Two Different Pathways Leading to Phenanthrenes and 9,10-Dihydrophenanthrenes of the Genus *Dioscorea*

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The bibenzyl batatasin III was formed *de novo* in aerial bulbils and tubes of Dioscoreaceae from dihydro-*m*-coumaric acid. In cell-free preparations, bibenzyl synthase activity and an O-methyltransferase converting 3,3′,5-trihydroxybibenzyl into batatasin III were detected.

High rates of incorporation of dihydro-*m*-coumaric acid into a 9,10-dihydrophenanthrene (hircinol) showed that a pathway being known to occur in orchids is also effective in Dioscoreaceae. The biosynthesis of hircinol could be stimulated by infecting the aerial bulbils with various fungi. In parallel to the dihydro-*m*-coumaric acid pathway another biosynthetic reaction sequence leading to phenanthrenes was found to occur. The pathway was established in its principal steps: *p*-coumaric acid – trihydroxystilbene (resveratrol) – phenanthrene (batatasin I or iso-batatasin I).

Introduction

Batatasins have been detected as the dormancy-inducing principle in bulbils of the yam, *Dioscorea batatas* [1, 2]. The chemical structures of four of these substances have been established. Several batatasins are dihydrostilbenes, batatasin I is a phenanthrene.

Batatasin I seems to be a constituent of tubers of many species within the genus *Dioscorea* [3, 4]; it was also isolated from *Tamus communis* [5], another Dioscoreaceae.

Batatasins may not only be seen as factors affecting the dormancy of plant tissues but also as preformed barriers [6] against fungal attack. This is proposed on the basis of the effects observed with other stilbenoids [7, 8]. Coxon *et al.* [9] isolated hircinol (2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene) and 7-hydroxy-2,4,6-trimethoxyphenanthrene from the peel of *Dioscorea rotundata*. They characterized these phenanthrenes by many chemical parameters and showed that the phenanthrenes have anti-fungal activity towards *Cladosporium cladoporioides* and a variety of yam soft rot pathogens.

Abbreviations: EDTA, ethylenediaminotetraacetate; PC, paperchromatography; TLC, thin layer chromatography.

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Present studies indicate that hircinol biosynthesis can be induced similar to the one of phytoalexins. Because of both the induction of its synthesis and its well known fungostatic properties [10], hircinol is considered as phytoalexin, now also in Dioscoreaceae.

Materials and Methods

TLC

Phenol fractions were separated by repeated TLC on silica gel. Established solvent systems were used. Solvent system I, chloroform – ethylacetate – formic acid (5:4:1) [11]; solvent system II, chloroform – acetic acid (20:1) [4, 12]; solvent system III, chloroform – methanol (94:4) [9].

PC

Good separation of bibenzyls from stilbenes with identical substitution pattern was achieved using acetic acid – water (1:2) [11].

Radiochemicals

L-[U-^{14}C]Phenylalanine (400 mCi/mmol), [methyl-^{14}C]-adenosyl methionine (10 mCi/mmol), [2-^{14}C]-malonyl-CoA (5 mCi/mmol) and [2-^{14}C]acetate (40 mCi/mmol) were products of the Radiochemical Centre, Amersham. [2-^{14}C]Malonic acid (10 mCi/

mmol) was purchased from NEN Chemicals, Dreieichenhain. [2-^{14}C]-Labelled cinnamic acids were synthesized from benzaldehydes and [2-^{14}C]malonic acid; reduction afforded the respective dihydrocinnamic acid. Labelled hydroxybibenzyls and hydroxystilbenes were synthesized enzymatically by using purified stilbene synthase [13] or bibenzyl synthase [11] and [2-^{14}C]malonyl-CoA as substrate.

Chemicals

Resveratrol (3,4′,5-trihydroxystilbene) was isolated from rhizome of *Veratrum album* [14]. Catalytic reduction with H_2 and Pd/C afforded dihydroresveratrol. 3,3′,5-Trihydroxystilbene and 3,3′,5-trihydroxybibenzyl were synthesized by condensation of 3,5-dimethoxybenzaldehyde with ethyl 3-methoxyphenylacetate [11]. Hircinol was isolated from orchid bulbs according to [15]. Batatasin I and batatasin III were prepared from tubes of *Dioscorea opposita* [4]. Permethylation was carried out with dimethyl sulfate in alkaline solution. Cinnamoyl-CoA esters were prepared from the respective succinimidyl derivatives.

Induction methods and feeding techniques

3 mm slices of aerial bulbils of *D. macruora* were induced by treating with fresh fungal mycel (10 mg per 2 cm^2 disc), then leaving for 10 h on moist filter paper. Radioactive precursors dissolved in H_2O were then pipetted onto the surface. The incubation was at 25 °C for 10 h.

In cases when only the content of hircinol was determined, slices were extracted immediately after the first incubation period. The ethyl acetate extract was separated into crude fractions by TLC in system I. An aliquot of the hircinol containing zone was further purified by TLC (solvent III) and eluted with methanol. Its amount was determined by UV: λ_{max} 292 nm, log $\varepsilon = 4.45$.

Preparation and assay of cell-free extracts

5 g of aerial bulbils and 1 g polyvinylpyrrolidone were homogenized in 7 ml buffer consisting of 50 mM HEPES-NaOH, pH 7.5, 1 mM dithiothreitol, and 0.1 mM EDTA. The homogenate was centrifuged at 20000 × *g* for 20 min. The cell-free preparation was partially purified by chromatography on Ultrogel AcA-34 and concentrated in a Visking dialysis tubing by embedding it in dry Sephadex G-100.

Enzyme assays were with [2-^{14}C]malonyl-CoA and CoA-esters of various phenylpropanoid acid according to [11, 16].

Results

Most species of the genus *Dioscorea* contain a series of batatasins. For the two species investigated in this work, aerial bulbils of *Dioscorea macruora* and tubers of *D. rotundata*, a preliminary survey showed that batatasin I and batatasin III were the dominating phenols. Hircinol became only detectable following induction.

Batatasin III is synthesized via dihydro-m-coumaric acid

Batatasin III is a bibenzyl and may thus either be formed from a stilbene by hydrogenation or may be synthesized, as it contains a *m*-hydroxy group in ring B, from a dihydroderivative of *m*-hydroxycinnamic acid.

Following administration of [4-^{3}H]phenylalanine to slices of aerial bulbs of *D. macruora*, the extracted phenols were subfractionated by TLC in system I, thus separating stilbenes and bibenzyls from other compounds. A further analysis of the stilbenoid fraction in system II afforded 3 peaks which could be assigned to resveratrol (3,4′,5-trihydroxystilbene), 3,3′,5-trihydroxybibenzyl, and batatasin III (3,4′-dihydroxy-5-methoxybibenzyl).

In another experiment, the efficiency was compared with which radioactive *p*-coumaric acid or *m*-coumaric acid, respectively, were incorporated into stilbenes and bibenzyls. Markedly different radioactivity profiles were observed when the phenol fraction after these two administration experiments were analyzed for stilbenoids. The radioactivity of *p*-coumaric acid was incorporated into resveratrol only, while batatasin III and 3,3′,5-trihydroxybibenzyl were the exclusive products from *m*-coumaric acid. Furthermore, it was found that by far the highest rate of incorporation of any cinnamic acid derivative was the conversion of dihydro-*m*-coumaric acid into batatasin III, *viz.* 10%.

Similar experiments with tubers of *D. rotundata* demonstrated that here, too, dihydro-*m*-coumaric acid was incorporated into batatasin III with high efficiency.

To provide further evidences for the route phenylalanine – *m*-coumaric acid – dihydro-*m*-coumaric acid – bibenzyl we combined the studies *in vivo* with experiments with cell-free preparations. Various CoA-esters of phenylpropanoic acids were tested, together with malonyl-CoA as second substrate, to find out whether stilbene synthase activities or bibenzyl synthase activities were detectable in Dioscoreaceae. As outlined in Table I, dihydro-*m*-coumaroyl-CoA was the best substrate, it afforded 3,3′,5-trihydroxybibenzyl as product.

The correct methylation of 3,3′,5-trihydroxybibenzyl leading to batatasin III was demonstrated *in vivo*. A crude preparation, as used for assaying bibenzyl synthase, was incubated with 100 μM 3,3′,5-trihydroxybibenzyl and 10 μM [methyl-^{14}C]S-adenosylmethionine (5 μCi) for 15 min. After extraction and chromatography of phenols the radioactivity profile exhibited only one peak, *viz.* the one corresponding to batatasin III (20% yield in radioactivity). This was confirmed (a) by comparing the chromatographic behaviour of the product with the two monomethylether of 3,3′,5-trihydroxybibenzyl, and (b) by permethylation of the radioactive product and subsequent recrystallization with authentic material, to constant specific activity.

Table I. Comparison of CoA-esters of phenylpropanoid acids as substrates. In a total volume of 250 μl, 17 μM [2-^{14}C]malonyl-CoA (0.1 μCi) and 100 μM aromatic CoA-ester were incubated with enzyme preparation. Products were identified by chromatography.

Second substrate	Product	Radioactivity [nCi]
m-Coumaroyl-CoA	3,3′,5-trihydroxy-stilbene	0.1
Dihydro-*m*-coumaroyl-CoA	3,3′,5-trihydroxy-bibenzyl	6.1
p-Coumaroyl-CoA	3,4′,5-trihydroxy-stilbene (resveratrol)	3.4
Cinnamoyl-CoA	3,5-dihydroxystilbene (pinosylvin)	0.2

Table II. Effects of various fungi on the induction of hircinol formation. The content of hircinol was determined, 24 h after inoculating the slices with the fungal mycel, by chromatography and spectroscopy. An increase of hircinol by a factor of 8–15 was denoted by +++. + is the symbol for a smaller but significant increase by factor 2–3. (+) denotes slight increases, but not sufficiently established.

Fungus	Effect on hircinol content
Phytophthora cambivora	+++
Rhizoctonia solani	+
Fusarium avenaceum	+
Sclerotina sclerotiorum	+
Cortitium rolfsii	+
Botrytis cinerea	(+)
Alternaria solani	(+)

Two different pathways leading to phenanthrenes and 9,10-dihydrophenanthrenes

A series of fungi was assayed for their capability of inducing the formation of stilbenoids. Among the fungi causing good induction of dihydrophenanthrene synthesis was *Phytophthora cambivora.*

Analyses of phenol extracts from aerial bulbils treated with fungal mycel demonstrated that this treatment led to a significantly enhanced stationary concentration of hircinol. The induction was rather selective with respect to hircinol. In cases when the amount of hircinol was increased by a factor of 10, the content of other phenolic products was only changed by a factor between 1 to 2. Differences were observed in the extent to which various fungi induce the formation of hircinol (Table II).

As the biosynthesis of phenanthrenes, *e.g.* batatasin I, does not appear to have been investigated previously we compared several aromatic compounds as to their function as precursors. After preliminary demonstration that both acetate and

Table III. Incorporation of radioactive precursors into hircinol and batatasin I (or its isomer). Slices of aerial bulbils were incubated with the mycel of *Phytophthora cambivora* for 10 h and subsequently with the radioactive compounds for another 10 h. Phenols were analyzed by repeated TLC.

Precursor	[nCi]	Product	[nCi]	Incorporation [%]
L-[U-^{14}C]Phenylalanine	150	batatasin I hircinol	3.0 1.2	2.0 0.8
L-[4-^{3}H]Phenylalanine	300	hircinol	1.2	0.4
[2-^{14}C]*p*-Coumaric acid	150	batatasin I hircinol	4.2 < 0.3	2.8 < 0.2
[2-^{14}C]*m*-Coumaric acid	70	batatasin I hircinol	< 0.1 1.5	< 0.1 2.1
[2-^{14}C]Dihydro-*m*-coumaric acid	40	hircinol	1.8	4.5
[α-^{14}C]3,4′,5-Trihydroxystilbene	50	batatasin I hircinol	2.6 < 0.1	5.2 < 0.2

phenylalanine were good precursors, *p*-coumaric acid, *m*-coumaric acid, and dihydro-*m*-coumaric acid were administered to cuttings of aerial bulbils in different experiments. The results summarized in Table III clearly demonstrate that *p*-coumaric acid is a proximal precursor of batatasin I or isobatatasin I. This phenanthrene was, however, not formed after application of *m*-hydroxylated phenylpropanes. In contrast, the 9,10-dihydrophenanthrene hircinol was labelled upon administration of radioactive *m*-coumaric acid or dihydro-*m*-coumaric acid, but not by *p*-coumaric acid.

Discussion

The data presented can be summarized in stating that (a) the "dihydro-*m*-coumaric acid pathway" previously detected in orchids [11] is also effective in another family, *i.e. Dioscoreaceae*, and (b) in *Dioscorea* a pathway *p*-coumaric acid – stilbene – phenanthrene (batatasin I) occurs in parallel to a route *m*-coumaric acid – dihydro-*m*-coumaric acid – bibenzyl – 9,10-dihydrophenanthrene (Fig. 1).

Batatasin III, a bibenzyl bearing a *m*-hydroxy group in ring B, is formed by O-methylation of 3,3′,5-trihydroxybibenzyl (Fig. 1). Data from experiments *in vivo* and *in vitro* are in agreement with analogous studies in various Orchidaceae. Taken together, the findings strongly argue that bibenzyls bearing a *m*-hydroxygroup generally originate from the respective dihydrocinammic acid and not *via* stilbenes.

The pathway to 9,10-dihydrophenanthrenes of the orchid type is demonstrated to take place also in Dioscoreaceae, but the gene expression obviously needs to be induced by an exogenous stimulus. In this respect, the present studies with *in vivo* labelling conform to the observation of Coxon *et al.* [9] who detected in the peel of yams (*D. rotundata*) two compounds with anti-fungal activity, a 9,10-dihydrophenanthrene (hircinol) and a phenanthrene, 7-hydroxy-2,4,6-methoxyphenanthrene. To detect antifungal compounds in the epidermis as a preformed barrier contrasts, or correlates, to the findings that the same compounds are detectable in other cells of this organism, but only subsequent to induction by fungi. This concept presents a com-

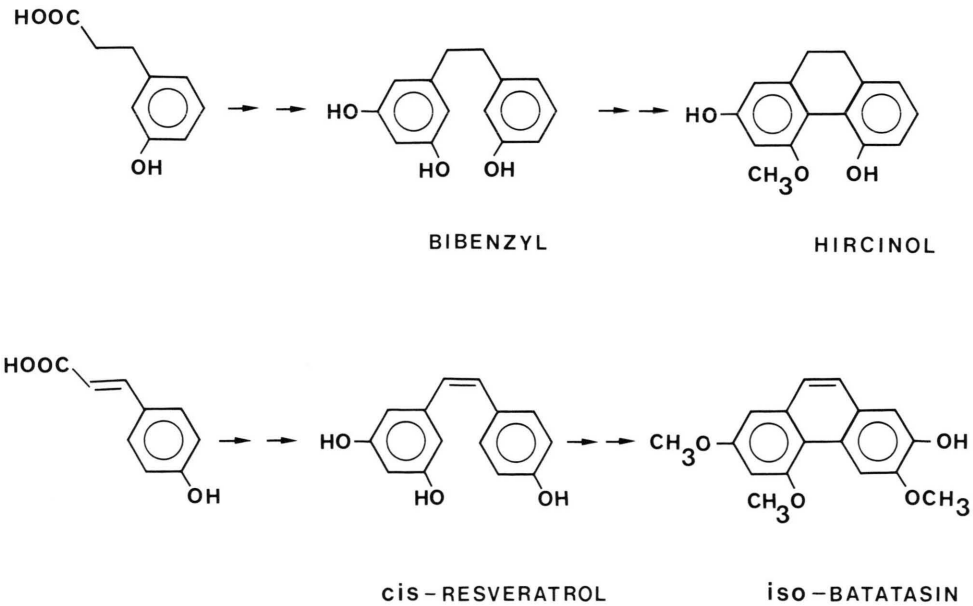

Fig. 1. Two independent pathways leading to the phenanthrene skeleton. Dihydro-*m*-coumaric acid (*m*-hydroxyphenylpropionic acid) originates from cinnamic acid *via m*-coumaric acid and is, as CoA-ester, substrate of a bibenzyl synthase. From the trihydroxybibenzyl the pathway branches to a monomethyl ether (batatasin III) or, by oxidative coupling, to 9,10-dihydrophenanthrenes. Resveratrol is synthesized from *p*-coumaroyl-CoA by the catalysis of a stilbene synthase and is the precursor of phenanthrenes.

bination of preformed barriers and elicited barriers, using the same tool but different controls.

In keeping with the studies on the natural constituents of the yam peel by Coxon *et al.* [9] it is necessary to address the question as to the structure of the phenanthrene that was studied in this work and denoted by batatasin I. We established the identity of this phenanthrene which should be batatasin I (6-hydroxy-2,4,7-trimethoxyphenanthrene) or iso-batatasin I (7-hydroxy-2,4,6-trimethoxyphenanthrene), by comparing its properties with the chromatographic properties of iso-batatasin I of Coxon *et al.*, and by subsequent permethylation and exact analysis at the stage of tetramethoxyphenanthrene. Although all data are strictly comparable with those for iso-batatasin I described in detail by Coxon *et al.* [9] we cannot rule out that the labelled product in our studies might have been contaminated with the isomer. It is, however, in terms of biosynthesis not essential to know which isomer of a monomethyl ether of a dihydroxy compound has been analyzed. It is evident that phenanthrenes containing a hydroxyl function in *p*-position to the side chain, *i.e.* the ethylene bridge, are synthesized *via* *p*-coumaric acid and resveratrol, and not by a pathway dihydrocinnamic acid – bibenzyl – dihydrophenanthrene – phenanthrene. We, therefore, postulate that independent pathways exist (a) leading to phenanthrenes *via* stilbenes, and (b) to dihydrophenanthrenes *via* bibenzyls. A third pathway from dihydro-*p*-coumaric acid to 3,4′,5-trihydroxybibenzyl and dihydrophenanthrenes (cannithrenes) [12] probably takes place in *Cannabis* species. Some of the details in phenanthrene biosynthesis may hold true as proposed by Stoessl [16]. But it is highly likely that at least three different pathways exist, each including at least 5 intermediates and branching out very early, *i.e.* at the stage of phenylpropanoids. Two of these sequences may function simultaneously in one cell, *viz.* in *Dioscorea* species.

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