Phytochemistry, 1973, Vol. 12, pp. 2957 to 2959. Pergamon Press. Printed in England.

INDUCTION AND IDENTIFICATION OF SATIVAN AND VESTITOL AS TWO PHYTOALEXINS FROM *LOTUS CORNICULATUS*

MORRIS R. BONDE, ROY L. MILLAR and JOHN L. INGHAM*
Department of Plant Pathology, Cornell University, Ithaca, NY 14850, U.S.A.

(*Received* 10 *June* 1973. *Accepted* 2 *July* 1973)

Key Word Index—*Lotus corniculatus*; Leguminosae; birdsfoot trefoil; sativan; vestitol; isoflavans; phytoalexins.

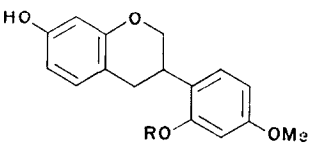
Abstract—Two phytoalexins, (−)-sativan, previously named sativin, [(−)-7-hydroxy-2′, 4′-dimethoxyisoflavan], and (−)-vestitol, [(−)-7,2′-dihydroxy-4′-methoxyisoflavan], were induced by a spore suspension of *Helminthosporium turcicum* Pass. to accumulate in leaves of birdsfoot trefoil (*Lotus corniculatus* L.).

INTRODUCTION

SEVERAL plant species respond to inoculation with various fungi by producing fungitoxic compounds that have been designated phytoalexins and postulated to have a primary role in disease resistance.

Many of the phytoalexins isolated to date are from species in the Lotoideae sub-family of the Leguminosae, and of these, most are isoflavanoids. A compound determined to be *R*-(−)-7-hydroxy-2′,4′-dimethoxyisoflavan (I), which also functions as a phytoalexin, was isolated recently from alfalfa (*Medicago sativa* L.); this compound was given the trivial name sativin.[1]

The name sativin, however, was pre-empted by the assignment of it by Tinline and Samborski[2] in 1959 to an unidentified pigment produced by *Cochliobolus sativus* (Ito and Kurib.) Drechs. ex Dastur and earlier to a specific protein.[3] We therefore propose that the trivial name sativan henceforth be used for the isoflavan *R*-(−)-7-hydroxy-2′,4′-dimethoxyisoflavan.



(I) R = Me
(II) R = H

We report herein the isolation of the two isoflavans (−)-sativan and (−)-vestitol from another species in the Lotoideae, namely *Lotus corniculatus* L. (birdsfoot trefoil). Both sativan and vestitol are induced in leaves of birdsfoot trefoil in response to inoculations with fungi and both may act as phytoalexins.

* Present address: Department of Botany, The University, Reading RG6 2AS, Berkshire, England.

[1] INGHAM, J. L. and MILLAR, R. L. (1973) *Nature* **242**, 125.
[2] TINLINE, R. D. and SAMBORSKI, D. J. (1959) *Mycologia* **51**, 77.
[3] HANKE, M. T. (1925) *J. Biol. Chem.* **66**, 489.

RESULTS

Sativan, [(−)-7-hydroxy-2′,4′-dimethoxyisoflavan], and vestitol, [(−)-7,2′-dihydroxy-4′-methoxyisoflavan] (II), were induced to accumulate in leaves of *Lotus corniculatus* L. (birdsfoot trefoil), cv. Viking, by means of the drop-diffusate technique.[4] Drops of a spore suspension of *Helminthosporium turcicum* Pass. were applied to trefoil leaves and 48 hr later the drops were removed, the spores separated by centrifugation, and the supernatant partitioned against EtOAc. The EtOAc extract was chromatographed on silica-gel TLC plates in a non-saturated atmosphere with Et_2O–hexanes (5:1) as the solvent system. R_f values were 0·7–0·8 and 0·4–0·6 for sativan and vestitol, respectively. Vestitol, however, always was eluted and chromatographed again in the same solvent system to ensure that it was not contaminated with a compound (unknown I) that migrated just behind it. No other solvent system was more effective in separating vestitol and unknown I. On plates sprayed with *p*-nitroaniline reagent, sativan appeared as an intense yellow spot, vestitol as a dark brown spot, and unknown I as a light brown spot.

Vestitol and sativan from trefoil were compared spectroscopically and chromatographically with authentic (+)-vestitol and (−)-sativan respectively. The UV data and colors formed upon reaction with *p*-nitroaniline reagent and R_f values, showed the compounds compared were indistinguishable. MS data for sativan were essentially identical with those previously reported by Ingham and Millar[1] for sativan from alfalfa ($M^+ = 286$, prominent fragments at *m/e* 164, 151, 149, 121) and the data for (+)-vestitol and vestitol from trefoil were also similar ($M^+ = 272$, prominent fragments at *m/e* 150, 138, 137, 135). UV absorption spectra for vestitol, regardless of the source had λ_{max}^{EtOH} (log ϵ) 208 (4·39), 223 (4·02), 281 (3·63) and 285 (3·62), and that for sativan, regardless of its source, was the same as that reported by Ingham and Millar,[1] λ_{max}^{EtOH} 208, 227, 280, 284 and 290 (sh.). Addition of 0·2 N NaOH to vestitol gave $\lambda_{max}^{EtOH+NaOH}$ 216, 242 (sh.) and 296, and to sativan gave $\lambda_{max}^{EtOH+NaOH}$ 216, 248 (sh.), 280 (sh.), 285 and 296 (sh.).

Sativan from trefoil has essentially the same optical activity $[\alpha]_D^{22} - 22°$ (*c* 2, mg/ml MeOH) as that reported by Ingham and Millar[1] for sativan from alfalfa. For vestitol, however, whereas the authentic sample was dextrorotatory (+21·5°),[1] trefoil vestitol was laevorotatory (−8°).

Vestitol (based on A at 281 nm, $\epsilon = 4233$) accumulated in the spore suspension to 92–176 μg/ml and sativan (A at 284 nm, $\epsilon = 4182$) to 90–125 μg/ml of spore suspension applied to the leaves. Ratios of (−)-vestitol to sativan in the spore suspension varied with experiments from 1:1 to 1·7:1.

Vestitol and sativan could be also extracted from the leaf tissue directly below the drops by extracting with 95% EtOH, followed by TLC on silica-gel plates with C_6H_6–MeOH (9:1, non-saturated atm.); R_f values were 0·3–0·4 and 0·7–0·8 for vestitol and sativan, respectively. Each compound was further purified by TLC using the Et_2O-hexanes solvent system. Vestitol and sativan concentrations in these tissues varied from 551–826 and 1615–1974 μg/g fresh wt of tissue, respectively. The ratio of vestitol to sativan varied from 1:5 to as high as 1:2 in inoculated tissue.

Bioassays for (−)-vestitol and sativan, 0–60 μg/ml of Czapek-Dox agar, demonstrated that both compounds are highly inhibitory to mycelial growth of *H. turcicum*. The amount of each phytoalexin required to inhibit mycelial growth to 50% of that obtained for controls in 48 hr was *ca.* 15 μg/ml medium for sativan and *ca.* 35 μg/ml for (−)-vestitol.

[4] HIGGINS, V. J. and MILLAR, R. L. (1968) *Phytopathology* **58,** 1377.

It has been suggested that alfalfa sativan may derive from 6*aR*, 11*aR*-(−)-demethylhomopterocarpin [(−)-medicarpin] and that (−)-vestitol may be an intermediate in this sequence.[1] All known (−)-pterocarpanoids have the 6*aR*, 11*aR* absolute configuration.[5] Apparently, however, there is no such correlation between direction of optical rotation and absolute configuration with the isoflavans. The optically active isoflavans (−)-duartin, (−)-mucronulatol, and (−)-equol, in addition to (+)-vestitol, possess the 3*S* configuration.[6] Because (+)-vestitol is known to have the 3*S* configuration, (−)-vestitol, therefore, must possess the 3*R* configuration. We suggest that (−)-sativan also possesses the 3*R* configuration on the basis that conversion of a ring OH to a OMe group is known not to affect the direction of optical rotation.[7]

Acknowledgements—We thank Professors W. D. Ollis and O. R. Gottlieb for samples of (+)-vestitol and Dr. J. W. Serum for assistance with mass spectral analyses. We also greatly appreciate advice and assistance provided to us by Drs. T. M. Jones and H. D. VanEtten.

[5] PELTER, A. and AMENECHI, P. I. (1969) *J. Chem. Soc.* **C,** 887.
[6] KUROSAWA, K., OLLIS, W. D., REDMAN, B. T., SUTHERLAND, I. O., GOTTLIEB, O. R. and MAGALHÃES ALVES, H. (1968) *Chem. Commun.* 1265.
[7] VERBIT, L. and CLARK-LEWIS, J. W. (1968) *Tetrahedron* **24,** 5519.