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DISTINCT KYNURENINASE AND HYDROXYKYNURENINASE ENZYMES IN AN ACTINOMYCIN-PRODUCING STRAIN OF *STREPTOMYCES PARVULUS*

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Summary

Extracts of *Streptomyces parvulus* ATCC 12434 contain enzyme activity which converts kynurenine and hydroxykynurenine to anthranilate and hydroxyanthranilate, respectively. The ratio of activity towards each substrate is variable depending on the age of the culture; kynurenine is preferentially hydrolysed by extracts from younger (zero h) mycelium whereas hydroxykynurenine is more readily hydrolysed by extracts from older (48 h) mycelium. Sephadex G-200 chromatography of a mixture of partially purified extracts from mycelium of both ages revealed a peak of activity toward kynurenine with a molecular weight of 82 000 and a peak of activity toward hydroxykynureninase possessing a molecular weight of 56 000. Thus, it is concluded that distinct kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) and hydroxykynurenine enzymes are produced by this organism.

Both enzymes are induced by the presence of tryptophan in the growth medium. However, just prior to the onset of actinomycin synthesis (in the absence of added tryptophan) only hydroxykynureninase activity increases significantly (50—60-fold) and high levels are subsequently maintained throughout the antibiotic production period. Growth in the presence of glucose or glycerol represses both antibiotic synthesis and the increase in hydroxykynureninase activity. A mutant unable to synthesize actinomycin D displays only a small and transient increase in this activity. It is concluded that hydroxykynureninase is a functional component of the actinomycin biosynthetic system.

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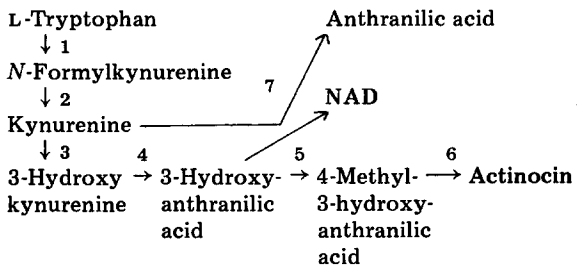
Introduction

Tryptophan can be utilized by microorganisms for both catabolic and anabolic reactions. The catabolic pathway gound in certain Pseudomonads [1] and *Bacillus megaterium* [2] proceeds via kynurenine to anthranilate (Scheme 1), which can subsequently be utilized as an energy source and for growth or be recycled to tryptophan. The anabolic pathway also proceeds via kynurenine, and leads ultimately to the syntheis of NAD; this pathway has been shown to operate in fungi [3,4] and the bacterial species, *Xanthomonas pruni* [5], as well as in higher animals [6]. Radioisotopic studies have also implicated tryptophan as the precursor of NAD in the filamentous bacterium, *Streptomyces antibioticus* [7].

In contrast, biosynthesis of NAD in most prokaryotes examined commences with aspartate and a three-carbon precusor [8,9]. It has been postulated by Gaertner and Shetty [10] that the anabolic pathway from tryptophan to NAD in higher animals has evolved from the catabolic pathway of tryptophan degradation found in prokaryotes. In support of this hypothesis, they have demonstrated a variation in properties of kynureninase-type enzymes in different organisms which follows an evolutionary trend: in lower organisms (i.e. in most prokaryotes) the enzyme is inducible and has a specificity primarily directed toward kynurenine, whereas in higher organisms the enzyme is constitutive and possesses a specificity primarily for hydroxykynurenine. The enzyme activity in organisms intermediate in evolution (e.g., fungi) has an intermediate substrate specificity and, in some instances, this can be attributed to the presence of two enzymes, each having activity primarily directed toward a single substrate. Activity preferentially against kynurenine (kynureninase) is



1. Tryptophan pyrrolase
2. Kynurenine formamidase
3. Kynurenine hydroxylase
4. Hydroxykynureninase
5. Methylase (?)
6. Phenoxazinone synthase
7. Kynureninase

Scheme I. Relationship of tryptophan to NAD and actinocin (actinomycin) biosynthesis.

inducible and that toward hydroxykynurenine (hydroxykynureninase) is constitutive. Thus, it was proposed that hydroxykynureninase may have evolved from kynureninase by a gene duplication followed by subsequent differential specialization of function of each gene.

As shown in Scheme I, the pathway from tryptophan is also operative during the biosynthesis of the phenoxazinone chromophore (actinocin) which is present in the antibiotic actinomycin. Radioisotope experiments have revealed that kynurenine and hydroxykynurenine are both intermediates in the metabolic sequence [11]. The investigation described herein demonstrates the existence of two inducible kynureninase-type enzymes in the actinomycin-producing organism, *Streptomyces parvulus*. One enzyme displays properties of a kynureninase and may have a catabolic function, while the second enzyme, a hydroxykynureninase, has properties consistent with a role in actinomycin synthesis. Since the hydroxykynureninase in *S. parvulus* is inducible, this may represent an early stage in evolution of the enzyme following duplication of the gene for kynureninase.

Materials and Methods

Materials. L-Kynurenine and L-3-hydroxykynurenine were obtained from Calbiochem-Behring and dithiothreitol was from Sigma. Sephadex G-200 was purchased from Pharmacia and DE-52 DEAE-cellulose from Whatman.

Organism and growth. Streptomyces parvulus ATCC 12434 (designated AM C1 in this laboratory) was cultivated in NZ-amine medium (medium 1) and then in glutamic acid/histidine/fructose medium (medium 2) as previously described [12]. Medium 2 was supplemented where stated with L-tryptophan (0.25 g/l) or D-glucose (5 g/l). When glycerol (15 ml/l) was present in this medium, it replaced D-fructose. The mycelium was harvested at the desired time by suction filtration on Whatman No. 2 paper, washed with an equal volume of saline (9.0 g NaCl/l) and stored at −20°C.

Preparation of cell extracts. All steps were carried out at 4°C. The mycelium was homogenised with 50 mM potassium phosphate buffer (pH 7.0) using a motor-driven Teflon pestle in a tissue grinding vessel. The resulting suspension was disrupted by passage twice through an Aminco French pressure cell at 12 000 lb inch^2 and then centrifuged at 27 000 × *g* for 20 min. The supernatant was decanted and served as the source of enzymes.

Purification procedures. Buffers used in the purification procedures were potassium phosphate of the concentration stated at pH 7.0, containing 0.5 mM each of dithiothreitol and EDTA. Nucleic acids were removed by addition of 0.1 ml 25% streptomycin sulphate solution (w/v) per ml of the cell extract. The precipitated material was removed by centrifugation (27 000 × *g*, 20 min) and the supernatant brought to 25% saturation with solid $(NH_4)_2SO_4$ added over a 45 min interval. The precipitate was again removed by centrifugation and the supernatant brought to 50% saturation with $(NH_4)_2SO_4$ as described above. The precipitate was collected by centrifugation and dissolved in one-tenth of the original volume of 50 mM buffer.

Desalting was accomplished using a Sephadex G-25 column (1.6 × 30 cm) equilibrated and eluted with 100 mM buffer. Approx. 1 ml of the ammonium

sulphate fraction (25—50% saturation) was diluted with 1 ml 100 mM buffer and applied to the column. Void fractions were collected and active fractions were pooled.

DEAE-cellulose chromatography employed a 0.9 × 14 cm column equilibrated with 100 mM buffer. A 5 ml desalted sample was applied and elution was effected with a 200 ml linear gradient of 100—200 mM buffer. Sephadex G-200 chromatography was performed using a 92 × 2.5 cm column developed by upward flow. A 5 ml desalted sample was applied and eluted with 100 mM buffer.

Assay of kynureninase and hydroxykynureninase activities. The assay for kynureninase and hydroxykynureninase activities depends on the conversion of L-kynurenine and L-3-hydroxykynurenine to anthranilate and 3-hydroxyanthranilate, respectively. Incubation mixtures for kynureninase contained 25 μmol potassium phosphate buffer (pH 6.5), 6 nmol pyridoxal phosphate, 1 μmol L-kynurenine and enzyme in 1.0 ml total volume. For hydroxykynureninase, assay mixtures contained 25 μmol potassium phosphate buffer (pH 7.5), 4 μmol pyridoxal phosphate, 1 μmol L-3-hydroxykynurenine and enzyme in 1.0 ml total volume. Reaction mixtures were preincubated for 5 min at 37°C and the reaction was initiated by addition of enzyme. Assays for kynureninase and hydroxykynureninase were incubated for 20 and 10 min, respectively. The reaction was stopped by addition of sufficient 1 N HCl to lower the pH to 2.5 and the reaction tubes were maintained at 0°C for 30 min. Anthranilate or hydroxyanthranilate were extracted with 2.0 ml ethylacetate and the amount formed was determined spectrophotofluorimetrically by comparison with appropriate standards. For both compounds an excitation wavelength of 340 nm was employed; for anthranilate emission at 400 nm, and for hydroxyanthranilate emission at 420 nm was measured in an Aminco-Bowman spectrophotofluorimeter. One unit of enzyme activity corresponds to 1 nmol product formed per min. Specific activity is given in units per mg protein.

Protein determination. Protein was precipitated with 5% (w/v) trichloroacetic acid and redissolved in 0.1 N NaOH. The amount was then determined by the method of Lowry et al. [13] using bovine serum albumin as standard.

Results and Discussion

Evidence for distinct kynureninase and hydroxykynureninase enzymes

Radioisotope experiments have shown that kynurenine and 3-hydroxykynurenine are intermediates in the metabolic pathway originating from tryptophan to the actinomycin chromophore [11]. Therefore, extracts derived from *S. parvulus* mycelium grown for various periods of time in medium 2 were tested for their ability to catalyze the hydrolysis of kynurenine and hydroxykynurenine to anthranilic acid and hydroxyanthranilic acid, respectively (Fig. 1). With hydroxykynurenine as substrate the activity observed is low during the early stages of cultivation but increases some 50—60-fold just prior to and during the onset of antibiotic synthesis and subsequently remains at an elevated level. This finding is consistent with a role for a hydroxykynureninase enzyme in actinocin (actinomycin) synthesis. Similar induction (derepression) of other enzymes (tryptophan pyrrolase, kynurenine formamidase, phenoxazi-

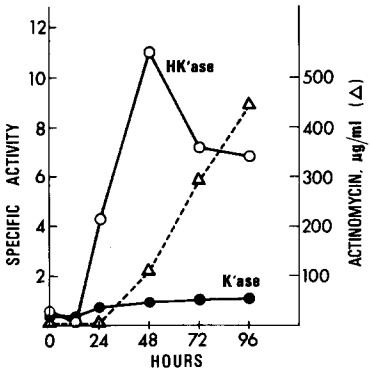


Fig. 1. Variation of kynureninase (●———●) and hydroxykynureninase (○———○) activities with age of *S. parvulus*, and actinomycin production (△———△). K'ase = kynureninase; HK'ase = hydroxykynureninase.

none synthase) in this biosynthetic sequence has been observed (Ref. 14, and unpublished results). By contrast, the activity with kynurenine as a substrate is relatively constant and low throughout the period examined. Thus, kynureninase is probably not involved in antibiotic biosynthesis but may serve a purely catabolic function in the metabolism of tryptophan (Scheme I).

Since kynurenine and hydroxykynurenine are structurally similar, there was the possibility that a single kynureninase-type enzyme was responsible for catalyzing the hydrolytic cleavage of both substrates. However, if this were the case, it might be expected that the ratio of enzyme activity against one substrate to activity against the other would be approximately constant regardless of the age of the mycelium. As shown in Fig. 1, the ratio of activity (kynurenine/hydroxykynurenine) is variable from about 1 : 0.6 at zero hour to 1 : 14 at 48 h suggesting that the enzyme in zero hour extracts was different from that found in extracts of 48-h old mycelium.

These results prompted a more detailed comparison of the activities present in extracts from zero and 48 h old mycelium. It is evident from Table I that no major differences in kinetic properties of the two activities were found. However, a calculation of the ratio of the transient times (γ) for each substrate (γ_K/γ_{HK}) as described by Gaertner and Shetty [10] gave a value of 0.13 for the enzyme from zero hour cells in contrast to values of 3.16 (crude extract) and 1.25 (partially purified) for the enzyme derived from 48 h cells. These data are consistent with the predominant activity in zero hour mycelium being a kynureninase and the major activity in 48 h cultures being a hydroxykynureninase. The decrease in γ_K/γ_{HK} during purification might be accounted for if the hydroxykynureninase is less stable than the kynureninase and this seems to be the case.

Attempts were then made to resolve the two activities in extracts from zero hour mycelium by various fractionation techniques. After removal of nucleic acids with streptomycin and fractionation by $(NH_4)_2SO_4$ precipitation, most of the enzyme activities (more than 95%) were recovered in the fraction precipitating between 25 and 50% saturation. When this fraction was desalted and chromatographed on a DEAE-cellulose column only a single peak of

TABLE 1

KINETIC PARAMETERS OF ACTIVITIES TO KYNURENINE AND HYDROXYKYNURENINE IN EXTRACTS FROM MYCELIUM HARVESTED AT ZERO AND 48 H

Activities were determined using crude extracts or extracts which had been partially purified using a DEAE-cellulose column (numbers in parentheses).

Extracts from mycelium harvested at:	Zero h		48 h	
	Kynurenine	Hydroxy-kynurenine	Kynurenine	Hydroxy-kynurenine
K_m (M)	$4.0 \cdot 10^{-5}$ ($2.5 \cdot 10^{-5}$)	n.t. * ($1.6 \cdot 10^{-4}$)	$6.0 \cdot 10^{-5}$ ($6.3 \cdot 10^{-5}$)	$1.8 \cdot 10^{-4}$ ($3.2 \cdot 10^{-4}$)
V (units/mg protein)	1.17 (16.8)	n.t. * (14.3)	0.63 (5.1)	5.98 (32.3)
Optimum concentration pyridoxal phosphate (μM)	6 (4)	n.t. * (4)	4 (4)	4 (4)

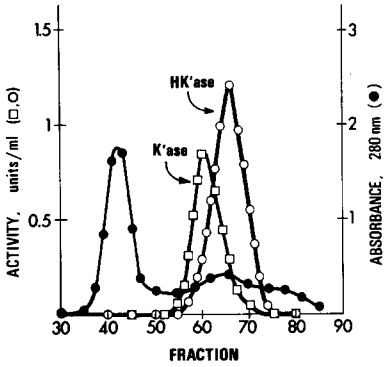
* n.t., not tested.

activity was found using kynurenine as the substrate;with hydroxykynurenine as substrate there was a coincident peak but of lower activity. Essentially similar results were obtained using 48-h old mycelium as the source of extract except that the activity in the peak eluting from the column was greater with hydroxykynurenine than with kynurenine as substrate. A mixture of desalted, partially purified enzyme from extracts of both zero and 48 h mycelium was then chromatographed on a similar DEAE-cellulose column. Again only a single peak of activity was observed using either substrate. Although the most active fraction with each substrate was again identical, the ratio of activity with hydroxykynurenine compared to kynurenine increased slightly in the latter part of the elution peak.

A similar mixture of desalted $(NH_4)_2SO_4$ fractions from zero and 48 h extracts was chromatographed on Sephadex G-200 and the peaks of activity with each substrate, though overlapping, were considerably different (Fig. 2). The peak of activity with kynurenine as substrate eluted first at a position corresponding to a protein with molecular weight of about 82 000 (Fig. 3) while the peak of activity with hydroxykynurenine eluted somewhat later at a position corresponding to a protein with molecular weight of about 56 000. These results provide definitive proof for separate kynureninase and hydroxykynureninase enzymes each preferentially catalyzing the breakdown of kynurenine and hydroxykynurenine, respectively, although it would appear that each enzyme has some capability to use the alternate substrate.

Regulation of expression

(1) Effect of L-tryptophan. Kynureninase enzymes are generally induced during growth with tryptophan (or a metabolite derived from it). The effect of the presence of L-tryptophan in the medium on the kynureninase and hydroxykynureninase activities in *S. parvulus* is shown in Table II. Both enzyme activities are significantly increased over the control after 12 h incubation (kynureninase 35-fold, and hydroxykynureninase 18-fold). The finding



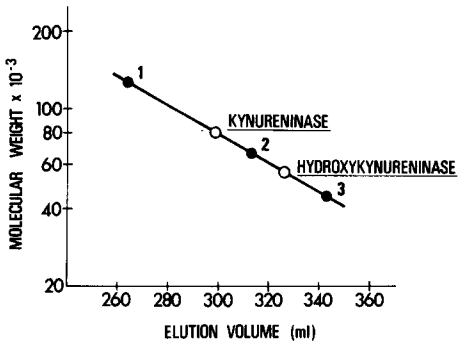


Fig. 2. Sephadex G-200 chromatography of a mixture of partially purified extracts from mycelium at zero and 48 h. Kynureninase activity (□———□); hydroxykynureninase activity (○———○).

Fig. 3. Determination of molecular weight of kynureninase and hydroxykynureninase by Sephadex G-200 chromatography. Standards employed were: 1, bovine serum albumin dimer (134 000); 2, bovine serum albumin monomer (67 000); and 3, ovalbumin (45 000).

that kynureninase is induced by tryptophan is consistent with the results obtained with other microorganisms [15—17]. However, the increase in activity against hydroxykynurenine was unexpected. It is too great to be accounted for by an increased level of kynureninase alone and must be attributed to an elevated level of hydroxykynureninase. Induction (18-fold) of hydroxykynureninase by tryptophan (or its metabolites) is unusual since such enzymes are generally constitutive [17]. This difference in regulation may reflect the different role of hydroxykynureninase in this organism, i.e in antibiotic formation, whereas hydroxykynureninases in other organisms have been implicated in NAD biosynthesis [10]. Alternatively it might be considered as an early stage in the evolution of a biosynthetic hydroxykynureninase enzyme from a catabolic, inducible kynureninase activity.

(2) Effect of glucose and glycerol. Glucose and glycerol have been shown to repress actinomycin synthesis in *S. antibioticus* [18] and *S. parvulus* [12]. These compounds may regulate actinomycin synthesis by catabolite repression control of the formation of enzymes required for antibiotic production. In

TABLE II

EFFECT OF GROWTH WITH L-TRYPTOPHAN ON THE LEVEL OF ACTIVITIES TOWARD KYNURENINE AND HYDROXYKYNURENINE

Culture age (h)	Apparent specific activity (units/mg protein)			
	Kynureninase		Hydroxykynureninase	
	No Trp	plus Trp	No Trp	plus Trp
0	1.34	1.34	0.30	0.30
12	0.18	6.26	0.22	4.02
48	1.02	1.40	16.04	14.52

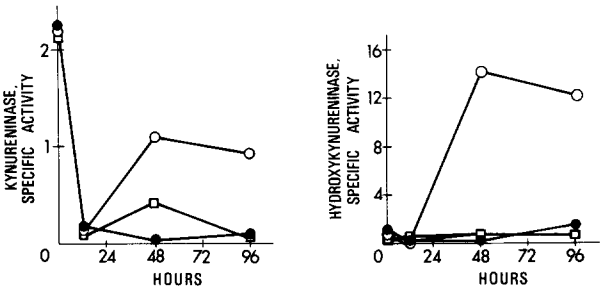


Fig. 4. Influence of glucose and glycerol on the synthesis of kynureninase and hydroxykynureninase activities by *S. parvulus*. Standard medium (○———○), medium containing glucose (●———●), and medium containing glycerol (□———□) were as described in Methods. Actinomycin titers (μg/ml) after 96 h were: control, 425; with glucose, 5; with glycerol, 7.

fact, studies have demonstrated that certain enzymes involved in actinomycin chromophore synthesis are repressed during cultivation in the presence of either of these carbon sources (e.g., phenoxazinone synthase in *S. antibioticus* and tryptophan pyrrolase and kynurenine formamidase in *S. parvulus*). The influence of glucose or glycerol in the growth medium on the levels of kynureninase and hydroxykynureninase activities in *S. parvulus* is illustrated in Fig. 4.

The striking increase in hydroxykynureninase activity seen in the control by 48 h is almost completely prevented by the presence of glucose or glycerol even after 96 h incubation. Actinomycin production was also prevented by these conditions. Moreover, the increase in activity with kynurenine as substrate is similarly repressed by glucose or glycerol. However, the low level of enzyme activity present in the control (after 12 h) may reflect the broad substrate specificity of the hydroxykynureninase enzyme rather than the activity of a kynureninase. Hence it is not possible from these data to state with confidence whether the synthesis of the kynureninase enzyme is affected also by growth with glucose or glycerol.

(3) Activity in an actinomycin non-producing mutant. Single colonies derived from a suspension of *S. parvulus* spores exposed to 8-methoxypsoralen plus near ultraviolet light as previously described [19] were examined for antibiotic production by the agar piece method [20]. One isolate (designated AM 8) which failed to produce antibiotic on an agar piece was shown to lack the ability to produce actinomycin in either chemically defined medium 2 or complex GYM * liquid medium. AM 8 sporulates and possesses growth properties similar to those of the parent strain. It was of interest to determine whether the inability of the organism to synthesize actinomycin was correlated with its failure to synthesize enzymes of the tryptophan → actinocin pathway. Kynureninase activity was in general lower in the mutant except at zero hour, but the differences amounted to no more than 4-fold (Fig. 5a). Hydroxykynureninase activity, on the other hand, was always lower (up to 30-fold) in the mutant (Fig. 5b). Of special interest was the elevated level of this enzyme in the mutant at 48 h which parallels a similar

* GYM medium contains 4 g D-glucose, 4 g yeast extract and 10 g malt extract per 100 ml deionized water, pH 7.2.

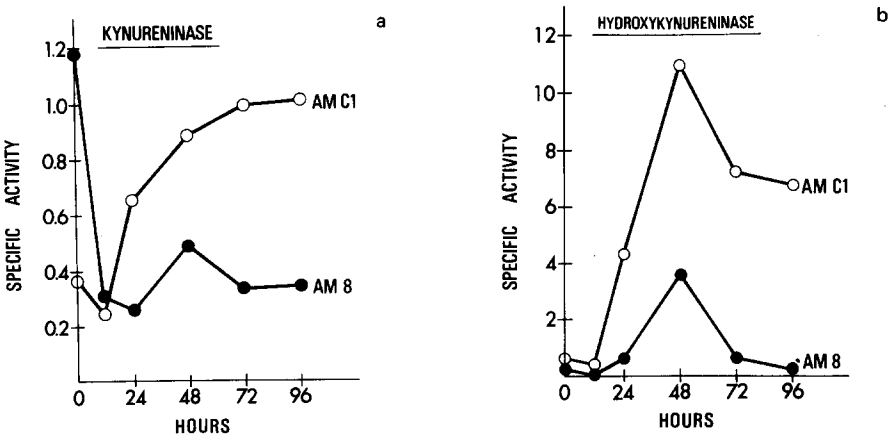


Fig. 5. Variation of kynureninase and hydroxykynureninase activities with growth of antibiotic-producing (○———○) and non-producing (●———●) strains of *S. parvulus*. The non-producing strain (AM 8) was derived from the producing strain (AM C1) by mutagenesis as described previously (19).

increase in the parental strain at this time. In contrast to the parent where a high level was subsequently maintained, the level in the mutant immediately declined. A similar pattern of activity of tryptophan pyrrolase has been observed in this mutant, and kynurenine formamidase, although present throughout growth, is at a markedly reduced level compared to that in the parent (unpublished results).

Although the nature of the mutation in AM 8 responsible for the absence of actinomycin production is unknown, it seems unlikely from these results that it is in a structural gene coding for one of the enzymes involved in tryptophan metabolism. Nevertheless, the expression of these genes must be tightly coupled to that of functional actinomycin synthesis, since in its absence, expression is transient and not maintained.

It is concluded that there are two distinct kynureninase-type enzymes in *S. parvulus*. One has a low γ_K/γ_{HK} value and is inducible by L-tryptophan; thus its properties are consistent with a kynureninase enzyme. As neither tryptophan pyrrolase nor kynurenine formamidase are inducible by L-tryptophan under the growth conditions examined thus far (unpublished results), the kynureninase may be the vestigial element of a pathway of tryptophan catabolism which is no longer operative per se, the earlier steps of the pathway (tryptophan → kynurenine) being regulated specifically to the specialized function of actinomycin biosynthesis. The other kynureninase-type enzyme, although inducible, had a higher γ_K/γ_{HK} which is consistent with it being a hydroxykynureninase enzyme. Induction (derepression) of this enzyme just prior to antibiotic synthesis, its repression by glucose and glycerol, and its transient expression in the AM 8 mutant indicate that the hydroxykynureninase is a functional component in the pathway from tryptophan leading to the biosynthesis of the actinomycin chromophore during antibiotic formation.

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