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EFFECTS OF ORCHINOL AND RELATED PHENANTHRENES ON THE ENZYMIC DEGRADATION OF INDOLE-3-ACETIC ACID

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Key Word Index—Phytoalexin; orchinol; hircinol; loroglossol; phenanthrenes; stilbenes; peroxidase; IAA-oxidase.

Abstract—Orchinol, hircinol, loroglossol and certain related phenanthrenes inhibited horseradish peroxidase-catalysed IAA degradation to a varied degree. Among the compounds tested, a hydroxy group at the 7-position conferred high inhibitor activity. Phenanthrenes without a hydroxy group had no inhibitor activity whereas trihydroxy compounds were highly inhibitory. Orchinol induced a lag period in IAA oxidation, the length of which was dependent on the concentrations of orchinol, IAA and enzyme, and on the timing of the addition of orchinol. Orchinol competed preferentially with IAA for the ferriporphyrin group of the enzyme, but was degradable soon after the enzyme had reacted with IAA. Thus, it served also as a substrate in the active IAA-oxidizing system.

INTRODUCTION

Orchinol (**1**), loroglossol (**2**) and hircinol (**3**) are natural products formed in the tubers of orchid species in response to fungal infection. They have been intensively investigated with respect to their biological properties [1, 2], chemical constitution [3–6] and laboratory synthesis [6–8]. The compounds and certain related phenanthrenes cause distortion or rupture of the tip of the germ tubes of fungal spores, indicating an interference with the growth processes of fungi [9]. Growth-regulating activity of the phenanthrene, batatasin I (**4**) and the related dihydrostilbene, batatasin III (**5**) has also been reported [10, 11]. Since many phenolic compounds are known to either activate or inhibit the enzymic oxidation of indole-3-acetic acid (IAA) [12–17] and since a possible relationship between IAA metabolism and disease resistance has not been conclusively established [18], it is of interest to investigate whether these compounds can affect the metabolism of IAA. This paper reports the relative activities of orchinol and 24 related phenanthrenes and stilbenes in the enzymic oxidation of IAA, and the kinetics and the mechanism of the inhibition of IAA oxidation by orchinol.

RESULTS

Inhibition of IAA degradation

Orchinol (**1**) caused a strong inhibition of IAA degradation catalysed by purified peroxidase. However, when orchinol concentrations were sufficiently low, the inhibition was only temporary and the duration was dependent on the concentration of orchinol (Fig. 1). The length of the lag period increased with, but was not proportional to, increasing concentrations of orchinol. Thus, with orchinol added at zero time to a final concentration of 0.8, 1.6, 2.4 or 3.2 μM. the delay for IAA degradation was 2.0, 5.5, 11.5 or 20.0 min, respectively. After the lag period, the rate of IAA degradation returned to the same level as that of the control without orchinol.

Structure–activity relationship

A group of related phenanthrenes (**1–3**, **6–25**), the dihydrostilbene, batatasin III (**5**) and a stilbene (**26**) were tested for activities in the IAA degradation system which contained 2,4-dichlorophenol and $MnCl_2$ as cofactors. Since some phenolic compounds interfere with the spectrophotometric measurements of IAA and its breakdown products, the reactions were followed by measuring [1-^{14}C] IAA decarboxylation. A summary of results for a 2 μM concentration of the compounds is presented in Table 1. Concentrations higher or lower than 2 μM were also tested for the relative activities of some compounds when necessary.

Orchinol at 2 μM inhibited the decarboxylation of IAA by 75% in a 10-min reaction. Orchinol was more inhibitory than the other phenanthrenes tested except the tri- and tetrahydroxy compounds which were also strong inhibitors of IAA degradation. Phenanthrenes without a hydroxy group had no inhibitor activity. Among the phenanthrenes tested, an OH group at the 7-position appears to be important for high inhibitor activity. For example, orchinol (**1**) was much more inhibitory than loroglossol (**2**) which has the OH at the 5-position. Compound **6** without a hydroxy group and **7** with a OCH_3 at the 7-position lacked inhibitor activity even at 20 μM concentration. Diphenols are well known inhibitors of IAA degradation, but the dihydroxyphenanthrenes **3**, **10** and **11**, with no hydroxy group at the 7-position, showed only moderate inhibitor activity but significantly less so than orchinol. The importance of the hydroxy group at the 7-position was also demonstrated among the trihydroxy compounds when tested at concentrations lower than 0.5 μM (Table 2).

Orchinol was more inhibitory to IAA degradation than dehydroorchinol [16]. An effect of the double bond bet-

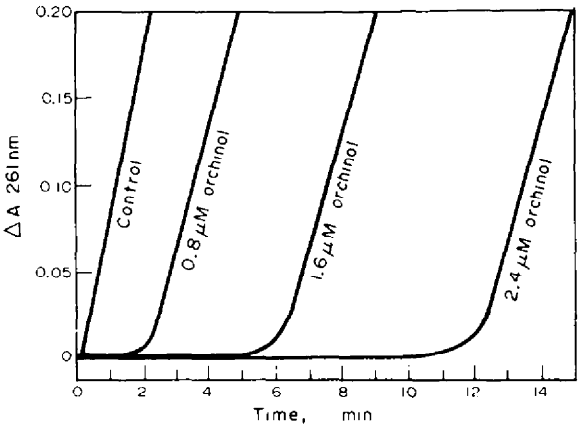
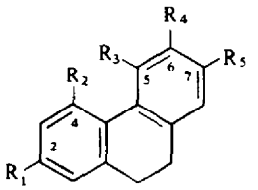


Fig. 1. Inhibition of IAA degradation by different concentrations of orchinol. The reaction soln had the molar composition: IAA (0.17 mM), 2,4-dichlorophenol (0.1 mM), $MnCl_2$ (0.1 mM), K-Pi buffer (30 mM, pH 6), orchinol (0 to 2.4 μM), and contained 0.2 μg peroxidase.



	R_1	R_2	R_3	R_4	R_5
1	OMe	OMe	H	H	OH
2	OMe	OMe	OH	H	H
3	OH	OMe	OH	H	H
6	OMe	OMe	H	H	H
7	OMe	OMe	H	H	OMe
8	OMe	OMe	H	OMe	H
9	OMe	OMe	OMe	H	OMe
10	OMe	OH	OH	H	H
11	OH	OH	H	H	H
12	OH	OH	H	H	OH
13	OH	OH	H	OH	H
14	OH	OH	OH	H	H
15	OH	OH	OH	H	OH

ween the 9 and 10 positions was also suggested by the results obtained with low concentrations of the trihydroxy compounds (Table 2). Compounds **12**, **13** and **14** were more inhibitory than the corresponding dehydro compounds.

Table 1. Activity of orchinol and related phenanthrenes in the inhibition of enzymic decarboxylation of IAA

Compound No.	% Inhibition of IAA decarboxylation	Compound No.	% Inhibition of IAA decarboxylation
1	75	16	15
2	5	17	9
3	31	18	0
6	0	19	0
7	0	20	0
8	0	21	0
9	0	22	48
10	35	23	100
11	38	24	47
12	100	25	98
13	64		
*14	99		
15	100		

The reaction soln (2.5 ml) had the molar composition: IAA (0.25 mM containing 0.17 μM IAA [1-^{14}C], 2,4-dichlorophenol (0.1 mM), $MnCl_2$ (0.1 mM), K Pi buffer (50 mM, pH 6), the phenanthrenes (2 μM), and contained 0.5 μg peroxidase. The reaction run at 25° for 10 min. $^{14}CO_2$ collected from the control without phenanthrenes was in the 7000–8000 cpm range.

The dihydrostilbene, batatasin III (**5**) and the stilbene **26**, in contrast to the phenanthrenes discussed above, increased IAA decarboxylation by 9–20%. When tested in the absence of 2,4-dichlorophenol, both compounds served as cofactors for IAA degradation, as expected of simple monophenols. Thus, the addition of the central ring changes the cofactor activity of a monophenol to inhibition. Orchinol (**1**) and the other monohydroxy compounds **2**, **16** and **17**, and the dimethoxy compounds **6** and **18**, were also tested for cofactor activity in the absence of 2,4-dichlorophenol, but no cofactor activity was detected.

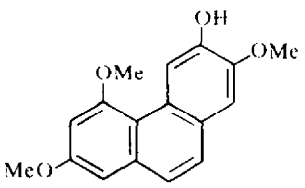
Stability of orchinol

The orchinol-induced inhibition of IAA degradation could be reversed to a varied degree by increasing the concentration of IAA or peroxidase. The effect of IAA

Table 2. Relative activity of trihydroxyphenanthrenes in the inhibition of enzymic decarboxylation of IAA

Compound No.	% Inhibition of IAA decarboxylation
12	99
13	36
14	65
23	49
24	30
25	35

The composition of the reaction soln and the conditions were the same as those of Table 1 except the concn of the trihydroxy compounds being 0.4 μM.



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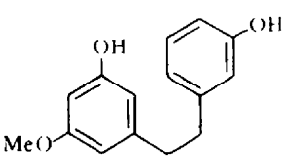

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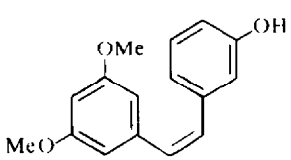

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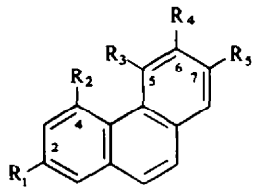

	R_1	R_2	R_3	R_4	R_5
16	OMe	OMe	H	H	OH
17	OMe	OMe	OH	H	H
18	OMe	OMe	H	H	H
19	OMe	OMe	H	H	OMe
20	OMe	OMe	H	OMe	H
21	OMe	OMe	OMe	H	OMe
22	OH	OH	H	H	H
23	OH	OH	H	H	OH
24	OH	OH	H	OH	H
25	OH	OH	OH	H	H

concentration is shown in Fig. 2. The lag time was inversely related to the concentration of IAA.

The lag period for IAA degradation induced by orchinol could also be shortened by a delay in adding orchinol to the reaction solution (Fig. 3). Under the experimental conditions, orchinol added 5 sec after the enzyme shortened the lag period by 40% as compared with the control in which orchinol was added before the enzyme. The lag priod was reduced practically to zero when orchinol was added 40 sec after the enzyme.

The kinetics of the inhibition as shown in Figs. 1 and 3 suggested that orchinol was itself unstable in the system in which IAA was degraded. This was confirmed by analysis of the reaction products by TLC, which showed that

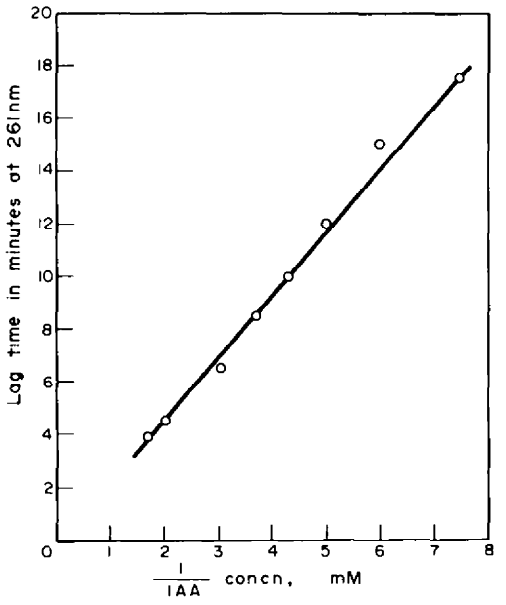

Fig. 2. Reversal of orchinol-induced inhibition of IAA degradation by higher concentrations of IAA. The composition of the reaction soln was the same as that shown in Fig. 1 except that the concns of orchinol and IAA were 2.4 μM and 133–600 μM, respectively.

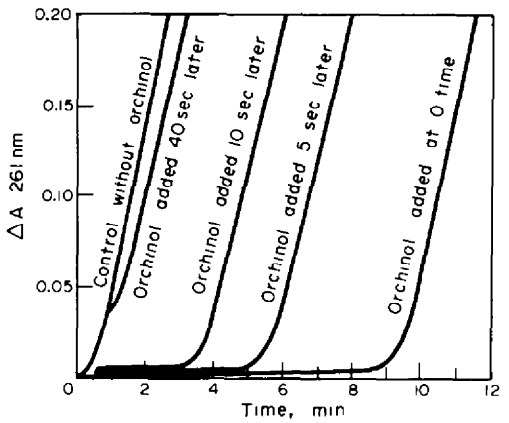

Fig. 3. Shortening of the lag period for IAA degradation by delaying the addition of orchinol. The composition of the reaction soln and the conditions were the same as shown in Fig. 1 except that orchinol in a final concn of 2 μM was added at different times as indicated.

the amount of recoverable orchinol decreased progressively during the lag period and was reduced to zero by the time the rate of IAA degradation was completely restored. The horseradish peroxidase alone did not induce the disappearance of orchinol under the experimental conditions. Clearly, orchinol was degraded in the reaction in which IAA was rapidly oxidized. The ending of the lag period for IAA degradation was therefore due to the depletion of orchinol in the active IAA-oxidizing system. Increasing the concentration of IAA or peroxidase accelerated the breakdown of orchinol.

Mechanism of action

It is known that under aerobic conditions IAA can react with peroxidase causing absorbance changes in the Soret band of the enzyme [19–21]. Certain phenolic inhibitors prevent such a reaction, thus inhibiting IAA degradation [22, 23]. A further experiment was therefore conducted to determine any effect of orchinol on the reactions between IAA and the ferriporphyrin group of the enzyme. The absorbance at 427 nm was used as an indicator for the overall change in the enzyme [24]. Adding IAA to the enzyme solution caused a rapid increase in $A_{427\,nm}$ which reached a peak in 40 sec (Fig. 4, line 1). However, when an appropriate amount of orchinol was added before IAA, no change in $A_{427\,nm}$ was observed. When orchinol was added 20 sec or less after IAA, $A_{427\,nm}$ immediately returned to zero and remained so for the entire 10-min period of the experiment (Fig. 4, line 2). Evidently, orchinol competed with IAA for the ferriporphyrin group of the enzyme, thus inhibiting IAA degradation.

Fig. 4 also shows that, under the experimental conditions, the effectiveness of orchinol as an inhibitor was dependent on when it was added. When orchinol was added 30 sec after IAA, the decrease in $A_{427\,nm}$ was rapid but lasted for only 30 sec after which $A_{427\,nm}$ increased (Fig. 4, line 3) at a rate similar to that of the control. Further delay in adding orchinol resulted in a less drastic decrease in $A_{427\,nm}$ and a shorter lag period. When orchinol was added 50 sec after IAA, the initial decrease and then increase in $A_{427\,nm}$ was too rapid to be detected with the instrument used. Since high concentrations of

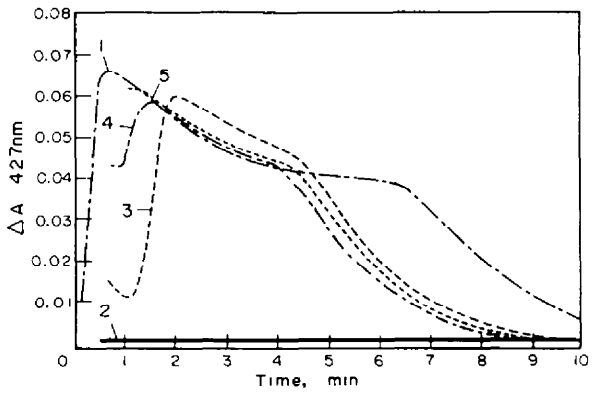

Fig. 4. Effect of orchinol on the spectral change in the ferriporphyrin group of horseradish peroxidase upon reacting with IAA. The reaction soln (3 ml) initially contained 0.20 mg peroxidase in K-Pi buffer (25 mM, pH 6). At zero time 10 μl of IAA (4 mM stock) were added alone (line 1) or followed by 5 μl of orchinol (0.5 mM stock) 20, 30, 40 or 50 sec later (line 2, 3, 4 and 5, respectively).

orchinol could reverse the IAA-induced spectral shift and prevent any further increase in $A_{427\,nm}$ for a prolonged period, the possibility that orchinol may not be inhibitory to IAA oxidation catalysed by enzyme intermediates after the initial step of reaction is ruled out. Thus, the results shown in Fig. 4 clearly indicate instability of orchinol in the active IAA oxidizing system and are consistent with those obtained from the experiments on IAA degradation (Fig. 3).

DISCUSSION

The kinetic behaviour of orchinol in the inhibition of IAA degradation (Figs. 1–4) is similar to that of 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran [17, 23]. The inhibition appears basically of a competitive type but the non-linear response to different levels of IAA suggests a complex nature. It is known that the oxidation of IAA takes place in several steps in which different intermediates of peroxidase are formed [19, 21, 24]. The kinetics of the inhibition by this type of inhibitor as shown previously [17, 23] suggest a possible participation of orchinol in different reaction steps in which the intermediates of peroxidase may have different affinities for orchinol and IAA or metabolites of IAA. The inhibition of IAA degradation by orchinol and the rapid disappearance of orchinol following the initial reaction of IAA with peroxidase indicate a preferential reactivity of orchinol with the enzyme. A similar behaviour has been reported for scopoletin [25] and quercetin [26] in the IAA–horseradish peroxidase system.

Certain peroxidase intermediates, rather than the native form, are believed to be responsible for the breakdown of certain phenolic inhibitors [17, 25, 27]. The effect of orchinol on the lag period of IAA degradation (Fig. 3) and on the spectral change of the enzyme (Fig. 4), and the disappearance of orchinol in the active IAA-oxidizing system lead to the same conclusion in the present case. During IAA oxidation catalysed by horseradish peroxidase, orchinol could serve as a substrate, coupling to the oxidation–reduction reactions with IAA or metabolites of IAA in the presence of certain peroxidase intermediates.

Because of the competitive nature, the inhibition of IAA degradation by orchinol was incomplete when a relatively large amount of IAA was present. Therefore, in the orchinol-induced lag period, IAA oxidation occurred though at a low rate. This active IAA-oxidizing system could initiate a chain reaction in which orchinol was broken down. As the concentration of orchinol gradually decreased as confirmed by TLC, the rate of IAA degradation increased, thus accelerating the further breakdown of orchinol. When the concentration of orchinol was sufficiently reduced, the rate of IAA oxidation rapidly reached the same maximum as without orchinol (Figs. 1 and 3).

The role of orchinol in the inhibition of IAA oxidation catalysed by peroxidase was to interfere with the reaction between IAA and the ferriporphyrin group of the enzyme (Fig. 4). Such an interaction has been previously demonstrated with 7-hydroxy-2,3-dihydrobenzofurans, catechol, protocatechuic acid, caffeic acid and ferulic acid [23].

Orchinol, hircinol and, to a lesser degree, loroglossol are inhibitors of IAA degradation (Table 1). The compounds are phytoalexins [9], but it is difficult to determine whether the inhibition of IAA degradation is related to their antifungal activity. The relative activities of orchinol and loroglossol in the inhibition of IAA oxidation *in vitro* are of the same order as in the inhibition of spore germination of the fungi *Monilinia fructicola* and *Phytophthora infestans* [9], but the order of activities of orchinol and dehydroorchinol is reversed. Since the synthesis of orchinol occurs in the host cells after fungal infection, it is reasonable to assume that it may affect the metabolism of IAA in both the host and the parasite cells. Shaw and Hawkins [28] reported an increase in the rate of IAA decarboxylation by leaf discs of a susceptible variety of wheat in the early stage of rust infection, followed by a sharp decrease of IAA decarboxylation and an increase in the level of IAA. These results were disputed by Daly and coworkers [29–31]. Pilet [32, 33] reported an association of IAA–oxidase activity and symptom development on the stem of *Euphorbia cyparissias* infected with *Uromyces pisi*. More IAA and less IAA–oxidase activity were found in infected than in healthy tissue; the differences were attributed to unidentified toxins as IAA–oxidase inhibitors in the infected tissue. Though different organisms are concerned, the results of the present study seem to support such a speculation. In general, increases in the concentration and number of phenolic compounds are well-known as a consequence of infection of plants by pathogenic organisms. However, in a later study Seevers and Daly [34, 35] emphasized an increase of peroxidase activity rather than a change of phenolic constituents in the regulation of IAA decarboxylation as an expression of rust resistance. Very recently, Gorham [36] has independently reported on the effect of batatasin III and related stilbenes on IAA oxidation, showing cofactor activity for batatasin III.

EXPERIMENTAL

Horseradish peroxidase (EC 1.11.1.7, Sigma type VI) with a RZ of 2.9 was used. IAA-[1-^{14}C] with a sp. act. of 60 μCi/μmol was obtained from New England Nuclear. The molar composition of the reaction solns are given in the figures and tables. The reactions were carried out at 25°. The rate of IAA degradation was determined by automatic recording of the increase in A at

Table 3. ^{1}H-NMR data for phenanthrenes*

Compound (solvent)	δ, $m(J')$								
	H_1	H_3	H_5	H_6	H_7	H_8	H_9	H_{10}	OMe
25	6.90	6.79	—	(7.75	to	7.04)	7.29†	7.57†	—
(MeOD-d_4)	*d*(2.5)	*d*(2.5)		(	– ABC multiplet –	)	*d*(9.0)	*d*(9.0)	
10	5.66	5.66	—	(7.15	to	6.75	2.66	2.66	3.79
(MeOD-d_4)	*s*(*br*)	*s*(*br*)		(	– ABC multiplet–	)	*s*(2)‡	*s*(2)‡	(3)‡
24	6.75	6.65	9.00	—	7.00	7.63	7.28‡	7.52†	—
(MeOD-d_4)	*d*(2.5)	*d*(2.5)	*d*(2.5)		*dd*(8.0 × 2.5)	*d*(2.5)	*d*(9.0)	*d*(9.0)	
8	6.45	6.45	7.88	—	6·70	7.13	2.73	2.73	3.88, 3.83
($CDCl_3$)	*s*	*s*	*d*(2.75)		*dd*(8.0 × 2.75)	*d*(8.0)	*s*(2)‡	*s*(2)‡	(3, 6)‡
13	6.30	6.25	7.93	—	6.54	6.98	2.63	2.63	—
(MeOD-d_4)	*d*(2.5)	*d*(2.5)	*d*(2.75)		*dd*(8.0 × 2.75)	*d*(8.0)	*s*(2)‡	*s*(2)‡	
23	6.84	6.76	9.66	7.15	—	7.27	7.51	7.51	—
(MeOD-d_4)	*d*(2.5)	*d*(2.5)	*q*§	*m*§		*m*§	*s*	*s*	
12	6.26	6.21	8.15	6.63	—	6.63	2.63	2.63	—
(MeOD-d_4)	*d*(2.5)	*d*(2.5)	*q*§	*m*§		*m*§	*s*(2)‡	*s*(2)‡	
21	6.86	6.77	—	6.77	—	6.86	7.52	7.52	3.98, 3.92
($CDCl_3$)	*d*(2.5)	*d*(2.5)		*d*(2.5)		*d*(2.5)	*s*	*s*	(6, 6)‡
9	6.47	6.47	—	6.47	—	6.47	2.64	2.64	3.85, 3.82
($CDCl_3$)	*s*	*s*		*s*		*s*	*s*(2)‡	*s*(2)‡	(6, 6)‡
15	6.36	6.36	—	6.36	—	6.36	2.58	2.58	—
(MeOD-d_4)	*s*	*s*		*s*		*s*	*s*(2)‡	*s*(2)‡	

* Chemical shifts (δ ppm from TMS as internal reference) were measured at the geometrical centre of multiplets, with observed splittings J' (= apparent coupling constant) in Hz;
† Or reversed assignments for compounds in the same row;
‡ Number of protons (where greater than 1);
§ ABX system.

261 nm with a spectrophotometer or by liquid scintillation counting of $^{14}CO_2$ released from IAA-[1-^{14}C] [37]. To detect the effect of orchinol on the reaction of IAA with the ferriporphyrin group of peroxidase, changes in *A* at 427 nm was followed because this wavelength marks the isosbestic point of the spectral curves from 3 intermediates of peroxidase and therefore can be used as an indicator for the overall change of the enzyme [24]. The stock solns of orchinol and related phenanthrenes were made in DMSO, and 5 μl aliquots were used in the reactions. Solubility of the compounds was confirmed by spectrophotometry. For TLC, orchinol was extracted 3 × from the reaction soln with purified Et_2O; the time for the extraction was determined by monitoring the reaction spectrophotometrically during the lag period. The concd extracts were applied to a Si gel plate and separated with $CHCl_3$–MeOH, 98:2. Orchinol was detected by spraying first with 15% KOH followed by 0.1% Gibb's reagent [38].

The stilbenes and phenanthrenes used were prepared by standard methods, analogous to those described previously [8], in combination with demethylation (HCl–Py at 200° unless otherwise noted) or methylation (CH_2N_2 or MeI–K_2CO_3) as appropriate. Except as noted, all known compounds had the mps reported in the earlier literature; their ^{1}H NMR spectra were employed as a further check on their identity and purity. Previously undescribed compounds are listed below and their ^{1}H NMR parameters are shown in Table 3.

Petrol was of bp 30–60°; solvents were redistilled. Precise mass determinations were carried out on a Varian MAT311A instrument; ^{1}H NMR spectra were obtained with Varian A60A or XL-100 spectrometers. Sublimations were done *in vacuo* at 150–160°.

4,5-*Dihydroxy*-2-*methoxy*-9,10-*dihydrophenanthrene* (*isohircinol*) (**10**) was isolable by preparative TLC (SiO_2; MeOH–$CHCl_3$ 2:98) from the demethylation product (BBr_3) from loroglossol (**2**). Mp 186–7° (from MeOH–$CHCl_3$). (Found: M^+, 242.0946 Calc. for $C_{15}H_{14}O_3$: M^+, 242.0943).

2,4,5,7-*Tetramethoxyphenanthrene* (**21**). (From 3,3′,5,5′-tetramethoxystilbene), mp 122–3° (from MeOH–$CHCl_3$). (Found: C, 70.47; H, 6.43. Calc. for $C_{18}H_{18}O_4$.0.5CH_3OH: C, 70.68; H, 6.41%) was hydrogenated to 2,4,5,7-*tetramethoxy*-9,10-*dihydrophenanthrene* (**9**), mp 109–110° (from MeOH–$CHCl_3$) (Found: C, 71.73; H, 6.78. Calc. for $C_{18}H_{20}O_4$: C, 71.98; H, 6.71%) which was demethylated to 2,4,5,7-*tetrahydroxy*-9,10-*dihydrophenanthrene* (**15**), mp 219–225° (sublimed) (Found: M^+, 244.0734. Calc. for $C_{14}H_{12}O_4$: M^+, 244.0737).

2,4,5-*Trihydroxyphenanthrene* (**25**). From dehydroloroglossol. Mp 215–6° (from MeOH–$CHCl_3$). (Found: M^+, 226.0633. Calc. for $C_{14}H_{10}O_3$: M^+, 226.0630).

2,4,6-*Trihydroxyphenanthrene* (**24**). From (**20**). Mp 219–221° (sublimed). (Found: M^+, 226.0632).

2,4,7-*Trihydroxyphenanthrene* (**23**). From dehydroorchinol (**16**). Mp 255° (from Et_2O–petrol). (Found: M^+, 226.0627).

2,4,6-*Trihydroxy*-9,10-*dihydrophenanthrene* (**13**). From (**8**). Mp 166–168° (from MeOH–$CHCl_3$). (Found: M^+, 228.0787 Calc. for $C_{14}H_{12}O_3$: M^+, 228.0786).

2,4,7-*Trihydroxy*-9,10-*dihydrophenanthrene* (**12**). From orchinol acetate. Mp 225–8° (sublimed). (Found: M^+, 228.0789).

2,4,6-*Trimethoxy*-9,10-*dihydrophenanthrene* (**8**), previously described as an oil [39], crystallized from petrol, mp 34–46°.

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