Isolation and Identification of Lathycarpin, a New Pterocarpan Phytoalexin from *Lathyrus sativus*

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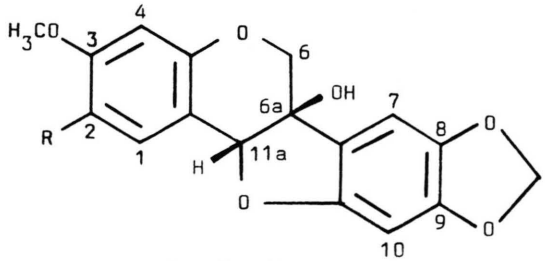
After treatment with the fungus *Helminthosporium carbonum* or aqueous copper sulphate, excised *Lathyrus sativus* leaflets produce pisatin and a second isoflavonoid phytoalexin (lathycarpin) identified as (+)-6a*R*; 11a*R*-2,3-dimethoxy-6a-hydroxy-8,9-methylenedioxypterocarpan.

Introduction

Earlier studies [1, 2] have demonstrated that a number of isoflavonoid (pterocarpan) phytoalexins [3] accumulate in excised, fungus-inoculated leaflets of species belonging to the genus *Lathyrus* (Leguminosae-Papilionoideae; tribe Vicieae). Apart from pisatin (3-methoxy-6a-hydroxy-8,9-methylenedioxypterocarpan, **1**), the most commonly encountered *Lathyrus* phytoalexin, small quantities of three other fungitoxic pterocarpans (medicarpin, maackiain and variabilin) are variously produced by several *Lathyrus* spp. [2]. In addition, nissolin and its 9-O-methyl ether (methylnissolin) are formed, together with medicarpin, by fungus-treated phyllodes of *L. nissolia* although as yet these two unusual 3,9,10-trisubstituted pterocarpans have not been discovered elsewhere in the genus [4]. Pisatin and the non-isoflavonoid chromones, lathodoratin and methyl-lathodoratin, occur as phytoalexins in tissues of *L. odoratus* (sweet pea) [5], whilst some evidence has been obtained to suggest that the Indian pulse *L. sativus* (grass pea; chickling pea) produces **1**, and a new pterocarpan (designated LS-2) of undetermined structure [1]. We have now re-examined the phytoalexin response of *L. sativus* and can confirm the ability of detached leaflets to accumulate **1** following treatment with a spore suspension of *Helminthosporium carbonum* [1, 2]. This fungus also stimulates the formation of a second antifungal isoflavonoid, probably identical with LS-2, which we propose to name *lathycarpin*. The characterisation of lathycarpin as (+)-6a*R*; 11a*R*-2,3-dimethoxy-6a-hydroxy-8,9-methylenedioxypterocarpan (**2**) is described in this report.



1: R = H

2: R = OCH_3

Results and Discussion

Pisatin and lathycarpin were initially isolated from *H. carbonum*-inoculated *L. sativus* leaflets (but not from those treated with de-ionised H_2O) using the drop-diffusate technique outlined in the Experimental section. This procedure was very tedious, however, and during later stages of the project was replaced by a modified diffusion method (see Experimental) in which leaflets were floated for 10 – 12 days on aqueous $CuSO_4$. After this period, the $CuSO_4$ solution was shaken with EtOAc to remove all isoflavonoid compounds, subsequent Si gel TLC purification of the organic phase yielding both **1** and **2** in milligram quantities. Pisatin was identified by UV, MS and Si gel TLC comparison with authentic material obtained from *Pisum sativum* [6]. Other compounds currently recognised as *Lathyrus* phytoalexins (see Introduction) were not produced by *L. sativus*.

The UV (EtOH) spectrum of chromatographically pure lathycarpin (λ max: 212, 235 sh, 303 nm) closely resembled that of 2,3-dimethoxy-8,9-methylenedioxypterocarpan (λ max: 212, 235 sh, 304 nm), and could be distinguished from those of spectroscopically similar 3,4-dioxygenated pterocarpans (*e.g.* 4-methoxymaackiain) by the absence of two

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slight shoulders between 270 and 290 nm [7]. Aqueous NaOH had no effect on the neutral UV spectrum of lathycarpin, whereas addition of conc. HCl resulted in rapid (1 min) dehydration to afford the corresponding pterocarpene (λ max: 353 and 368 nm). This acid-mediated spectroscopic change is characteristic of pterocarpans (*e.g.* pisatin and variabilin) containing a labile C-6a OH group [7, 8]. Further evidence for tertiary hydroxylation of **2** (High Resolution MS, M^+ 344.0892; $C_{18}H_{16}O_7$; 55%) was provided by the mass spectrum which exhibited a major fragment at *m/z* 326.0803 ($M^+ - H_2O$; 100%) corresponding to $C_{18}H_{14}O_6$ (*cf.* **1**, M^+ 314 (28%), *m/z* 296 ($M^+ - H_2O$; 100%)). Other prominent ions in the MS of **2** appeared at *m/z* 345 ($M^+ + 1$; 11%), 329 ($M^+ - CH_3$; 5%), 327 (22%), 325 (31%), 316 (16%), 311 (16%), 301 (6%), 285 (13%), 283 (7%), 199 (9%), 163 (26%), 151 (9%), 149 (14%) and 133 (10%). On TLC plates sprayed with chromotropic acid reagent, lathycarpin gave a purple-pink colour indicative of methylenedioxy substitution [9, 10].

In view of its co-occurrence with **1**, the above data suggested that lathycarpin was a methoxy-pisatin, and this was confirmed by ^{1}H NMR spectroscopy (Table I) which revealed that pisatin and lathycarpin differed only in that the latter compound possessed an extra methoxyl substituent, and lacked a C-2 proton. The new phytoalexin is thus 2-methoxypisatin (2,3-dimethoxy-6a-hydroxy-8,9-methylenedioxypterocarpan, **2**), a structure supported by the loss, relative to pisatin, of significant coupling in the H-1 and H-4 signals and by the upfield shift of H-1 consequent upon *ortho*-oxygenation [11].

Table I. ^{1}H NMR data for pisatin and lathycarpin[a].

Proton	Pisatin (**1**)	Lathycarpin (**2**)
H-1	7.36 d, 1H (J = 8.5 Hz)	6.96 s, 1H
H-2	6.63 q, 1H (J = 8.5, 2.5 Hz)	–
H-4	6.39 d, 1H (J = 2.5 Hz)	6.43 s, 1H
H-6ax/eq	4.11 s, 2H	4.06 d, 2H (J = 1.5 Hz)
H-7	6.89 s, 1H	6.89 s, 1H
H-10	6.35 s, 1H	6.36 s, 1H
H-11a	5.29 s, 1H	5.26 s, 1H
O–CH_2–O	5.93 q, 2H (J = 2.9, 0.9 Hz)	5.92 q, 2H (J = 2.9, 0.9 Hz)
OCH_3	3.75 s, 3H	3.76 s, 3H 3.78 s, 3H

[a] Solvent, $(CD_3)_2CO$; chemical shifts are given as δ values (TMS reference); figures in parentheses refer to coupling constants.

Lathycarpin has $[\alpha]_{589\,nm}^{21}$ + 232° (1.2 mg in 1 ml MeOH) and can therefore be assigned the 6a*R*; 11a*R* absolute configuration depicted in **2** [12]. Pisatin from *L. sativus* is also dextrorotatory, $[\alpha]_{589\,nm}^{21}$ + 292° (1.05 mg in 1 ml MeOH).

In TLC plate bioassays [13, 14], lathycarpin (20 µg) was clearly inhibitory to the spore germination/germ tube growth of *Cladosporium herbarum*. More precise measurements against radial mycelial growth of *H. carbonum* [14] gave an ED_{50} value of about 45 µg/ml, comparable with that reported earlier for pisatin [15]. On average, diffusates from excised *L. sativus* leaflets treated with a spore suspension of *H. carbonum* contained pterocarpans **1** and **2** at concentrations of 18 and approx. 30 µg/ml (based on ε = 7244 at 309 nm for **1** [8]) respectively. Abiotic induction using droplets of aqueous $CuSO_4$ was less efficient yielding (over four experiments) only 5–14 µg/ml of **1**, and 8–20 µg/ml of **2**. Leaf diffusates invariably contained more lathycarpin than pisatin.

Experimental

Plant material. Seeds of an unnamed variety of *Lathyrus sativus* L. (supplied by Dr. L. J. G. Van der Maesen, I.C.R.I.S.A.T., Hyderabad, India) were sown in John Innes No. 1 compost, and the resulting plants grown (20 – 24 °C) for about 6 weeks before individual leaflets were removed for treatment with either *H. carbonum* or aqueous $CuSO_4$. At later stages of growth, the plants were routinely deflowered to encourage leaf production.

Isolation and purification of pisatin (**1**) *and lathycarpin* (**2**). a) *Standard drop-diffusate technique.* 2–5 droplets of aqueous $CuSO_4$ (0.25 g/100 ml de-ionised H_2O, plus 0.5 ml Tween 20 as a wetting agent) or *H. carbonum* spore suspensions (also containing Tween 20) [16, 17] were placed along the upper surface of excised *L. sativus* leaflets floating on tap H_2O. After incubation (20 °C) for 72 h, the droplets (diffusate) were collected, extracted (× 3) with EtOAc, and the pooled organic fractions reduced to dryness (*in vacuo*, 40 °C). Si gel TLC (Merck, F-254,

layer thickness 0.25 mm) of the extracts ($CHCl_3$ – MeOH, 50:1, 12 h equilibration at 20 °C) gave pisatin + lathycarpin as a broad, fluorescence-quenching band (R_F 0.67). This band was removed and the pterocarpans eluted (EtOH) prior to separation by Si gel TLC in *n*-hexane – acetone, 2:1, 1 h equilibration [1] (**1**, R_F 0.37; **2**, R_F 0.28). Final TLC purification of pisatin and lathycarpin was undertaken using *n*-pentane-Et_2O-glacial HOAc, 75:25:3, 1 h equilibration (**1**, R_F 0.31; **2**, R_F 0.10). b) *Modified diffusion technique.* In a typical experiment about 600 *L. sativus* leaflets were cut into short (1 – 3 cm) sections and floated (10 – 12 days) on aqueous $CuSO_4$ (about 2 l; see above for composition) in transparent-plastic sandwich boxes covered with clear, cling-film food wrap. The leaf material was ultimately discarded, and the $CuSO_4$ solution shaken (× 2) with equal volumes of EtOAc. Si gel TLC of the organic phase was undertaken as described above. Pterocarpan yields ranged from 0.5 – 1.1 mg for pisatin, and from approx. 1.2 – 1.9 mg for lathycarpin.

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