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IDENTIFICATION OF TWO CHROMONE PHYTOALEXINS IN THE SWEET PEA, *LATHYRUS ODORATUS*

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Key Word Index—*Lathyrus odoratus*; Vicieae; Leguminosae; sweet pea; phytoalexins; chromones; 5,7-dihydroxy-3-ethylchromone; fungitoxicity.

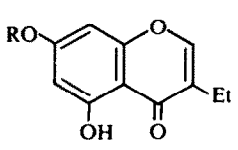
Abstract—A major phytoalexin isolated from the *Helminthosporium carbonum*-inoculated leaflets and pods of *Lathyrus odoratus* has been identified by spectroscopic procedures as 5,7-dihydroxy-3-ethylchromone (lathodoratin). Small amounts of the corresponding 7-*O*-methyl ether (methyl-lathodoratin) are also formed by this plant. Both compounds similarly occur as phytoalexins in the closely related legume *L. hirsutus* but are absent from the other *Lathyrus* species examined. The unusual 3-substitution of the chromone nucleus appears to be essential for fungitoxicity since the synthetic isomer 5,7-dihydroxy-2-ethylchromone is apparently inactive.

INTRODUCTION

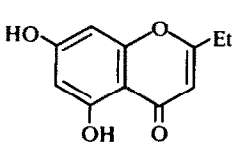
During a survey of phytoalexin production by species belonging to the tribe Vicieae (Leguminosae–Papilionoideae) [1, 2], two compounds of a type new to the family were isolated from fungus-inoculated leaflets and other tissues of the sweet pea (*Lathyrus odoratus* L.). These compounds—lathodoratin and methyl-lathodoratin—were accompanied by varying amounts of pisatin and variabilin, two isoflavonoid phytoalexins of widespread occurrence in the genus *Lathyrus* [1]. This paper describes the isolation of lathodoratin (**1**) and methyl-lathodoratin (**3**) from tissues inoculated with *H. carbonum* and *Botrytis cinerea*, and presents evidence to support their respective characterization as 5,7-dihydroxy-3-ethylchromone and the corresponding 7-*O*-methyl analogue.

RESULTS AND DISCUSSION

Lathodoratin (**1**) was initially isolated from diffusates [3] obtained when spore suspensions of *H. carbonum* were incubated for 48 hr on excised leaflets of *L. odoratus* cv Air Warden. Chromatographic examination (Si gel TLC, $CHCl_3$–MeOH, 50:1) of diffusate extracts revealed the known phytoalexins pisatin + variabilin (R_f 0.50) together with two further fluorescence-quenching compounds, lathodoratin (R_f 0.24) and methyl-lathodoratin (R_f 0.63), of unknown constitution. Although methyl-lathodoratin was only a very minor diffusate component, much larger quantities were subsequently obtained from the immature pods of *L. odoratus*. Unlike pisatin and variabilin, these new compounds reacted immediately when TLC plates were sprayed with either diazotized *p*-nitroaniline (orange/yellow) or Gibbs reagent (blue).



1 R = H
3 R = Me



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Synthesis of lathodoratin was entirely characteristic of *L. odoratus* being formed in excised or intact leaflets, cotyledons, roots, etiolated epicotyls and pod endocarps variously inoculated with fungal spores. The phytoalexin was isolated from all six cultivars tested (Air Warden, Frolic, Galaxy, Johnson's Giant Waved, Noel Sutton and Swan Lake) and additionally from four 'wild' *L. odoratus* accessions. Thorough investigation of 30 other *Lathyrus* species revealed that lathodoratin was only produced by the related *L. hirsutus* L. (3 accessions tested) [1]. Besides being formed in the presence of *Helminthosporium carbonum*, lathodoratin also accumulated when detached *L. odoratus* leaflets were inoculated with spore suspensions of *Botrytis cinerea*, *Ascochyta pisi* and *Alternaria brassicicola* or treated abiotically with actinomycin D (8×10^{-6} M) or UV (254 nm; 30 min exposure) light. Attempts to induce lathodoratin formation with aqueous $HgCl_2$ (1×10^{-4} M) were unsuccessful. On no occasion was lathodoratin (or any other *Lathyrus* phytoalexin) isolated from control (H_2O treated) tissues in more than trace amounts.

Lathodoratin was obtained from *H. carbonum*-induced leaf diffusates in quantities entirely adequate for full chemical characterisation. The UV (EtOH) spectrum closely resembled that of the 5,7-dioxygenated chromone, eugenin [4], and exhibited bathochromic shifts in the presence of NaOH, NaOAc (C-7 OH) and $AlCl_3$ (C-5 OH) (Table 1). High resolution mass spectrometry established the molecular formula as $C_{11}H_{10}O_4$. The ^1H NMR of lathodoratin was particularly informative showing a one

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Table 1. UV and ^{1}H NMR spectral data for the chromones 1–3

	Lathodoratin 1	Synthetic 2	Methyl-lathodoratin 3
Solvent/reagent		UV Spectral Maxima	
EtOH	212, 231, 252, 259, 296, 328 sh	210, 229, 250, 257, 297, 322 sh	210, 232, 251, 258, 292, 326 sh
EtOH–NaOH	210, 228 sh, 268, 335	214, 268, 342	244, 260 sh, 266, 355
EtOH–NaOAc	261, 268 sh, 332	260 sh, 268, 335	251, 258, 292, 326 sh
EtOH–$AlCl_3$	258 sh, 267, 284 sh, 312, 368	254 sh, 266, 310, 365	260 sh, 266, 284 sh, 308, 368
Signal assignment		^{1}H NMR data*	
2-H	7.83 *s*	—	7.94 *s*
3-H		6.03 *s*	—
6-H/8-H	6.14 *d*; 6.24 *d*	6.28 *d*; 6.36 *d*	6.34 *d*; 6.50 *d*
2-Et	—	1.32 *tr*/2.68 *q*	—
3-Et	1.17 *tr*/2.42 *q*	—	1.19 *tr*/2.44 *q*
7-OMe	—	—	3.86 *s*

* δ values (TMS reference); 1 and 3 in MeOH-d_4 (360 MHz), 2 in MeOH-d_4 + $CDCl_3$ (100 MHz).

proton singlet (δ 7.83), two doublets attributable to the *meta*-coupled H-6 and H-8 protons, and further signals indicative of an ethyl group (Table 1). The proton represented by the signal at δ 7.83 was readily assigned to C-2 (cf. the corresponding δ values for H-2 of isoflavones in a comparable solvent such as DMSO-d_6 with that for H-3 in either eugenin [4] or an appropriate flavone [5]). Finally, the fact that lathodoratin could be easily differentiated from the otherwise closely similar 2-ethyl isomer (**2**) by TLC, MS (see Experimental) and ^{1}H NMR (H-3 proton signal at δ 6.03; Table 1) confirmed that **1** was identical with 5,7-dihydroxy-3-ethylchromone.

The second new phytoalexin, methyl-lathodoratin (**3**), was principally isolated from *H. carbonum*-treated endocarps of immature *L. odoratus* pods; only traces of **3** were present in leaf diffusates. The phytoalexin had M^+ 220 and was spectroscopically (UV in EtOH) similar to **1** (Table 1). However, the neutral UV spectrum was unaffected by addition of NaOAc and this, together with the presence of a 3 proton ^{1}H NMR singlet (δ 3.86; OMe) suggested that methyl-lathodoratin was the 7-*O*-methyl ether of **1**. This structure was established by selective (C-7) methylation of **1** using CH_2N_2 to give a product indistinguishable (UV, MS, TLC) from natural **3**.

The synthetic 2-ethyl analogue (**2**) of lathodoratin was readily prepared by standard procedures [6] from 2,4,6-trihydroxyacetophenone, propionic anhydride and sodium propionate. However, all attempts to synthesize **1** were unsuccessful presumably because of the relatively unreactive methylene group adjacent to the carbonyl in the appropriate ketonic starting material. Thus, 2-hydroxy-4,6-dimethoxybutyrophenone failed to condense with ethyl formate in the presence of powdered sodium. Similarly, 2,4,6-trihydroxybutyrophenone was recovered unchanged after treatment with ethoxalyl chloride in pyridine [6].

The fungitoxicity of phytoalexins **1** and **3** was established by means of a standard TLC bioassay using *Cladosporium herbarum* [7]; in this test the isomer **2** proved to be essentially inactive at levels comparable with those of **1**. In a more precise bioassay against mycelial growth of *H. carbonum*, **1** had an ED_{50} of 8 μg/ml. This value is considerably lower than the corresponding ED_{50} (*ca* 39 μg/ml) recorded for the pterocarpan phytoalexin pisatin [2]. Lack of material precluded similar tests on methyl-lathodoratin. Apart from its high fungitoxicity, it seems clear that lathodoratin plays a significant role in protecting *L. odoratus* from fungal invasion since this chromone is formed in amounts which frequently exceed those of pisatin. The relative concentrations of **1** and pisatin in diffusates, leaf tissues and cotyledons of *L. odoratus* after inoculation with *H. carbonum* or *B. cinerea* are shown in Table 2. Curiously, in abiotic systems, variabilin replaced pisatin as the major pterocarpan accompanying lathodoratin. Thus, in diffusates from leaflets treated with aqueous actinomycin D (8×10^{-6} M), **1** was present at a concentration of *ca* 7 μg/ml, together with variabilin (6 μg/ml) and pisatin (*ca* 3 μg/ml). In *H. carbonum*-challenged pod endocarps (cv Johnson's Giant Waved), the levels of **1**, **3** and pisatin were 330, 106 and 158 μg/g fresh weight, respectively.

Chromone phytoalexins have not previously been isolated from any member of the family Leguminosae although some constitutive compounds of this type have been described [8, 9]. Indeed, prior to this report, chromone induction in higher plants was apparently restricted to carrot (*Daucus carota*; Umbelliferae) root tissues infected with various fungi, notably *Ceratocystis fimbriata* and *B. cinerea*; here, 5,7-dihydroxy-2-methylchromone and its 7-*O*-methyl ether (eugenin) accumulate together with two isocoumarin derivatives [4]. Neither lathodoratin nor methyl-lathodoratin has hitherto been described as a natural product. These compounds are very unusual in that both not only lack a 2-substituent but additionally possess an ethyl group at C-3. Biosynthetically, these *Lathyrus* chromones could have a polyketide origin, the extra carbon fragment required for heterocyclic ring formation being inserted at a late stage in synthesis. Their possible formation by a degradative pathway from a 2′,5′-dioxygenated isoflavone through

Table 2. Relative concentrations of lathodoratin and pisatin in diffusates, leaf tissues and cotyledons of *L. odoratus*

Time after inoculation (hr)	Phytoalexin concentration					
	Diffusate*‡ (μg/ml)		Leaf tissue*‡ (μg/g fr. wt)		Cotyledon tissue†‡ (μg/g fr. wt)	
	L	P	L	P	L	P
12	na	na	na	na	–	–
24	6	7	51	–	–	–
36	11	10	na	84	–	–
48	18	13	67	119	–	5
72	24	15	66	133	38	32
96	na	na	na	na	79	58
120	na	na	na	na	85	78

L = lathodoratin; P = pisatin; na = data not available; – = not detected.

* Inducer = *Helminthosporium carbonum.*

† Inducer = *Botrytis cinerea.*

‡ Phytoalexin concentrations were calculated using the following extinction coefficients: lathodoratin, log ε = 4.24 at 259 nm; pisatin, log ε = 3.86 at 309 nm [12].

oxidative cleavage of the aromatic B-ring should not, however, be completely ruled out as precedents for such a route have been described [10].

EXPERIMENTAL

Plant sources. Seeds of the *L. odoratus* accessions were either purchased in the Reading area (named cultivars) or obtained from various European botanic gardens.

Induction and isolation of chromone phytoalexins. (a) *Diffusates.* Si gel TLC ($CHCl_3$–MeOH, 50:1, overnight equilib.) [11] of *H. carbonum*-induced diffusate extracts (EtOAc) gave pisatin (or variabilin + pisatin when using abiotic induction), lathodoratin (**1**) and traces of methyl-lathodoratin (**3**) at R_f 0.50, 0.24 and 0.63 respectively. Chromone **1** was eluted (EtOH) and purified by TLC in xylene–Me_2CO, 4:1 (R_f 0.40) and/or *n*-pentane–Et_2O–glacial HOAc (PEA), 75:25:3 (R_f 0.26) prior to quantification and structural analysis. Eluates of the pisatin/variabilin zone were normally quantified without additional purification. (b) *Pod endocarp and leaf tissues.* Inoculated tissues were excised and thoroughly extracted with EtOH. TLC ($CHCl_3$–MeOH, 50:1) of these extracts yielded methyl-lathodoratin (**3**), **1** and pisatin, the latter compounds being treated as outlined above; compound **3** was additionally chromatographed in *n*-hexane–Me_2CO, 2:1 (R_f 0.43) followed by PEA, 75:25:3 (R_f 0.54). (c) *Cotyledons.* Imbibed seeds were treated with a spore suspension of *B. cinerea*, extracted after 3–5 days [11] and the various phytoalexins separated as described under (a) and (b).

5,7-Dihydroxy-3-ethylchromone **1** *(lathodoratin).* MS *m/e* (rel. int.): 207 (11), 206 (M^+; 100), 205 (83), 204 (13), 191 (22), 178 (16), 177 (8), 163 (14), 153 (7), 152 (7), 137 (9), 124 (11). High resolution MS gave the molecular ion at 206.0561 ($C_{11}H_{10}O_4$). For UV and ^1H NMR data see Table 1.

7-O-Methyl ether (CH_2N_2). UV and MS as given for **3**.

5-Hydroxy-7-methoxy-3-ethylchromone **3** *(methyl-lathodoratin).* MS *m/e* (rel. int.): 221 (13), 220 (M^+; 100), 219 (77), 218 (8), 205 (20), 192 (11), 191 (11), 177 (17), 167 (6), 151 (5), 149 (10), 138 (5). UV and ^1H NMR, see Table 1.

Synthesis of 5,7-dihydroxy-2-ethylchromone (**2**). 2,4,6-Trihydroxyacetophenone (2.5 g), propionic anhydride (8 ml) and sodium propionate (1 g) were refluxed together for 4 hr. The product, 5,7-dipropionyloxy-2-ethylchromone, recrystallized from EtOH as plates, mp 82–84°. Deacylation with 1N NaOH at room temp. for 3 hr gave 5,7-dihydroxy-2-ethylchromone as colourless needles from aqueous EtOH, mp 218–222°. MS *m/e* (rel. int.): 207 (12), 206 (M^+; 100), 205 (2; cf. the $M^+ - 1$ ion of chromones **1** and **3**), 178 (10), 177 (5), 163 (34), 153 (6), 152 (15), 124 (16). See Table 1 for UV and ^1H NMR data. Lathodoratin and synthetic **2** were resolved by TLC in PEA, 75:25:3 (**1**, R_f 0.26; **2**, R_f 0.15).

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