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Metabolic Products of Microorganisms. 192.* The Anthraquinones of the *Aspergillus glaucus* Group. II. Biological Activity

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Abstract. From the mycelia of *Aspergillus cristatus* the following anthraquionic pigments were isolated: catenarin, emodin, erythroglaucin, rubrocristin, physcion, physcion-9-anthrone, questin, viocristin, and isoviocristin. The latter two do not belong to the 9,10-anthraquinone series but to the 1,4-anthraquinones, and so far they have not been reported among naturally occurring quinones.

Emodin, catenarin, viocristin, and isoviocristin showed antibacterial activity with minimal inhibitory concentrations ranging from 1–10 µg/ml. In *Bacillus brevis* catenarin and emodin inhibited the incorporation of uracil and leucine preferentially. At higher concentrations the incorporation of thymidine into the trichloroacetic acid-precipitable fraction of cells was also affected. In the presence of viocristin or isoviocristin all three macromolecular syntheses came to a halt. Rubrocristin, erythroglaucin, and physcion showed no significant inhibitory effects.

In Ehrlich ascites carcinoma cells catenarin, emodin, and viocristin inhibited the incorporation of uridine and thymidine. The incorporation of leucine was hardly affected.

In vitro, inhibition of DNA-dependent RNA polymerase from *Escherichia coli* by catenarin and to a lesser extent by emodin was observed, whereas rubrocristin (catenarin-8-methyl ether), physcion, and erythroglaucin were not active.

Key words: Antibiotics – Polyhydroxyanthraquinones – Catenarin – Emodin – Erythroglaucin – Physcion – Rubrocristin – Viocristin – *Aspergillus cristatus* – *Bacillus brevis*.

A number of anthraquinone pigments was isolated by us from cultures of *Aspergillus cristatus*, namely: catenarin, emodin, erythroglaucin, rubrocristin, physcion, physcion-9-anthrone, questin, and viocristin. Catenarin showed antibacterial activity, whereas rubrocristin (catenarin-8-methyl ether) was not active (Anke et al., 1980).

Anthraquinones are known to exhibit a variety of biological effects, like growth inhibition of tumors (Powell, 1944), toxicity to cockerels (Wells et al., 1975) to earthworms (Lagrange, 1946), hepatoxicity (Ueno et al., 1968; Ueno and Saheki, 1968), mutagenicity (Brown and Brown, 1976; Brown and Dietrich, 1979), and inhibition of bacterial and fungal growth (Fujikawa et al., 1952, 1953; Buckelew et al., 1972). Since little is known of the antibiotic effect and mode of action of the above mentioned anthraquiones, we investigated the biological activities of the anthraquinones isolated by us as well as those of two structurally related compounds, 1,4,6,8-tetrahydroxyanthraquinone and catenarin-6,8-dimethyl ether.

Parts of the results have been presented at the Annual Meeting of the American Society for Microbiology in Los Angeles, May 4–8, 1979 (Abstract 0 25) and at the Meeting of the Gesellschaft für Biologische Chemie in Tübingen, September 26–29, 1979 (Hoppe-Seyler's Z. Physiol. Chem. **360**, 1166–1167 (1979).

Materials and Methods

The cultivation of *Aspergillus cristatus*, the isolation, and identification of the anthraquinones have been reported in the preceding paper (Anke et al., 1980).

Measurement of the incorporation of precursors into trichloroacetic acid (TCA)-insoluble fractions of *Bacillus brevis* and Ehrlich

Abbreviations. MIC = minimal inhibitory concentration; TCA = trichloroacetic acid; ECA = Ehrlich ascites carcinoma

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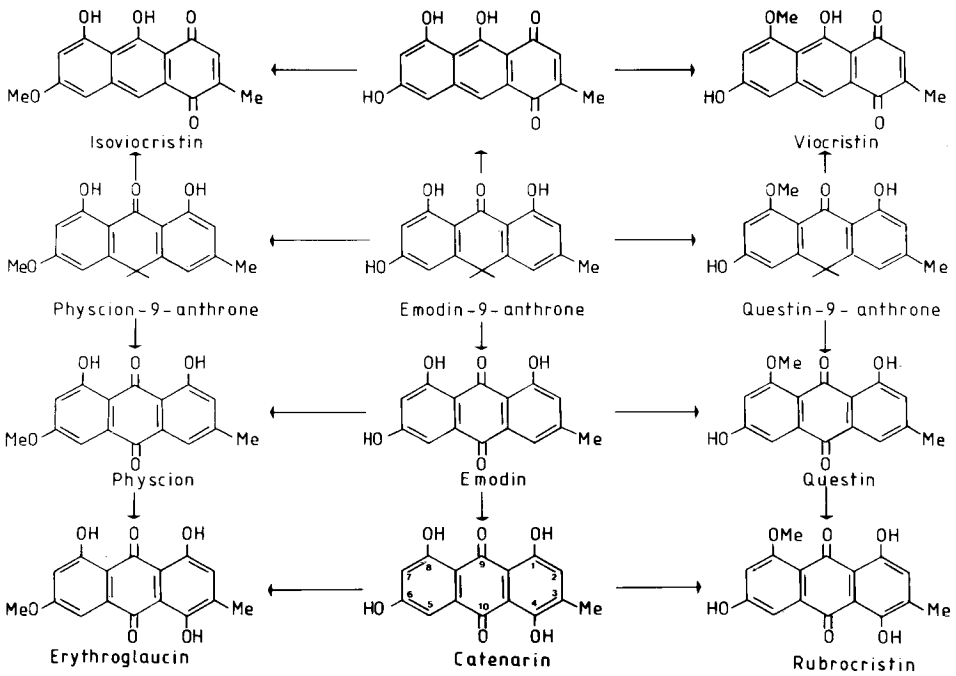

Fig. 1. Structural relationship of the anthraquinones of the *Aspergillus glaucus* group (genus *Eurotium*). All horizontal arrows stand for methylation-reactions, and all vertical arrows for hydroxylation-reactions

ascites carcinoma (ECA) cells was carried out as described before (Anke, 1979).

DNA-dependent RNA polymerase from *E. coli* was tested according to Fuchs et al. (1967). The test system contained 20 µg DNA per ml as template.

The binding of the pigments to DNA was followed according to the method of Swanbeck (1966). The pH of the buffer was 7.2. The optical density was recorded in a Zeiss PMQII apparatus. For the measurement of physcion and erythroglaucin cuvettes with 5 cm light path were used.

Mice bearing Ehrlich ascites carcinomas were a gift from H. G. Probst, Tübingen.

Catenarin-6,8-dimethyl ether was obtained by methylation of erythroglaucin with methyl iodide – silver oxide.

1,4,6,8-Tetrahydroxyanthraquinone was a gift from Bayer AG, Leverkusen. Calf thymus DNA, DNA-dependent RNA polymerase, and other biochemicals were purchased from Boehringer and Söhne, Mannheim. Radioactive compounds were purchased from Amersham Buchler.

Results and Discussion

Structural Relationship of the Anthraquinones Isolated from Strains of the Aspergillus glaucus Group

The anthraquinones of the *Aspergillus glaucus* group (genus *Eurotium*) and their structural relationship are given in Fig. 1. So far, we have isolated and identified nine of the twelve listed compounds. The structures of viocristin and isoviocristin have not been reported before, and the structure elucidation of these two compounds will be published elsewhere. Emodin anthrone and questin anthrone have not yet been found to occur together with other anthraquinones, and we are presently looking for them in the quinone mixtures of other strains of the *A. glaucus* group. The absence of these anthrones in naturally occurring anthraquinone mixtures has led to the conclusion that physcion anthrone A is derived from physcion rather than being a precursor of the latter (Turner, 1971). Therefore, the search for these anthrones is also stimulated by biosynthetical reasons.

Table 1. Antibacterial activity of catenarin, catenarin-6,8-dimethyl ether, emodin, erythroglaucin, rubrocristin, physcion, 1,4,6,8-tetrahydroxyanthraquinone, viocristin, and isoviocristin in the serial dilution assay. Testorganism: *Bacillus brevis* (10^6 cells/ml) in nutrient broth

Substance	MIC (µg/ml)
Catenarin	1
Catenarin-6,8-dimethyl ether	>20[a]
Emodin	5
Erythroglaucin	>20[a]
Rubrocristin	>25
Physcion	>20[a]
Tetrahydroxyanthraquinone	1
Viocristin	5–10
Isoviocristin	8–10

[a] The compound was not completely dissolved; after the incubation formation of some precipitate was observed

Antibacterial Activity

As shown in Table 1, catenarin exhibited the highest antibacterial activity. The MIC against *Bacillus brevis*

Table 2. Effect of tetrahydroxyanthraquinone, catenarin, rubrocristin, emodin, viocristin, and isoviocristin on the incorporation of uracil, leucine, and thymidine into TCA-precipitable fraction of cells of *Bacillus brevis*

Compound	(μg/ml)	Incorporation (% of the control without inhibitor)		
		Uracil	Leucine	Thymidine
Tetrahydroxyanthraquinone	1	12	10	58
	10	2	2	22
Catenarin	1	14	16	61
	10	2	3	25
Rubrocristin	1	90	105	110
	10	85	92	106
Emodin	1	12	40	69
	10	3	12	46
Viocristin	1	96	84	85
	10	13	2	2
Isoviocristin	1	120	96	110
	10	16	8	8

in complex media was 1 μg/ml. 1,4,6,8-Tetrahydroxyanthraquinone was as active as catenarin, indicating that the methyl group in position 3 has no effect on the antibiotic activity. Methylation of the hydroxyl groups in position 6 or 8 leads to antibiotically inactive compounds – erythroglaucin and rubrocristin or catenarin-6,8-dimethyl ether were not inhibitory at concentrations up to 20 μg/ml towards *B. brevis* and other bacteria. In the agar diffusion assay with *B. brevis* as test organism 20 μg of erythroglaucin per disc caused a small inhibition zone. The same effect is observed with methylated emodin: physcion was not active, whereas emodin showed antibiotic activity. Podojil and coworkers (1978) have isolated physcion and erythroglaucin from mycelia of *Eurotium repens* and reported that physcion exhibited antibacterial activity in the agar plate diffusion assay. The high amount (125 μg/disc) used by these authors, compared to 20 μg/ml in our tests, includes the possibility of impurities exhibiting antimicrobial activity. For the same reason we do not consider erythroglaucin to be antibiotically active. The amounts of questin were too small to allow the evaluation of the biological activities, however, fractions containing questin did not exhibit inhibitory activity towards *B. brevis*.

The effect of catenarin, tetrahydroxyanthraquinone, rubrocristin, emodin, viocristin and isoviocristin on the macromolecular syntheses in exponentially growing cells of *B. brevis* is shown in Table 2. The addition of 1 μg/ml of catenarin or tetrahydroxyanthraquinone resulted in an almost complete inhibition of the incorporation of uracil and leucine, whereas the incorporation of thymidine was less affected. Rubrocristin, not inhibiting growth, had no effect on the incroporation of precursors. Emodin, at low concentrations (1 μg/ml), affected preferentially the incroporation of uracil. The incorporation of leucine was inhibited at higher concentrations (10 μg/ml); this is rather interesting since it indicates different effects of these structurally related polyhydroxyanthraquinones on bacteria.

Viocristin and isoviocristin affected all three macromolecular syntheses in *B. brevis*. The antibiotic activity of isoviocristin is similar to the one of viocristin, as indicated by the spectrum of activity (data not shown) and the same rate of inhibition of the macromolecular syntheses. The amounts of isoviocristin produced were rather low e.g. 1–2 mg per l of culture, therefore further experiments were conducted with viocristin only. As 1,4-quinones, both compounds are capable of Michael-type additions, and react with SH-groups resulting in an inhibition of various enzyme reactions.

Figure 2 shows the effect of catenarin and rubrocristin on the incorporation of uracil, leucine, and thymidine at low concentrations. In *B. brevis*, the incorporation of uracil and leucine was inhibited at concentrations as low as 0.1 μg/ml.

Inhibitory Effects Towards ECA Cells

In ECA cells catenarin and tetrahydroxyanthraquinone inhibited preferentially the incorporation of uridine as shown in Table 3. At higher concentrations DNA-synthesis was also impaired. Incorporation of leucine was not affected by the tested anthraquinones. Methylation of the hydroxyl groups of catenarin and emodin resulted in the loss of activity, again rubrocristin, erythroglaucin, and physcion showed no significant inhibitory activity.

Podojil et al. (1978) have reported physcion to be cytotoxic to Hela cells at very low concentrations (0.1 μg/ml), and emodin was found to be toxic to 1-day

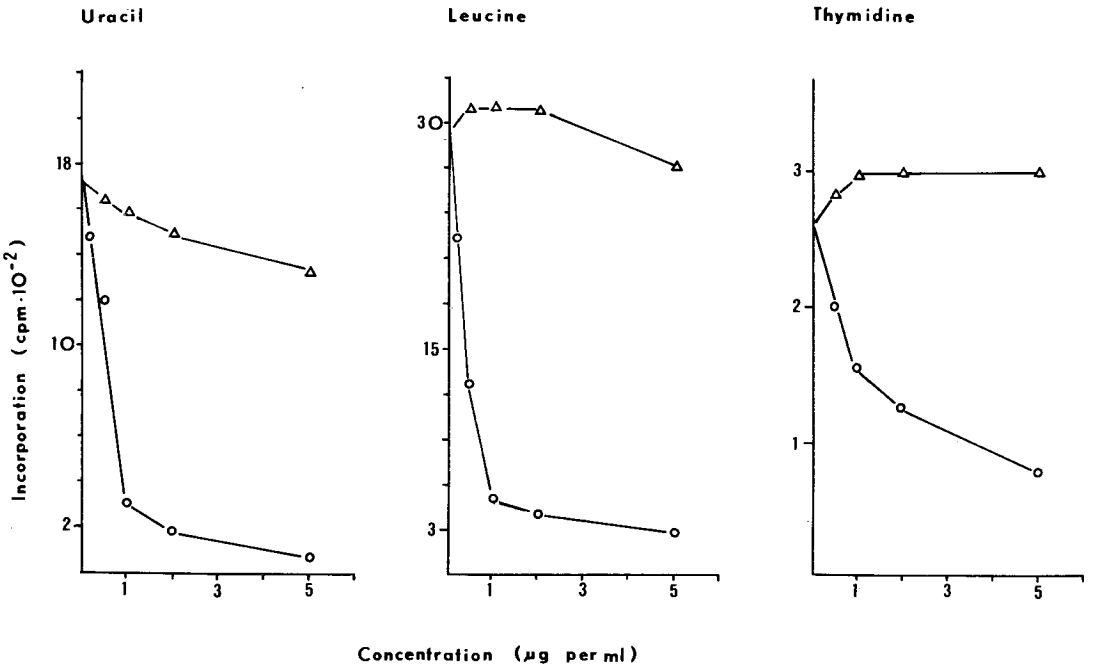

Fig. 2. Effect of catenarin and rubrocristin (catenarin-8-methyl ether) on the incorporation of uracil, leucine, and thymidine into TCA-precipitable material of exponentially growing cells of *Bacillus brevis*. ○ ——○ catenarin; △– – △ rubrocristin

Table 3. Effect of polyhydroxyanthraquinones on the incorporation of leucine, uridine, and thymidine into TCA-precipitable material in ECA cells. $3 \cdot 10^6$ Cells were incubated with the antibiotics in buffered saline for 10 min. The radioactive precursors were added and the incubation continued for 20 min. The incorporation was calculated in % of the control containing no antibiotic

Compound	(µg/ml)	Incorporation (%)		
		Leucine	Uridine	Thymidine
Erythroglaucin	10	100	82	100
Physcion	10	100	84	101
Rubrocristin	10	102	75	89
Emodin	10	99	40	89
Tetrahydroxyanthraquinone	10	68	6	41
Catenarin	1	91	39	100
Catenarin	2	90	30	72
Catenarin	10	75	7	43
Viocristin	1	100	48	82
Viocristin	2	98	38	73
Viocristin	10[a]	98	26	67

[a] The antibiotic was not completely dissolved

old cockerels (Wells et al., 1975). Unfortunately erythroglaucin or other anthraquinones were not tested. In ECA cells both compounds, physcion and erythroglaucin, had no significant inhibitory effect on macromolecular syntheses, whereas catenarin, tetrahydroxyanthraquinone and emodin preferentially inhibited the incorporation of uridine. Catenarin and tetrahydroxyanthraquinone were equally effective and emodin was less active. All three compounds showed higher inhibitory activity towards *B. brevis* than against ECA cells.

Inhibition of DNA-Dependent RNA Polymerase from Escherichia coli and Binding to DNA

When DNA-dependent RNA polymerase from *E. coli* (E.C. 2.7.7.6) was tested, catenarin and tetrahydroxyanthraquinone were found to be inhibitory but rubrocristin, physcion, dimethyl catenarin, and erythroglaucin had no effect on the enzyme activity (Table 4); emodin was slightly inhibitory. The concentrations needed for 50% inhibition of the enzyme activity were $3.5 \cdot 10^{-5}$ M for catenarin and $3 \cdot 10^{-5}$ M for tetrahydroxy-

Table 4. Inhibition of DNA-dependent RNA polymerase from *Escherichia coli* by polyhydroxyanthraquinones

Compound	(µg/ml)	AMP incorporated (% of the control)
Catenarin	10	65
Catenarin	20	38
Catenarindimethyl ether	10	111
Emodin	10	84
Emodin	20	76
Erythroglaucin	10	115
Rubrocristin	10	100
Rubrocristin	20	89
Physcin	10	112
Tetrahydroxyanthraquinone	10	62
Tetrahydroxyanthraquinone	20	33

Table 5. Inhibition of DNA-dependent RNA polymerase from *Escherichia coli* by catenarin and 1,4,6,8-tetrahydroxyanthraquinone

Inhibitor	(µg/ml)	AMP incorporated (nMol/ml)	
None	0	138	
Rifampicin	5	3	
Tetrahydroxyanthraquinone	1	110	
Tetrahydroxyanthraquinone	5	95	
Tetrahydroxyanthraquinone	10	65	
Tetrahydroxyanthraquinone	20	56	
None	0	106	
Rifampicin	5	5	
Catenarin	1	83	
Catenarin	5	69	
Catenarin	10	51	
Catenarin	20	42	
Catenarin	20	62	2 × DNA
Catenarin	20	115	4 × DNA

anthraquinone in an assay containing 20 µg/ml T4-DNA. Emodin at a concentration of $8 \cdot 10^{-5}$ M caused only 25% inhibition. Due to the low solubility of these compounds higher concentrations could not be tested.

The inhibition of RNA polymerase by catenarin strongly depends on the DNA concentration in the test system. The inhibition could be reversed by raising the DNA content of the assay as shown in Table 5, indicating an interaction of catenarin with DNA. The binding of anthraquinone derivatives to DNA has been studied in connection with the intercalating activity of anthracycline antibiotics (Swanbeck and Zetterberg, 1971; Berg and Eckhardt, 1970; Kersten and Kersten, 1965). The simplest method for following the interaction of anthraquinones with DNA is to measure the absorption maximum of the dye in the visible range: binding to DNA results in a red-shift. Data on the binding to DNA of catenarin and the other anthraquinones isolated from *A. cristatus* are not available, only emodin was reported to bind to DNA (Swanbeck and Zetterberg, 1971). The binding depended on the pH of the buffer solution. At neutral pH, emodin did not interact with DNA, only under acidic conditions a red-shift of the visible spectrum of the dye was observed, when DNA was added to an aqueous solution of emodin. Table 6 shows that emodin, erythroglaucin, catenarin-6,8-dimethyl ether, and rubrocristin gave no red-shift in the presence of DNA. Catenarin and tetrahydroxyanthraquinone interacted immediately with DNA under the chosen conditions (pH 7.2) – this is in agreement with our finding that only catenarin and tetrahydroxyanthraquinone inhibited RNA polymerase significantly. These two compounds also showed the highest antibacterial activity. The results obtained with emodin and physcion were not unambiguous. The addition of DNA to a solution of emodin resulted in a blue-shift and a weak inhibitory effect on RNA polymerase was observed. Physcion showed some binding to DNA noticeable after 24 h, but no inhibition of RNA polymerase was observed,

Table 6. Red-shift of polyhydroxyanthraquinones in the presence of DNA

Compound	Red-shift			
	immediately		after 24 h	
	$\lambda^{max.}$	$\lambda^{max.}$ + DNA	$\lambda^{max.}$	$\lambda^{max.}$ (nm) + DNA
1, 4, 6, 8-Tetrahydroxyanthraquinone	466	494	464	490
Catenarin	455	475	445	480
Rubrocristin	512	512	515	515
Catenarin-6,8-dimethyl ether	490	490	492	492
Erythroglaucin	450	456	448	448
Physcion	420	430	420	440
Emodin	462	455	475	470

even when physcion was preincubated with DNA for several hours. All tests with physcion and erythroglaucin were hindered by the insolubility of these compounds and only concentrations of 10 µg/ml could be tested.

Berg and Eckhardt (1970) could not find any influence of the number and position of the chromophore hydroxyl groups on the binding ability of polyhydroxyanthraquinones to DNA, whereas Swanbeck and Zetterberg (1971) noticed that substitution of anthraquinone with hydroxy- and methoxy groups increases the degree of binding to DNA except, if the substitution makes the compound a negative ion at pH 7.2 no binding occurs. This is the case for hydroxysubstitutions in positions 2, 3, 6 or 7 (β-positions). Our findings are partially in agreement with these latter findings: emodin has three hydroxyl groups, two of them, in positions 1 and 8, promote binding to DNA, the third one hinders this binding. In physcion (emodin-6-methyl ether) the hydroxyl group which hinders binding to DNA, is methylated and weak binding was found. Compared to emodin, catenarin possesses a forth hydroxyl group in position 4 which promotes binding, and as a result of three promoting and one hindering groups, catenarin binds to DNA immediately. Rubrocristin with two hydroxyl groups in promoting and one in hindering positions should not bind to DNA – no binding was found. However, erythroglaucin with three hydroxyl groups in binding-promoting positions and the forth one methylated showed no binding to DNA neither did catenarin-6,8-dimethyl ether, which possesses to binding-promoting hydroxyl groups and two methoxy groups. Therefore methoxy groups should not be considered to increase the degree of binding to DNA, except if they substitute for hydroxyl groups in β-positions and the formation of a negative ion is prevented. On the other hand, methylation of hydroxyl groups decreases the solubility of the compounds drastically. This might be the reason for the lacking of biological activity of erythroglaucin and catenarin-6,8-dimethyl ether.

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