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STRUCTURE AND ANTIFUNGAL ACTIVITY OF HIRCINOL, LOROGLOSSOL AND ORCHINOL*

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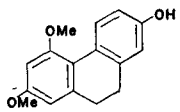
Key Word Index—*Orchis militaris*; *Loroglossum hircinum*; Orchidaceae; phytoalexins; antifungal activity; structure

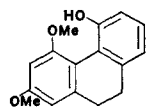
Abstract—The structure of hircinol, a phytoalexin isolated from *Loroglossum hircinum* has been established as 2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene. This phytoalexin, the second to be identified in the Orchidaceae, is less effective than orchinol, and its isomeric co-metabolite, loroglossol, 5-hydroxy-2,4-dimethyoxy-9,10-dihydrophenanthrene, is inactive.

INTRODUCTION

OF THE twenty known phytoalexins, three have been isolated from European orchids;[1] these are all trisubstituted dihydrophenanthrenes. Orchinol, isolated from *Orchis militaris*,[2–4] has been identified as 2,4-dimethoxy-7-hydroxy-9,10-dihydrophenanthrene (I).[5–7] Hircinol[8] (IIIb) and loroglossol[5,8] (II), two other phenols isolated from *Loroglossum hircinum* share the dihydrophenanthrene skeleton, but their structures have not been determined. Reinvestigation of these substances by modern spectural means has permitted structural assignments to be made.



(I)



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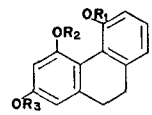
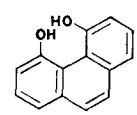


(IIIa) $R_1 = R_2 = H$; $R_3 = Me$
(IIIb) $R_1 = R_3 = H$; $R_2 = Me$
(IIIc) $R_2 = R_3 = H$; $R_1 = Me$



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RESULTS AND DISCUSSION

Orchinol has been assigned as 2,4-dimethoxy-7-hydroxy-9,10-dihydrophenanthrene (I), partly on the basis of negative evidence. In our spectra of orchinol, two points un-

* Part I in the series "Orchid Phytoalexins". For a review see M. H. FISCH, Y. SCHECHTER and J. ARDITTI, *Bull. Am. Orch. Soc.* **41**, 605 (1972).

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[1] E. GÄUMANN, *Phytopathol.* **49**, 211 (1963).
[2] A. BOLLER, H. CORRODI, E. H. GÄUMANN, E. HARDEGGER, H. KERN and N. WINTERHALTER-WILD, *Helv. Chim. Acta* **40**, 1062 (1957).
[3] E. GÄUMANN and H. KERN, *Phytopath. Z.* **35**, 347 (1959).
[4] E. GÄUMANN and H. KERN, *Phytopath. Z.* **36**, 1 (1959).
[5] E. HARDEGGER, N. SCHELLENBAUM and H. CORRODI, *Helv. Chim. Acta* **46**, 1171 (1963).
[6] E. HARDEGGER, H. R. BILAND and H. CORRODI, *Helv. Chim. Acta* **45**, 1354 (1963).
[7] E. HARDEGGER, N. RIGASSI, J. SERES, CH. EGLI, P. MÜLLER and K. O. FITZI, *Helv. Chim. Acta* **46**, 2543 (1963).
[8] J. URECH, B. FECHTIG, J. NÜESCH and E. VISCHER, *Helv. Chim. Acta* **46**, 2758 (1963).

ambiguously confirm the suggested structure. A moderately strong band at 829 cm^{-1} (12·07 μ) corresponds to two adjacent aromatic hydrogens and the weak band for the isolated proton is detectable at ~880 cm^{-1} (11·36 μ).[9] In the NMR spectrum, the two protons at C_6 and C_8 appear as a multiplet (2H) centered at 6·68 δ. The two protons of the tetrasubstituted ring, both isolated, fortuitously have approximately the same chemical shift and appear as a singlet at 6·43 δ.

The two line pattern of the remaining proton (centered at 8·13 δ) is characteristic of phenanthrene hydrogen at the 4 or 5 position.[10] In addition, the resonance is obviously half of an *AB* pattern which requires that there be two adjacent protons at C-5 and C-6, thus placing the hydroxyl at C-7. This interpretation is consistent with the relatively large coupling of the adjacent aromatic hydrogens (J_{AB} = 9·4 Hz). Hircinol and loroglossol have one and two methoxyl groups, respectively, and form the same trimethoxyphenanthrene.[8]

On the basis of the analogies in the UV spectra, it has been suggested that hircinol, orchinol, and loroglossol all have methoxyl or hydroxyl substituents at the 2 and 4 positions, although there is no proof of this. Since loroglossol and orchinol are different, it has been suggested that loroglossol is 5-hydroxy-2,4-dimethoxy-9,10,-dihydrophenanthrene[6,7] but no definitive evidence has heretofore been available.

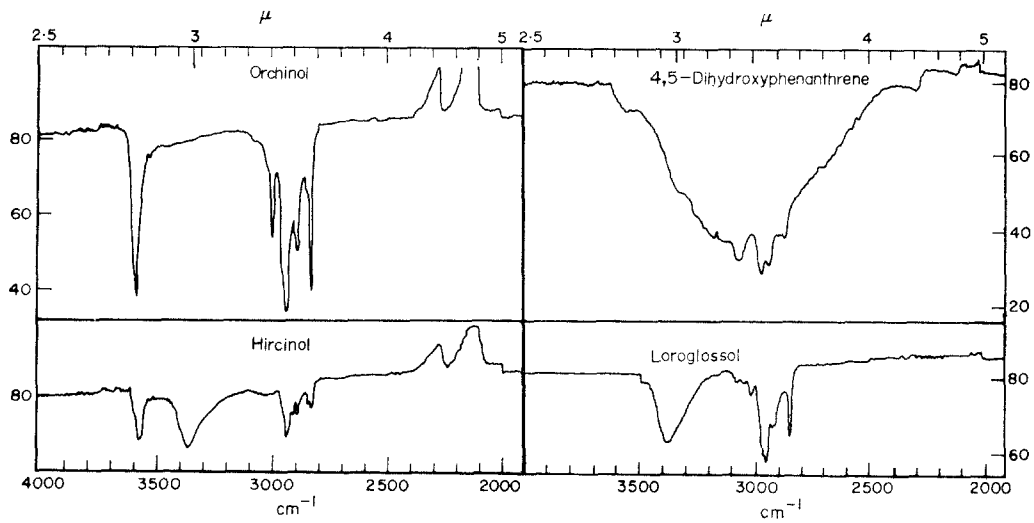


FIG. 1. IR SPECTRA OF ORCHINOL, HIRCINOL, LOROGLOSSOL AND 4,5-DIHYDROXYPHENANTHRENE.

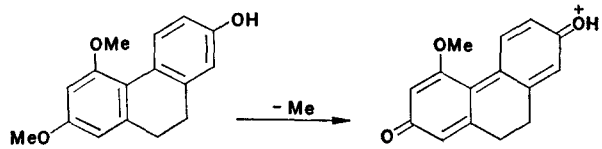
The location of the three functional groups follows easily from consideration of the IR and NMR spectra of hircinol. The low-field resonance of orchinol at 8·1 δ is absent, and all five aromatic protons resonate between 6·4 and 7·2 δ. Thus, there is substitution of both C-4 and C-5. The IR shows bands at 785 cm^{-1} (3 adjacent H),[9] but no band corresponding to two vicinal hydrogens (c.f. orchinol). Again, there is a two-proton singlet at 6·47 δ corresponding to two isolated aromatic hydrogens which fortuitously have the same chemical shift. The remaining three protons resonate in a complicated *ABC* pattern (6·7–7·2 δ)

[9] D. J. PASTO and C. R. JOHNSON, *Organic Structure Determination*, Chapter 4. Prentice-Hall, Englewood Cliffs, New Jersey (1969).
[10] P. M. G. BAVIN, K. D. BARTLE and J. A. S. SMITH, *Tetrahedron* **21**, 1087 (1965).

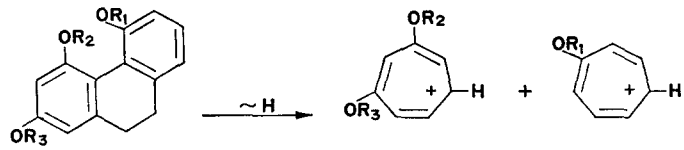
which resembles the analogous spectrum of 6-methoxysalicylaldehyde.[11] Thus, loroglossol and hircinol have substituents at C-2, C-4, and C-5. It remains only to specify the locations of the hydroxyl groups.

Biosynthetically, one may easily envisage loroglossol as arising via cyclization of desmethoxydihydrorhapontigenin.[12,13] The IR shows only intramolecularly bonded hydroxyl in the region 3000–4000 (Fig. 1) which requires that the free OH be at C-4 or C-5 in order to hydrogen bond with the proximate methoxyl. By analogy with the known substitution pattern in orchinol, we prefer structure II to the isomeric 4-hydroxy-2,5-dimethoxy alternative.

Hircinol (≡ desmethyloroglossol) is restricted to one of three possible structures, IIIa–IIIc. We conclusively eliminate IIIc by consideration of the MS. Aryl methyl ethers readily lose methyl if the resulting positive ion, $(p\text{-Me})^+$ is stabilized by quinonoid structures.[14] There are ample possibilities for such fragmentation pathways here as illustrated below for orchinol. As a result, the usual decomposition of 9,10-dihydrophenanthrenes, loss of hydrogen followed by loss of acetylene to yield biphenylene,[15] is suppressed and a strong peak appears at $m/e = 227$ corresponding to $(p\text{-Me})^+$.



A plausible alternate pathway is a fragmentation with hydrogen shift to yield a substituted tropylium ion. The charge would be expected to remain with the more substituted fragment. In orchinol, as expected, there is a significant peak at $m/e = 151$ but not at $m/e = 121$. To the extent that this fragmentation pathway is general, we may use it in the case of hircinol also. Structures IIIa and IIIb would yield a significant peak at $m/e = 137$ whereas IIIc would lead to a fragment of $m/e = 123$. Inspection of the hircinol MS shows a peak at m/e 137, but not at $m/e = 123$ in keeping with structures IIIa and IIIb only. Since these are also the preferred structures by analogy, we can eliminate IIIc from further consideration.



In the NMR spectrum, hircinol has signals corresponding to two types of OH: one at 5·33 δ (free) and the other at 7·95 δ (bonded). This observation suggests structure IIIb, since if both C-4 and C-5 were OH substituted, one would expect only intramolecular hydrogen

[11] S. Forsen, B. Akermak and T. Alm, *Acta. Chem. Scand.* **18**, 2313 (1964).
[12] D. H. R. Barton and T. Cohen, *Festschrift A. Stoll.* p. 117, Birkhauser, Basel (1957).
[13] H. Erdtman and C. A. Wachtmeister, *Festschrift A. Stoll.* p. 144, Birkhauser, Basel (1959).
[14] J. H. Beynon, R. A. Saunders and A. E. Williams, *The Mass Spectra of Organic Molecules*, p. 171, Elsevier, Amsterdam (1968).
[15] E. Dynesen, S. -O Lawessen, G. Schroll, J. H. Bowie and R. G. Cooks, *Ark. Kemi.* **26**, 379 (1967).

bonding. In addition, the protons of the tetrasubstituted ring have identical chemical shifts as in orchinol. It is likely that demethylating the C-4 methoxyl would affect the C-1 and C-3 ring proton shifts differently since one is *ortho* and one is *para* to C-4, whereas demethylation of the C-2 methoxyl would affect the C-1 and C-3 hydrogens similarly (both are *ortho*). Since the tetrasubstituted ring in orchinol has almost identical chemical shifts for its ring protons, the same is more likely to be true for IIIb than for IIIa. NMR evidence in favor of IIIb is confirmed by analysis of the hydroxyl region in the IR. The free OH in orchinol is at 3595 cm^{-1}, whereas loroglossol shows only an intramolecularly hydrogen-bonded OH centered at $\sim$3400 cm^{-1} (Fig. 1). Hirchinol shows *both* types of OH (3595 cm^{-1} and 3375 cm^{-1}). To rule out the possibility that C-4 and C-5 could give rise to both free and bonded hydroxyl absorption we examined the model compound 4,5-dihydroxyphenanthrene (IV).[16] Only bonded OH was observed (Fig. 1). Hircinol is unequivocally 2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene (IIIb).

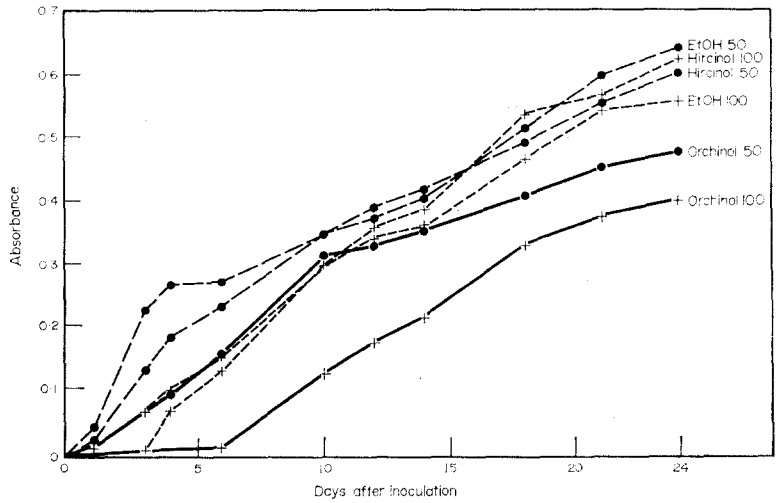
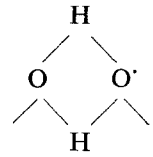


FIG. 2. GROWTH OF *Candida lipolytica* ON ORCHINOL 100 AND 50 ppm, HIRCINOL 100 AND 50 ppm AND ETHANOL 100 ppm AND 50 ppm.

At the concentrations used, intermolecular hydrogen bonding is not expected. The absence of free OH in 4,5-dihydroxyphenanthrene is presumably due to double internal bonding,



Orchinol at either 50 or 100 ppm is considerably more active than hircinol against *Candida lipolytica* BY 17 (Fig. 2). At 100 ppm, orchinol inhibits growth completely for the first 6 days. After that, the cultures lag noticeably behind the control or hircinol assays. Although less effective at 50 ppm, orchinol still inhibits fungal growth. Hircinol at 50 ppm

[16] M. S. NEWMAN and R. L. CHILDERS, *J. Org. Chem.* **32,** 62 (1967).

slows down growth during the first 6 days, but later its inhibition is greatly reduced. At 100 ppm, hircinol completely inhibits growth during the first 3 days, parallels the effects of 50 ppm orchinol until the tenth day, but is no longer effective thereafter. In 10 days of growth on 100 ppm orchinol, the absorbance is equal to that of a 5-day-old culture on 50 ppm. In this range, inhibition may be linear with phytoalexin concentration.

Following prolonged growth on 100 ppm, cultures accelerate. The convergence of 50 and 100 ppm growth curves is even more evident with hircinol (Fig. 2), which, after 13 days of culture, merge with the controls. This implies that both orchinol and hircinol are being degraded by *Candida lipolytica*. This was confirmed by examining filter sterilized media which had been used in a previous assay for thirteen days; when reinfected, they were indistinguishable from the controls.

Theoretical considerations suggest that in mycorrhizal relationships, slowly degradable phytoalexins could be most effective. If degradation is too slow, the fungi might be so inhibited as to destroy the association; if too rapid, the orchid would be parasitized. A continuous production of reasonably degradable phytoalexin(s) would be optimal in that it would keep the infection within acceptable limits without damaging the fungus. Both extremes as well as intermediate cases have been observed.[17,18]

Orchis militaris and *Loroglossum hircinum* are closely related to each other within the Orchidaceae and it is not surprising that their phytoalexins are similar. Whether phytoalexins from other orchids, e.g. *Cymbidium*, will also be dihydrophenanthrenes remains to be seen. Work with Leguminosae indicates that phytoalexins are host, rather than fungus, specific.[19,20] The same seems to be true for the Orchidaceae, at least for *Orchis militaris* and *Loroglossum hirchinum*.

EXPERIMENTAL

Phytoalexins. Samples of orchinol (I), hircinol (IIIb) and loroglossol (II) were obtained through this courtesy of Dr. H. Kern, Eidgenössische Technische Hochschule, Zürich.

Model compound. 4,5-Dihydroxyphenanthrene (IV) was prepared by cleavage of 4,5-dimethoxyphenanthrene by fusion with pyridine hydrochloride.[16,21]

Spectra. IR spectra were measured in CS_2 or CCl_4 and calibrated with polystyrene film at 1028, 1601, and 3027 cm^{-1} (Fig. 1). NMR spectra were determined in $CDCl_3$ by use of microcell techniques. MS were obtained at 70 eV electron energy. Variations down to 20 eV had little effect.

TLC. The phytoalexins were spotted on 300 μ silica gel G (E. Merck) plates and developed with MeOH–$CHCl_3$ (1:49) to a height of 12·5 cm. They were visualized by spraying with conc. H_2SO_4–95% EtOH (1:19) and then heating at 80° for 5 min. Orchinol had R_f 0·60 (light grey in fluorescent light, dark blue in UV) and hircinol had R_f 0·45 (light green in fluorescent light, dark blue in UV).

Bioassays. Orchinol and hircinol at concentrations of 50 ppm and 100 ppm were tested for activity against *Candida lipolytica* (BY 17) by measure of fungal growth spectrophotometrically (Fig. 2). In addition, the phytoalexins were spotted on silica gel G plates, sprayed with a spore suspension of *Cladosporium cucumerinum* and oversprayed with half strength Difco potato dextrose agar.[22,23] Inhibition of fungal growth was evident 36 hr after spraying as light colored zones on a dark background.

Acknowledgements—Supported by Office of Naval Research Contract NR 108-796. We thank Dr. H. Kern, Eidg. Tech. Hochschule, Zürich for samples of hircinol, orchinol, and loroglossol; Dr. M. S. Newman, Ohio State University, Columbus, for a gift of 4,5-dimethoxyphenanthrene; G. Osmond U.S. Navy Depot, Crane, Indiana for a *Candida lipolytica* BY17 culture, Robert Ernst for many fruitful discussions, A. Gotcher for technical assistance.

[17] N. Bernard, *Ann. Sci. Nat. Bot. Ser.* 9, **9**, 1 (1909).
[18] H. Burgeff, *Samenkeimung der Orchideen*, G. Fischer, Jena (1936).
[19] I. A. M. Cruikshank, *A. Rev. Phytopathol.* **1**, 350 (1963).
[20] I. A. M. Cruikshank, D. R. Biggs and D. R. Perrin, *J. Ind. Bot. Soc.* **50A**, 1 (1971).
[21] L. F. Fieser, *Experiments in Organic Chemistry*, 3rd Edn, p. 337, Heath, Boston (1957).
[22] W. L. Klarman and J. B. Sanford, *Life Sci.* **7**, 1095 (1968).
[23] N. T. Keen, J. J. Simms, D. Erwin, D. C. Rice and J. E. Partridge, *Phytopath.* **68**, 1084 (1971).