

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

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Z. Naturforsch. **37c**, 724 – 726 (1982);
received March 22, 1982

Leguminosae, *Lathyrus*, Isoflavonoids, Pterocarpans, Phytoalexins

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 (+)-6aR; 11aR-2,3-dimethoxy-6a-hydroxy-8,9-methylenedioxypterocarpans.

Introduction

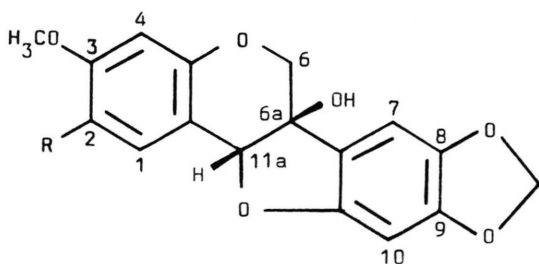
Earlier studies [1, 2] have demonstrated that a number of isoflavonoid (pterocarpans) phytoalexins [3] accumulate in excised, fungus-inoculated leaflets of species belonging to the genus *Lathyrus* (Leguminosae-Papilionoideae; tribe Viciaeae). Apart from pisatin (3-methoxy-6a-hydroxy-8,9-methylenedioxypterocarpans, **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, to-

gether 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 pterocarpans (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 (+)-6aR; 11aR-2,3-dimethoxy-6a-hydroxy-8,9-methylenedioxypterocarpans (**2**) is described in this report.

Results and Discussion

Pisatin and lathycarpin were initially isolated from *H. carbonum*-inoculated *L. sativus* leaflets (but not from those treated with de-ionised H₂O) 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 CuSO₄. After this period, the CuSO₄ 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-methylenedioxypterocarpans (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



- 1: R = H
2: R = OCH₃

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0341-0382/82/0700-0724 \$ 01.30/0



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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 pterocarpanes (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 1H 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-methoxypisa-

tin (2,3-dimethoxy-6a-hydroxy-8,9-methylenedioxy-pterocarpan, **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].

Lathycarpin has $[\alpha]_{589\text{ nm}}^{21} + 232^\circ$ (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, $[\alpha]_{589\text{ nm}}^{21} + 292^\circ$ (1.05 mg in 1 ml MeOH).

In TLC plate bioassays [13, 14], lathycarpin (20 μ g) 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 μ g/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 pterocarpanes **1** and **2** at concentrations of 18 and approx. 30 μ g/ml (based on $\epsilon = 7244$ 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 μ g/ml of **1**, and 8–20 μ g/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 ($\times 3$) with EtOAc, and the pooled organic fractions reduced to dryness (*in vacuo*, 40 °C). Si gel TLC (Merck, F-254,

Table I. 1H 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	$\left\{ \begin{array}{l} 3.76 \text{ s, 3H} \\ 3.78 \text{ s, 3H} \end{array} \right.$

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

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 pterocarpan 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-Et₂O-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 ulti-

mately discarded, and the CuSO_4 solution shaken ($\times 2$) 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.

Acknowledgements

Part of the work described in this communication was undertaken (by J.L.I.) at the National Research Centre (Cairo) during an exchange visit sponsored by the Royal Society/Egyptian Academy of Sciences. Financial assistance (to J.L.I.) from the S.E.R.C. is also acknowledged. We thank Dr. H. Wong for determination of ^1H NMR spectra.

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