.1–0.5 M). Fractions of 10 ml were collected.

Chemicals

Chorismate was prepared by the method of Gibson [13], prephenate according to Dayan and Sprinson [14]. Arogenate was isolated from the culture supernatant of a triple mutant of *Neurospora crassa* ATCC 36373 as described by Lingens *et al.* [15].

All chemicals were of the highest purity available.

Results

Chorismate mutase

This allosteric enzyme common to both phenylalanine and tyrosine biosynthesis was detected in all organisms of this study (Table II).

Chorismate mutase activity was sensitive to feedback inhibition by L-tyrosine and partially by L-tryptophan.

The chorismate mutase of all members of the term Actinoplanetes except *Actinoplanes missouriensis* and *Actinoplanes philippinensis* was found to be product inhibited by L-tryptophan. The enzyme of all members of the term Maduromycetes except *Planomonospora venezuelensis* was not inhibited by L-tryptophan. L-Phenylalanine inhibited only the chorismate mutase of *Dactylosporangium thailandensis*.

Prephenate dehydratase/arogenate dehydratase

Dual pathways for phenylalanine biosynthesis were indicated by the presence of both prephenate dehydratase and arogenate dehydratase in *Micromonospora echinospora* and *Dactylosporangium*

Table II. Specific activities and relative chorismate mutase activities in Sporo-Actinomycetales. (Assays as described in Methods, assays without further supplementation were set 1, addition to the enzyme standard assay: 1 mM L-phenylalanine, 1 mM L-tyrosine or 1 mM L-tryptophan.)

Organism	Specific activity [mU/mg]	Relative activity L-phenylalanine	L-tyrosine	L-tryptophan
Actinoplanes missouriensis	5.0	1.2	0.6	1.0
Actinoplanes philippinensis	1.4	1.8	0.8	0.9
Amorphosporangium auranticolor	2.3	1.9	0.4	0.5
Ampullariella campanulata	8.4	0.8	0.4	0.2
Pilimelia terevasa	1.5	0.9	0.3	0.4
Dactylosporangium thailandensis	2.5	0.4	0.4	0.6
Micromonospora echinospora	5.0	0.9	0.7	0.8
Planobispora longispora	2.6	1.2	0.8	1.0
Planomonospora venezuelensis	6.7	1.0	0.4	0.4
Spirillospora albida	3.9	1.0	0.2	1.0
Streptosporangium roseum	6.1	1.0	0.1	1.0
Actinomadura citrea	7.0	1.4	0.4	0.7
Actinopycnidium caeruleum	15.7	1.2	0.6	0.6

thailandensis. In these strains an arogenate dehydratase activity of 0.4 mU/mg and 0.7 mU/mg, respectively, was found. In all other strains no arogenate dehydratase was detectable.

All data of prephenate dehydratase activities and feedback inhibition or activation by L-phenylalanine and L-tyrosine are summarized in Table III.

The prephenate dehydratase was found to be product inhibited by L-phenylalanine, except for the two Actinoplanes strains and *Planomonospora venezuelensis* and in several cases *(Amorphosporangium auranticolor, Planobispora longispora, Planomonospora venezuelensis)* strongly activated by L-tyrosine.

Arogenate dehydrogenase/prephenate dehydrogenase

In all bacteria tested arogenate dehydrogenase was detected but not prephenate dehydrogenase. Arogenate dehydrogenase activity required NAD^+, which could not be replaced in all organisms by $NADP^+$ (see Table IV). Arogenate dehydrogenase activity was not sensitive to feedback inhibition, neither by L-phenylalanine nor by L-tyrosine.

Table III. Specific activities and relative prephenate dehydratase activities in Sporo-Actinomycetales. (Assays as described in Methods, assays without further supplementation were set 1, addition to the enzyme standard assay: 1 mM L-phenylalanine or 1 mM L-tyrosine.)

Organism	Specific activity [mU/mg]	Relative activity L-phenylalanine	L-tyrosine
Actinoplanes missouriensis	1.7	1.0	0.9
Actinoplanes philippinensis	1.6	1.0	1.9
Amorphosporangium auranticolor	0.6	0.4	8.3
Ampullariella campanulata	1.2	0.2	3.6
Pilimelia terevasa	1.4	0.3	1.1
Dactylosporangium thailandensis	4.6	0.2	2.3
Micromonospora echinospora	1.0	0.7	0.7
Planobispora longispora	0.2	0.0	8.8
Planomonospora venezuelensis	1.5	1.9	6.3
Spirillospora albida	1.4	0.2	1.6
Streptosporangium roseum	4.8	0.0	2.0
Actinomadura citrea	1.5	0.6	0.5
Actinopycnidium caeruleum	30.0	0.8	0.5

Table IV. Specific activities and relative arogenate dehydrogenase activities in Sporo-Actinomycetales. (Assays as described in Methods, assays without further supplementation were set 1, addition to the standard enzyme assay: 1 mM L-phenylalanine or 1 mM L-tyrosine.)

Organism	Specific activity with NAD^+ [mU/mg]	Specific activity with $NADP^+$ [mU/mg]	Relative activity with NAD^+ + L-phenylalanine	+ L-tyrosine
Actinoplanes missouriensis	400	–	1	1
Actinoplanes philippinensis	402	–	1	1.2
Amorphosporangium auranticolor	408	1	1	1.4
Ampullariella campanulata	739	–	1	1.2
Pilimelia terevasa	287	–	1.05	1.1
Dactylosporangium thailandensis	314	–	1	1.3
Micromonospora echinospora	5	0.7	1	1
Planobispora longispora	1233	–	1	1.3
Planomonospora venezuelensis	930	–	0.95	1.1
Spirillospora albida	695	–	1.05	1.1
Streptosporangium roseum	21	–	1	1
Actinomadura citrea	9.5	–	1	1
Actinopycnidium caeruleum	300	1.6	1	1

Elution characteristics of tyrosine and phenylalanine biosynthetic enzymes on DEAE-cellulose

A crude extract of *Actinoplanes missouriensis*, *Planomonospora venezuelensis* and *Streptosporangium roseum*, respectively, was fractionated by chromatography on DEAE-cellulose.

The elution positions of chorismate mutase, prephenate dehydratase and arogenate dehydrogenase are shown in Fig. 2–4. One peak of activity was obtained for chorismate mutase. Chorismate mutase of *Actinoplanes missouriensis* and *Planomonospora venezuelensis* were found in the wash eluate, whereas chorismate mutase of *Streptosporangium roseum* was eluted with the salt gradient. Prephenate dehydratase activity of *Streptosporangium roseum* was recovered in the salt gradient too. Only chorismate mutase and prephenate dehydratase of *Planomonospora venezuelensis* were found to co-elute. Arogenate dehydrogenase activity of all three species was found to elute in the gradient.

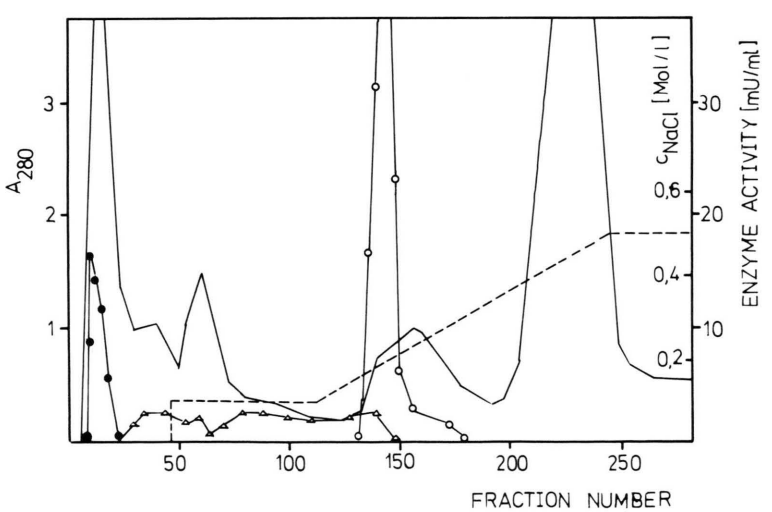

Fig. 2. Chromatography of a crude extract of Actinoplanes missouriensis DSM 43046 on DEAE-cellulose (DE-52). Protein (———), NaCl-salt gradient (– – – –), chorismate mutase (●—●—●), prephenate dehydratase (△—△—△), arogenate dehydrogenase (○—○—○).

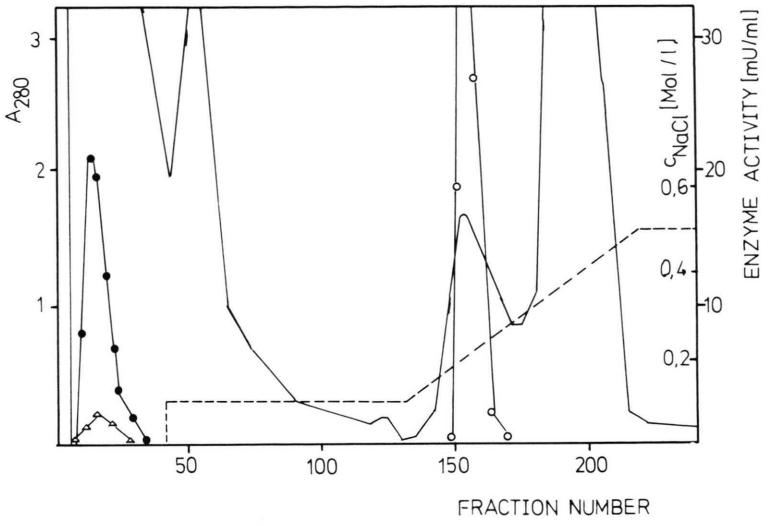

Fig. 3. Chromatography of a crude extract of Planomonospora venezuelensis DSM 43178 on DEAE-cellulose (DE-52).
Protein (———), NaCl-salt gradient (– – – –), chorismate mutase (●—●—●), prephenate dehydratase (△—△—△), arogenate dehydrogenase (○—○—○).

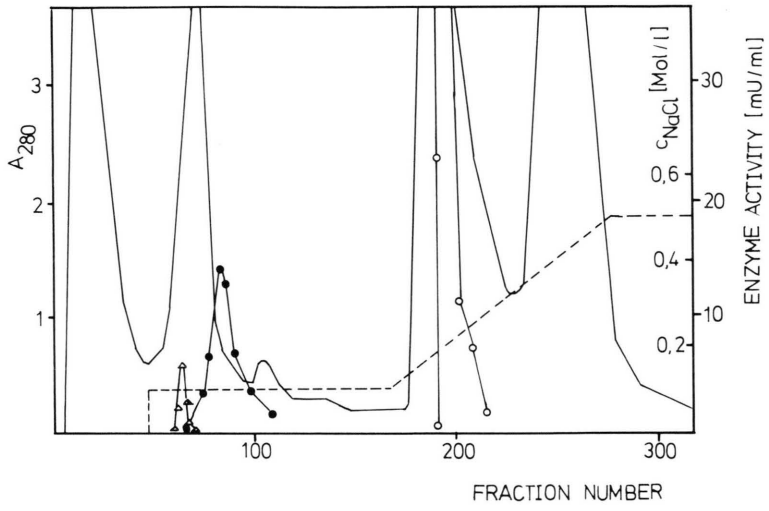

Fig. 4. Chromatography of a crude extract of Streptosporangium roseum DSM 43021 on DEAE-cellulose (DE-52).
Protein (———), NaCl-salt gradient (– – – –), chorismate mutase (●—●—●), prephenate dehydratase (△—△—△), arogenate dehydrogenase (○—○—○).

Discussion

Pathways

Within the order Actinomycetales only a small variety of Streptomycetes [6] has already been investigated and compared in their characteristics as to the biosynthesis of phenylalanine and tyrosine. Tyrosine is synthesized exclusively *via* arogenate, while phenylalanine is synthesized *via* phenylpyruvate. The investigations of this paper to characterize the aromatic biosynthesis of 13 members of the Sporoactinomycetes resulted in similar data: tyrosine is synthesized exclusively *via* arogenate and phenylalanine *via* phenylpyruvate.

Only *Dactylosporangium thailandensis* and *Micromonospora echinospora* possess a very low arogenate dehydratase activity, which agrees with the separate status of the morphologically distinct genera from the Actinoplanetes [1].

In contrast to the results of Keller *et al.* [6] arogenate dehydrogenase of the most Streptomycetes is feedback inhibited by L-tyrosine, not however, in all Sporoactinomycetes presented in this paper.

Enzyme regulation

Actinoplanes missouriensis, Actinoplanes philippinensis (Actinoplanetes) and *Planomonospora venezuelensis* (Maduromycetes) show some differences in the regulatory patterns of chorismate mutase and prephenate dehydratase, when compared with the other strains in the respective term. Chorismate mutase of the Actinoplanes strains is not feedback inhibited by L-tryptophan and prephenate dehydratase is not product inhibited by L-phenylalanine and L-tyrosine. Chorismate mutase of *Planomonospora venezuelensis* is inhibited by L-tryptophan and prephenate dehydratase activated by L-phenylalanine.

Actinopycnidium caeruleum, a Streptomycetes, possesses the highest specific activity of chorismate mutase and prephenate dehydratase. Chorismate mutase is product inhibited by L-tyrosine and L-tryptophan as in the Maduromycetes, and prephenate dehydratase is inhibited by L-tyrosine in contrast to the Maduromycetes and Actinoplanetes. Arogenate dehydrogenase activity of *Actinopycnidium caeruleum* requires NAD^+, but it could be replaced by $NADP^+$, a further result indicating differences in the aromatic amino acid biosynthesis of Streptomycetes and Maduromycetes.

Enzyme chromatography

Chorismate mutase and prephenate dehydratase from *Actinoplanes missouriensis* and *Streptosporangium roseum* are not associated, since we get two distinct activity peaks by chromatography on DEAE-cellulose. There are a lot of examples showing that chorismate mutase and prephenate dehydratase are separate proteins: *Streptomyces phaeochromogenes* [16], *Phenylobacterium immobile* [17], *Pseudomonas aureofaciens* ATCC 15926 [18].

The arogenate dehydrogenase from *Actinoplanes missouriensis, Streptosporangium roseum* and *Planomonospora venezuelensis* is also not complexed with any other terminal enzyme of the biosynthetic pathway to L-phenylalanine or L-tyrosine contrary to the result described for the chorismate mutase/prephenate dehydrogenase complex in *E. coli* [19]. Only one chromatographic step (DEAE-cellulose) was necessary to separate the arogenate dehydrogenase from the other terminal enzymes, whereas separation of chorismate mutase/arogenate dehydrogenase from *Streptomyces phaeochromogenes* [16] and *Phenylobacterium immobile* [17] required a gel permeation chromatography step.

Studies on the aromatic biosynthesis in other members of the order Actinomycetales as well as in some taxonomically not unequivocally identified Actinomycetales are in progress. At the same time our efforts go towards enzyme purification and characterization, especially for arogenate dehydrogenase.

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[1] M. Goodfellow, M. Mordarski, and S. T. Williams (eds.), The Biology of the Actinomycetales, Academic Press, London 1983.
[2] H. Prauser, Zbl. Bakt. Hyg., I Abt., Supplement **11,** 17–24 (1981).
[3] E. Stackebrandt and C. R. Woese, Current Microbiology **5,** 197–202 (1981).
[4] E. Stackebrandt, B. Wunner-Füssl, V. J. Fowler, and K. H. Schleifer, Int. J. Syst. Bacteriol. **31,** 420–431 (1981).
[5] M. Goodfellow and T. Pirouz, J. Gen. Microbiol. **128,** 503–527 (1982).
[6] B. Keller, E. Keller, H. Görisch, and F. Lingens, Hoppe-Seyler's Z. Physiol. Chem. **364,** 455–459 (1983).
[7] R. E. Buchanan and N. E. Gibbons (eds.), Bergey's Manual of Determinative Bacteriology, 8th ed., Williams and Wilkins, Baltimore 1974.
[8] O. H. Lowry, J. N. Rosebrough, A. L. Farr, and R. N. Randall, J. Biol. Chem. **193,** 254–275 (1951).
[9] R. G. H. Cotton and F. Gibson, Biochim. Biophys. Acta **100,** 76–88 (1965).
[10] R. G. H. Cotton and F. Gibson, Meth. Enzymol. **17A,** 564–574 (1970).
[11] R. G. H. Cotton and F. Gibson, Biochim. Biophys. Acta **147,** 222–237 (1967).
[12] C. L. Shapiro, R. A. Jensen, K. A. Wilson, and J. R. Brown, Analyt. Biochem. **110,** 27–30 (1981).
[13] F. Gibson, Meth. Enzymol. **17A,** 362–364 (1970).
[14] J. Dayan and D. B. Sprinson, Meth. Enzymol. **17A,** 559–561 (1970).
[15] F. Lingens, E. Keller, and B. Keller, Meth. Enzymol. **142,** 513–518 (1987).
[16] B. Keller, E. Keller, and F. Lingens, Biol. Chem. Hoppe-Seyler **366,** 1063–1066 (1985).
[17] E. Mayer, S. Waldner-Sander, B. Keller, E. Keller, and F. Lingens, FEBS Letters **179,** 208–212 (1985).
[18] E. Blumenstock, O. Salcher, and F. Lingens, J. Gen. Microbiol. **117,** 81–87 (1980).
[19] F. Gibson and J. Pittard, Bacteriol. Rev. **32,** 465–492 (1968).