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(−)-PISATIN, AN INDUCED PTEROCARPAN METABOLITE OF ABNORMAL CONFIGURATION FROM *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; phytoalexin; pterocarpan; pisatin; maackiain; biosynthesis.

Abstract—Pea (*Pisum sativum*) tissues, on treatment with aqueous $CuCl_2$ synthesize the 6a-hydroxypterocarpan phytoalexin (+) - (6aR, 11aR) - pisatin. By supplying (−) - (6aR, 11aR) - maackiain during this induction process, significant quantities of (−) - (6aS, 11aS) - pisatin are produced, immature pods being most effective. Pisatin levels are considerably reduced when compared with the normal induction process, but may contain as much as 92% (−)-pisatin. This confirms that the 6a-hydroxylation of maackiain during the biosynthesis of pisatin must proceed with retention of configuration at C-6a.

INTRODUCTION

The 6a-hydroxylation of pterocarpans is an established conversion in the pathway of microbial metabolism for some pterocarpan phytoalexins[1]. Thus, phaseollin [2, 3], medicarpin [4, 5] and maackiain[5] are all capable of being metabolized to 6a-hydroxy derivatives, prior to further modification. Although the chirality of these products was not reported, it is logical to assume that this hydroxylation occurs with retention of configuration at C-6a and this has been confirmed in some instances[6]. Any inversion process would necessitate the additional inversion at C-11a since pterocarpans have a *cis*-fused ring system[7, 8]. Recent feeding experiments in the garden pea, *Pisum sativum*[9], have demonstrated a similar 6a-hydroxylation step during the biosynthesis of the phytoalexin (+) - (6aR, 11aR) - pisatin (**4**)* from maackiain via (+) - (6aR, 11aR) - 6a - hydroxymaackiain (**3**). Experiments utilizing (±) - [^{14}C]maackiain had shown excellent incorporation of activity into (+)-pisatin, and it was anticipated that only (+) - (6aS, 11aS) - maackiain (**2**)* had served as a precursor. Subsequent studies, however, established that (−) - (6aR, 11aR) - [^{14}C]maackiain (**5**) was also well-incorporated into pisatin [Dewick, P. M. and Banks, S. W., unpublished results]. The results reported here demonstrate that this isomer is almost certainly incorporated into (−) - (6aS, 11aS) - pisatin (**6**), with retention of configuration at C-6a.

RESULTS AND DISCUSSION

Feeding experiments with radioactive labelled precursors[9] employed either young pods or 4-day-old seedlings of *Pisum sativum*. Phytoalexin synthesis was initiated by treating the roots of seedlings with dilute aqueous cupric chloride, or by injecting a similar solution into the pods. After 12 hr, the $CuCl_2$ was replaced by a solution of the appropriate labelled compound, and the plant tissue was worked-up after a further 36 hr metabolism period. In both plant systems, (−) - [^{14}C]maackiain was incorporated into pisatin (incorporations 1–10%) [Dewick, P. M. and Banks, S. W., unpublished results]. A check of the optical activity of the pisatin produced indicated significantly lower $[\alpha]_D$ values than for (+)-pisatin ($[\alpha]_D = +288°$), particularly in those samples derived from the immature pod tissue, suggesting a partial synthesis of (−)-pisatin (**6**) in addition to (+)-pisatin (**4**). Accordingly, a more extensive study of the accumulation of pisatin from (−)-maackiain was initiated.

Young pods (*ca* 6, 10–15 g) were injected and filled with 3×10^{-3} M aq. $CuCl_2$, then left for 12 hr under conditions of high humidity. A solution of unlabelled (−)-maackiain in 2-methoxyethanol-Tween 20-H_2O was then injected into the pods, which had by then absorbed all the $CuCl_2$ solution. Pisatin was isolated after a further 36 hr, purified by repeated TLC, and its optical rotation measured. The results are shown in Table 1.

In virtually all cases, pisatin of lower positive specific rotation or of negative specific rotation was isolated. However, total pisatin synthesis was considerably reduced compared with the standard induction process (*ca* 160–200 μg/g). Older pod tissue appeared to be less effective at producing detectable

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*Pterocarpans having a large negative $[\alpha]_D$ are assigned the (6aR, 11aR) configuration[10]; (−) - 6a - hydroxypterocarpans are regarded as having the same absolute configuration[11], but the priority rules give a (6aS, 11aS) nomenclature.

quantities of (−)-pisatin then immature flat pods, although radioactivity from (−) - [^{14}C]maackiain was still incorporated. Also, increasing the amount of (−)-maackiain fed to the pods strangely had little reproducible effect on (−)-pisatin content. From the specific rotation measurements, the proportion of (−)-pisatin produced could be estimated; figures as high as 92% were observed.

That the compound produced was in fact pisatin was clearly established from its ^{1}H NMR spectrum, which was in all ways identical to that of authentic (+)-pisatin. Other spectral and chromatographic data were also identical. The addition of (−)-maackiain to the hypersensitized (+)-pisatin-synthesizing pea tissue thus also initiates synthesis of (−)-pisatin. The 6a-hydroxylation step must therefore proceed with retention of configuration at C-6a.

(+)-Pisatin is unusual amongst pterocarpan phytoalexins in having the opposite absolute configuration to all other reported examples. Other (+) - 6a - hydroxypterocarpans and (+) - pterocarpans are known[1, 6, 12], but not as phytoalexins. It is also unusual that other pterocarpan phytoalexins isolated from *P. sativum* have the opposite (normal) configuration. Thus (−) - (6aR, 11aR) - maackiain [13], (−) - (6aR, 11aR) - 3 - hydroxy - 2, 9 - dimethoxypterocarpan[14], (−) - (6aR, 11aR) - 2,3,9 - trimethoxypterocarpan[14] and (−) - (6aR, 11aR) - 4 - hydroxy - 2,3,9 - trimethoxypterocarpan[14] have all been identified in infected pea tissue. The last three were thought to be microbial transformation products of other pea metabolites since they were formed only in the *P. sativum–Fusarium solani* f. sp. *pisi* interaction[15]. Recently they have been identified in $CuCl_2$-treated pea seedlings along with afrormosin, a likely isoflavone precursor[16]. We have confirmed that maackiain produced as a minor phytoalexin on $CuCl_2$ treatment of pea seedlings indeed has the (6aR, 11aR) configuration ($[\alpha]_D = -229°$).

P. sativum utilizes the planar 2′,7 - dihydroxy - 4′,5 - methylenedioxyisoflavone (**1**) to synthesize maackiain and hence pisatin[9, 17], and must carry out stereospecific reductions on this isoflavone to give either (−)-maackiain which may be extracted from the plant tissue, or (+)-maackiain which must be almost exclusively channelled into (+)-pisatin synthesis. If the pool size of (+)-maackiain is extremely small, the maackiain obtained would appear to be of (−) - (6aR, 11aR) configuration within the experimental limits of optical rotation measurements, but under 'normal' hypersensitized conditions, it would seem that the synthesis of (+)- and (−)-maackiain in pea is compartmentalized. This is in keeping with the observation that the mechanism of isoflavone reduction to (+)- and (−)-pterocarpan products appears to be different[18]. A number of other plants are known that constitutively accumulate pterocarpans in both enantiomeric forms [1, 12].

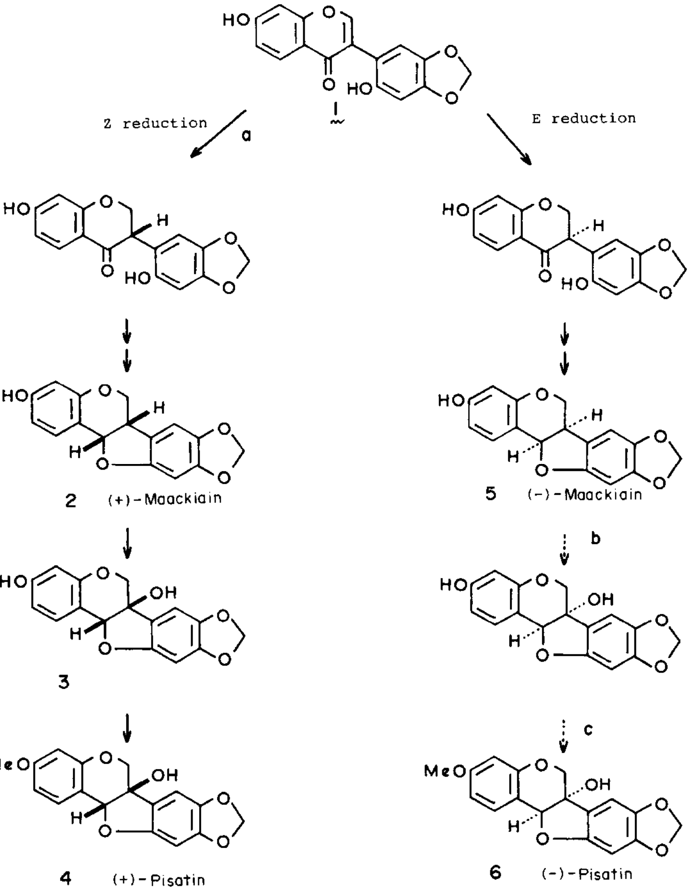
By supplying endogenous (−)-maackiain, the synthesis of (−)-pisatin is stimulated, but how this is achieved at the enzymic level must await detailed investigation. Although (+)- and (−)-pterocarpans have the same overall shape, and their molecular models can be superimposed [19], the 6a-positions are not coincident, but separated by two bonds. It is unlikely, therefore, that the 6a-hydroxylating enzyme is non-stereoselective and capable of accepting both isomers as substrates. The experimental data are best accommodated if further induced enzymes catalysing the hydroxylation and methylation reactions **b** and **c** respectively are produced by the addition of excess (−)-maackiain (see Scheme 1). In very young pod tissue, feed-back repression of the enzymes of the 'normal' induction process relative to the new enzymes occurs and a reduced level of pisatin synthesis, but mainly of (−)-pisatin, is observed. Even though relatively large amounts of (−)-maackiain were added in these experiments, considerable dilution of radioactive label was noticed, so the tissue must also have been synthesizing greatly increased amounts of (−)-maackiain *de novo*. It is likely then that the repression occurs after the planar isoflavone stage, and it is probable that the reduction step **a** could be the one repressed.

Table 1. Pisatin production by *P. sativum* pods induced with $CuCl_2$ and fed (−)-maackiain

(−)-Maackiain fed (mg/g)	Pisatin produced (μg/g)	$[\alpha]_D$ (EtOH)	%(−)-pisatin*	[^{14}C] incorporation (%)
Immature pods				
0.008	40	−107°	69	3.6
0.010	32	−41°	57	—
0.013	55	+82°	36	8.9
0.018	98	−245°	92	7.5
0.28	26	−217°	88	—
0.34†	80	+196°	16	—
Mature pods				
0	161	+288°	0	—
0.008	97	+207°	14	1.3
0.009	167	+265°	4	1.7
0.12	235	+263°	4	—
0.14	214	+239°	8	—

*Calculated using $[\alpha]_D$ +288° for (+)-pisatin.

†In this experiment (−)-maackiain was fed 6 hr before induction.



Scheme 1. Biosynthesis of (+)- and (−)-pisatin in *P. sativum*.

EXPERIMENTAL

General. Pea pods were obtained from plants (cvs Little Marvel and Kelvedon Wonder) grown under normal garden conditions. TLC was carried out using 0.5 mm layers of Si gel (Merck TLC-Kiesel gel 60GF$_{254}$). Me$_2$CO (analar) was used for elution of TLC zones.

Pisatin production in pea pods. Pea pods (*ca* 6) were induced by injecting and filling with aq. CuCl$_2$ (3×10^{-3} M), then leaving for 12 hr. Immature pods were essentially flat, whereas mature pods were expanded, though containing still immature peas. (−)-Maackiain (0.2–5 mg) was dissolved in 2-methoxyethanol (0.2 ml), then in Pi buffer (0.1 M, pH 7.0, 2 ml), Tween 20 (1 drop), and sufficient H$_2$O to fill the pods (cf. CuCl$_2$ vol. used) was added. This soln was injected into the pods, which were left for 36 hr in a 12 hr light–12 hr dark regimen under high humidity. The pods were homogenized in a mortar with powdered glass and a little H$_2$O, then poured into cold EtOH (100 ml), stirred and left at room temp. for 10 min. The mixture was filtered, and the solids re-extracted with cold EtOH (2 × 100 ml). The combined extracts were evaporated to dryness, treated with H$_2$O (50 ml), and extracted with Et$_2$O (100 ml, then 3 × 50 ml). The evaporated Et$_2$O extract was separated by TLC (C$_6$H$_6$–EtOAc–propan-2-ol, 90:10:1), and the pisatin band purified further by TLC using the solvent systems CHCl$_3$–MeOH (25:1); hexene–Me$_2$CO (2:1); hexane–EtOAc–MeOH (60:40:1). Pisatin content was assayed by its UV absorption at 307 nm, lit. [20] log ϵ 3.86. UV $\lambda_{\max}^{\mathrm{EtOH}}$ nm: 279, 284, 307; ^{1}H NMR (250 MHz, CDCl$_3$, TMS): δ 7.37 (1H, *d*, $J = 8.5$ Hz, H-1), 6.80 (1H, *s*, H-7), 6.65 (1H, *dd*, $J = 8.5$, 2.4 Hz, H-2), 6.45 (1H, *d*, $J = 2.4$ Hz, H-4), 6.39 (1H, *s*, H-10), 5.94 (1H, *d*, $J = 1.2$ Hz, O–CH$_2$–O), 5.90 (1H, *d*, $J = 1.2$ Hz, O–CH$_2$–O), 5.28 (1H, *br s*, H-11a), 4.18 (1H, *dd*, $J = 11.6$, 0.6 Hz, H-6eq), 4.01 (1H, *d*, $J = 11.6$, H-6ax), 3.77 (3H, *s*, OMe).

(−) - (6a*R*, 11a*R*) - *Maackiain.* (−)-Maackiain was isolated from red clover (*Trifolium pratense*) roots essentially as described [21]. Homogenized roots were allowed to autolyse for 2 days at room temp. The mixture was poured into hot EtOH, filtered, and the residue re-extracted twice more. The filtrate was evaporated, treated with H$_2$O and extracted with Et$_2$O (× 3). (−)-Maackiain was isolated by TLC (hexane–EtOAc, 3:2), then recrystallized twice from aq. MeOH. mp 179–180°, lit. [21] 179.5–180°; $[\alpha]_D - 227°$ (EtOH; *c* 0.723), lit. [21] −214°, [13] −220°. UV $\lambda_{\max}^{\mathrm{EtOH}}$ nm: 279, 284, 308; ^{1}H NMR (250 MHz, (CD$_3$)$_2$CO, TMS): δ 7.30 (1H, *d*, $J = 8.5$ Hz, H-1), 6.89 (1H, *s*, H-7), 6.55 (1H, *dd*, $J = 8.5$, 2.4 Hz, H-2), 6.40 (1H, *s*, H-10), 6.36 (1H, *d*, $J =$ 2.4 Hz, H-4), 5.93 (1H, *d*, $J = 1.2$ Hz, O–CH$_2$–O), 5.90 (1H, *d*, $J = 1.2$ Hz, O–CH$_2$O), 5.49 (1H, *d*, $J = 6.7$ Hz, H-11a), 4.28 (1H, *m*, approx. *dd*, $J = 9.6$, 3.3 Hz, H-6eq), 3.63 (1H, approx. *t*, $J = 10.0$ Hz, H-6ax), 3.56 (1H, *m*, H-6a).

Isolation of (+) - (6a*R*, 11a*R*) - *pisatin and* (−) - (6a*R*, 11*aR*) - *maackiain from pea seedlings*. 5-Day-old pea seedlings (cv Kelvedon Wonder, 500 g) grown in moist Vermiculite at 25° with a 12 hr light–12 hr dark cycle, were treated via the roots with aq. $CuCl_2$ (3×10^{-3} M) for 18 hr. They were transferred to distilled H_2O and grown on for 48 hr prior to extraction as with the pods above. Pisatin and maackiain were separately eluted after the first TLC system (C_6H_6 – EtOAc – propan - 2 - ol, 90:10:1), and each was purified further by TLC using the solvent systems above. Maackiain was finally purified by gel filtration (Sephadex LH-20, column 25 × 1.5 cm, EtOH, flow rate 12 ml/hr), being collected in the 68–81 ml eluate. Yields (by UV): (+)-pisatin, 50 mg, $[\alpha]_D + 288°$ (EtOH; *c* 0.912) lit. [20] $[\alpha]_{578} + 280°$; (−)-maackiain, 1.1 mg, $[\alpha]_D - 229°$ (EtOH; *c* 0.112).

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