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Biosynthesis of Shermilamine B[1]

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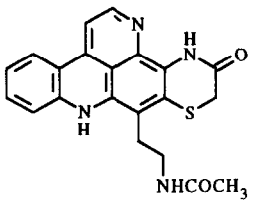
Abstract: The biosynthesis of shermilamine B is proposed which involves tryptophan, dopamine and cysteine.

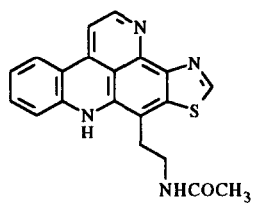
In recent years several benzo-3,6-phenanthroline alkaloids have been isolated, not only from tunicates but also from sponges.[2] Almost all compounds are pharmaceutically active and, due to their complex heterocyclic structure represent interesting targets for organic syntheses.[3] In this paper we report results on the biosynthesis of shermilamine B.

Results and Discussion

The investigations of the biosynthesis were made on the colonial tunicate *Cystodytes dellechiajei* which is frequently found in the Mediterranean Sea as a deep-blue coating on submarine rocks at a depth between 6 and 25 m. As well as some other pigments, *C. dellechiajei* contains mainly shermilamine B[4a] and kuanoniamine D.[4b]



shermilamine B

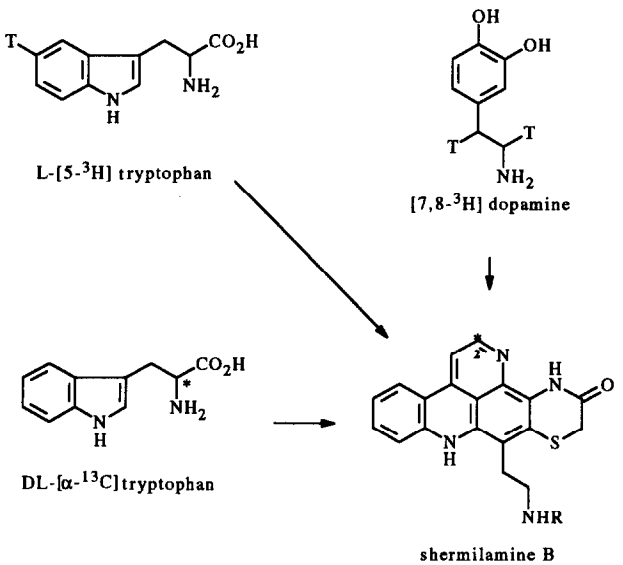


kuanoniamine D

In the course of intensive studies almost all components of the tryptophan and 3-hydroxytyrosine metabolic pathways have been isolated. This gave rise to the idea of feeding the tunicates with labelled tryptophan and dopamine. Some problems emerged in this connection. Initially the tunicates only survived for a period of 3 - 4

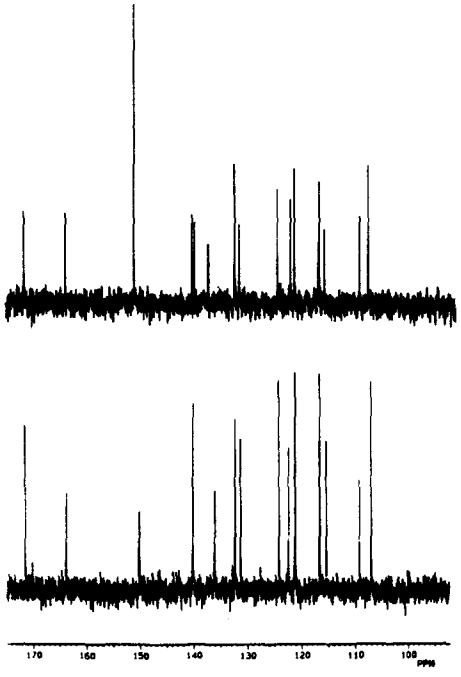
weeks in the aquarium or continued to exist as more or less resting form without recognizable metabolism. Only after several experiments it was possible to keep *C. dellechiajei* for up to two years and even to reproduce through larvae or through stolonifers.

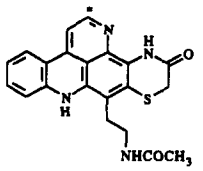
In order to get an idea of uptake and distribution of the precursor the tunicates (31g wet weight) were kept for one day in a small tank with seawater (0.4 liter) to which 3 ml L-[5-^{3}H] tryptophan (3 μCi = 11.1 x 10^4 Bq = 6.66 x 10^6 dpm) was added. Subsequently they were put in an aquarium with a constant flow of fresh seawater. After 20 days the specimens were harvested and ground in a turbomixer with methanol/chloroform (2:1) and repeatedly extracted with the same solvent. The organic layer was evaporated to dryness and subjected to solvent partitioning (toluene vs water). Repeated chromatography of the evaporated toluene layer on a Sephadex LH-20 column (eluents: chloroform/methanol 1:1 and then methanol) yielded shermilamine B.



Evaluation of the results by measurement of radioactivity was performed in a scintillation counter. However, the intensively red pigments absorbed the photons generated in the scintillation cocktail to a great extent. Therefore calibration curves for scintillation measurement were produced which, dependent on the concentration of the pigment and the added tritium standard, provided information about the absorption of light by the respective pigment and thus allowed an exact determination of the incorporation rate.

Activity measurement showed that [5-^{3}H] tryptophan was incorporated into the pigment but rates were low and only reached eight to ten times the level of background (shermilamine B: 4850 dpm; background: 450 dpm). Similar results were obtained with [7,8-^{3}H] dopamine. This indicates clearly the slow rate of metabolism shown by this invertebrates compared with other organisms like bacteria or algae. One reason for this is certainly the low uptake of primary metabolites by tunicates and sponges.[5]





shermilamine B

Figure 1

Better incorporation rates and shorter times were obtained by using a cell free extract of the tunicate prepared according to the recent work of Djerassi et al.[6] on the biosynthesis of sterols in sponges. Grinding of *C. dellechiajei* (80 g wet weight) in Hepes buffer, preparation of the microsomal fraction and incubation in an *in vitro* assay, conventional extraction and chromatography after addition of a small amount of cold shermilamine B indeed produces in relatively short time a better incorporation rate of [5-^{3}H] tryptophan and [7,8-^{3}H] dopamine into shermilamine B and desacetylshermilamin B, respectively.

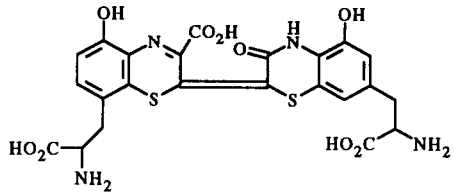
activity administered: 3 µl L-[5-^{3}H] tryptophan, 3 µCi, 6.66x10^6 dpm
activity recovered: shermilamine B: 36,880 dpm
background: 45 dpm

activity administered: 3 µl [7,8-^{3}H] dopamine, 3 µCi, 6.66x10^6 dpm
activity recovered: shermilamine B: 38,120 dpm
background: 39 dpm

Although these experiments permit the conclusion that both tryptophan and dopamine could be involved in the biosynthesis of shermilamine B, it is not clear how these compounds are used by the organism. In order to answer this question C. *dellechiajei* was kept in a 150 liter seawater aquarium, to which 800 mg DL-[α-^{13}C] tryptophan, encapsulated in liposomes,[7] was added in small portions. Ten weeks later, those colonies which exibited

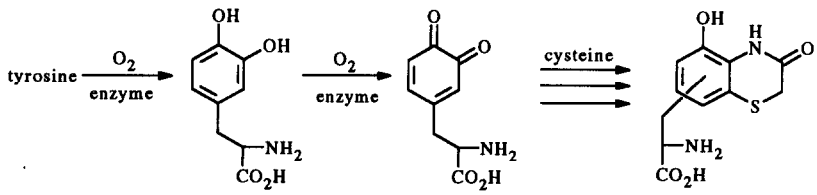
noticeable growth were harvested. Now a significantly stronger signal C-2 (δ = 150.67 ppm, DMSO-d_6) in the ^{13}C NMR spectrum of shermilamine B becomes apparent (Figure 1). This long lasting experiment not only shows that tryptophan was incorporated, it also indicates the position of the label in the molecule.

In this context the structures of the trichochromes are very interesting, which Prota et al.[9] have isolated for example from red human hair and from urine of patients with melanoma.



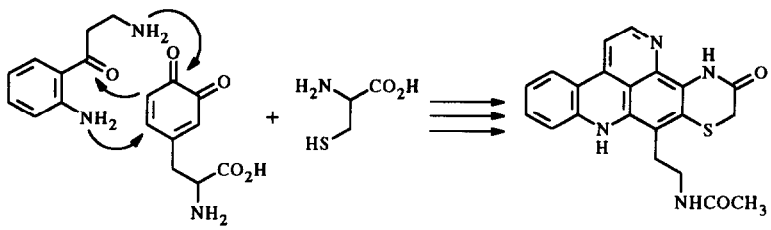
trichochrome B

A mechanism has been formulated for the formation of the benzothiazinon ring system,[9] which is also a principal intermediate in the biosynthesis of brown phaeomelanins.



dopa

Bearing this in mind part of the biosynthesis of shermilamine B can be outlined. The pigment is probably formed from the amino acids tryptophan, dopa and cysteine. Before or after combination with dopa or the preformed benzothiazinon system tryptophan is transformed into kynurenine and kynuramine respectively and then produces the benzo-3,6-phenanthroline system.



shermilamine B

Experiments with other precursors labelled at different sides will not only provide further arguments for the proposed biosynthesis but may also give insight into the sequence of individual steps leading to shermilamine B.

We believe that the proposed biosynthesis is a good rationale for the biomimetic synthesis of shermilamine B and other benzo-3,6-phenanthroline alkaloids. Experiments in this direction gave promising results and will be published elsewere.

Experimental

Specimens of *Cystodytes dellechiajei* were collected at various sites in the Mediterranean using SCUBA (-18 m). The tunicates were identified by Dr. F. Lafargue, Laboratoire Arago, Banyuls/Mer, France.

C. dellechiajei was kept in 80 l of seawater contained in a 150 l aquarium. The uptake of metabolites was made easier by means of a well controlled flow which also eliminated contamination of the surface of the tunicates. To maintain a concentration of organic material similar to the value of dissolved organic carbon (DOC)[12] the tunicates were pulse fed with 100 mg portions of liposomes containing DL-[α-^{13}C] tryptophan during ten weeks.

^{13}C NMR spectra (125 MHz) were recorded in DMSO-d_6 on a Bruker AMX 500 spectrometer using the solvent peak of DMSO-d_6 at 39.5 ppm as internal reference.

2-^{13}C-Diethylacetamidomalonate was purchased from Fa. Aldrich, Steinheim and Instant scint. Gel from Fa. Packard, Australia; all other chemicals were purchased from Fa. Sigma, Deisenhofen, Germany. Instrumentation: scintillation counter Minaxi Tri-Carb 4000 Series, Fa. United Technologies Packard, Canberra, Australia; ultracentrifuge L 7-65 Beckman Instruments, Palo Alto, USA; Polytron, Fa. Kinematica, Kriens/ Luzern, Schweiz.

DL-[α-^{13}C] tryptophan was prepared according to the literature[10]: gramine methiodide[11] was condensed with 2-^{13}C-diethylacetamidomalonate under basic condition, saponification and decarboxylation gave the product in 40% overall yield.

The efficiency η was determined according to the method of the internal standart with a calibrated T_2O probe. 50 ml T_2O with an activity of 48.665,1 dpm were added respectively. In pure methanol the η-value is 0.565. In a mixture of chloroform and methanol (1 ml, 1:1 v/v, in 10 ml scintillation cocktail) η amounts to 0.194. Addition of shermilamine B (in mg) produce the corresponding η-values (Tab. 1). For low concentrations the left part of the curve approaches the η-axis like an asymptote. For relevant concentrations the middle part of the curve (0.049 - 0.245 mg) behaves linearely and can be used for calibration. In the actual biosynthetic experiment the content of shermilamine B after work-up and chromatography was determined photometrically.

Table 1: The efficiency η is plotted against the content (in mg) of shermilamine B in the scintillation cocktail.

η	0.141	0.130	0.111	0.091	0.075	0.061	0.055	0.029
mg	0.004	0.009	0.024	0.049	0.098	0.147	0.196	0.245

Cell-free extracts of *C. dellechiajei* (80g wet weight) were prepared at 4°C by homogenization (Polytron) in 200 ml buffer consisting of 100 mM Hepes (N-2-hydroxylethylpiperazine-N'-2-ethanesulfonic acid), 10% PVP40 (polyvinylpyrrolidone 40), 0.75% BSA (bovine serum albumine, fraction V), 2 mM DTT (dithiothreitol),

1 mM ascorbic acid and at pH 7.4. As protease inhibitors pepstatin A and PMSF (phenylmethylsulfonyl fluoride) were added at final concentrations of 0.01 mg/ml and 0.1 mg/ml, respectively. Homogenates were filtrated through Nylon gauze (250 µm). The 10,000 x g supernatants (10 min, 4°C) of the filtrates were centrifuged at 125,000 x g (60 min, 4°C) to give a microsomal pellet which was suspended in 6 ml of the same buffer without protease inhibitors and immediately used in the *in vitro* assay system. 2 ml of the microsomal preparation were supplemented with 1% Tween 80 and incubated with either 3 µCi tryptophan or 3 µCi dopamine or cold tryptophan for 4 h at room temperature.

Liposomes (MLVs, multilamellar vesicles)[7b] were prepared from in chloroform/methanol (2:1 vol/vol, 5 ml) dissolved egg yolk L-α-phosphatidylcholine (100 mg) and cholesterol (40 mg) in a molar ratio of 1.0 : 0.9. 60 mg DL-[α-^{13}C] tryptophan was dissolved in 5 ml 0.5 m phosphat-buffer (pH 7.4) and added to the preformed vesicles. The resulting mixture was chromatographied over Sephadex G 40 in the same buffer to separate MLVs with entraped tryptophan from unreacted material. Entrapments of 25-30 % were achieved.

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