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(+)-MAACKIAIN AND (+)-MEDICARPIN AS PHYTOALEXINS IN *SOPHORA JAPONICA* AND IDENTIFICATION OF THE (−) ISOMERS BY BIOTRANSFORMATION

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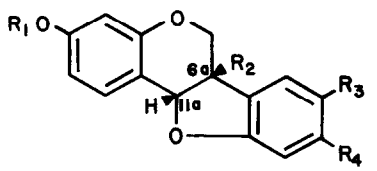
Key Word Index—*Sophora japonica*; Leguminosae; *Nectria haematococca*; *Fusarium solani*; phytoalexins; pterocarpans; optical isomers; medicarpin; maackiain; biosynthesis; catabolism.

Abstract—Leaves of *Sophora japonica* produced the pterocarpanoid phytoalexins medicarpin and maackiain in response to inoculation with the fungus *Helminthosporium carbonum*. The optical rotation of the phytoalexin samples indicated that the (+) enantiomers were the major optical isomers produced along with a small amount of the (−) enantiomers. This appears to be the first case in which (+) enantiomers of 6aH pterocarpans have been reported as phytoalexins. The presence of (−) enantiomers in the phytoalexin samples was substantiated by stereospecific biotransformations with the fungus *Nectria haematococca*. The fungus converted optically pure standards of (−)-medicarpin and (−)-maackiain to their (−)-6a-hydroxy derivatives but no 6a-hydroxy derivatives accumulated when (+)-medicarpin and (+)-maackiain were used as the substrates. When the phytoalexin samples from inoculated *S. japonica* leaves were metabolized by *N. haematococca*, (−)-6a-hydroxypterocarpans were produced confirming the presence of the (−) enantiomers of medicarpin and maackiain in the phytoalexin samples. Similar analysis of preformed maackiain in root tissue of *S. japonica* indicated the presence of both optical isomers, as has been reported previously.

INTRODUCTION

Pterocarpans are isoflavanoids with the basic ring structure illustrated in **1**–**5**. Since these compounds possess asymmetric carbons at 6a and 11a four diastereomeric structures are possible. However, the 6a–11a junction has been established to be a *cis*-fusion of the two heterocyclic rings [1–4] resulting in only one pair of naturally occurring enantiomers [5, 6]. All (+) isomers are proposed to have the absolute configuration illustrated while (−) isomers are believed to have the opposite configuration [7].

Pterocarpans have attracted considerable attention because they are produced as phytoalexins by a number of Leguminosae in response to microbial infection [6]. However, pterocarpans are also found in heartwood and bark and, as glycosides, in root tissue of uninfected plants [5]. Maackiain (**1**) and medicarpin (**2**) are two pterocarpans found in such diverse sources and both (+) and (−) enantiomers of these pterocarpans have been isolated [5, 6, 8, 9]. Both enantiomers also have antimicrobial activity [10, 11]. Commonly medicarpin and maackiain occur together when these pterocarpans are produced as phytoalexins. However, as has been previously observed with almost all the pterocarpans, only the (−) enantiomers have been detected as phytoalexins [6].



1 R_1, R_2 = H; R_3, R_4 = OCH_2O
2 R_1, R_2, R_3 = H; R_4 = OMe
3 R_1 = H; R_2 = OH; R_3, R_4 = OCH_2O
4 R_1 = Me; R_2 = OH; R_3, R_4 = OCH_2O
5 R_1, R_3 = H; R_2 = OH; R_4 = OMe
(The same notation is used for (+) and (−) pterocarpans **1**–**5**).

Both (+)- and (−)-maackiain have been detected as performed constituents in the roots of the Leguminous tree *Sophora japonica* [9]. The free form of maackiain was a racemate while (+) maackiain was found as the aglycone of the glycoside sophojaponicin. The purpose of the present study was to determine if *S. japonica* would produce maackiain and/or medicarpin as phytoalexins and, if so, to determine what optical form would be produced by a plant with the capacity to synthesize both enantiomers*. We also reinvestigated the optical properties of preformed maackiain found in roots, and demonstrated that differential catabolism of pterocarpanoid enantiomers by the fungus *Nectria haematococca* could help establish the presence of the (−) enantiomer in enantiomeric mixtures.

RESULTS

Medicarpin and maackiain as phytoalexins in S. japonica

Both medicarpin and maackiain were produced by leaf tissue of *S. japonica* in response to inoculation with spores

*While this research was in progress, Ingham [6] listed an unpublished report of (−)-medicarpin and (−)-maackiain as phytoalexins in *S. japonica*.

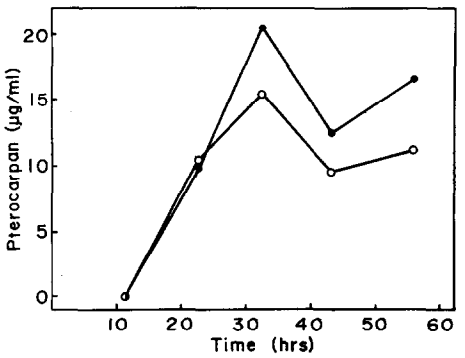


Fig. 1. Accumulation of medicarpin (○) and maackiain (●) in drop diffusates on detached young leaves of *Sophora japonica* inoculated with *Helminthosporium carbonum*. Drop diffusates from leaves treated with the control solution (0.5% Tween 20) and extracted after 72 hr contained 4.8 μg/ml maackiain and 2.4 μg/ml medicarpin.

of *Helminthosporium carbonum* (Fig. 1). Young expanding leaves produced *ca* equal amounts of the two pterocarpans (Fig. 1) while fully differentiated leaves tended to produce medicarpin as the predominant pterocarpan (medicarpin: maackiain ratios as high as 7:1). When fresh leaf tissue was extracted immediately, or after grinding in water and incubating overnight, no medicarpin or maackiain was detected, and only small amounts of the pterocarpans were detected in diffusates of uninoculated leaves. This implies that the pterocarpans or their glycosides were not present as preformed compounds in leaves, but were produced as phytoalexins in response to *H. carbonum*.

The $[\alpha]_D^{25}$ values of the medicarpin and maackiain samples from *H. carbonum*-inoculated leaves were strongly dextrorotatory but the specific rotation for medicarpin (+120°) and maackiain (+152°) was of lower magnitude than for the reference samples of (−)-medicarpin isolated from fenugreek ([α] = −195°) and (−)-maackiain isolated from red clover ([α] = −244°).

Maackiain from roots of S. japonica

Because the specific rotation values of medicarpin and maackiain produced as phytoalexins by *S. japonica* leaf tissue suggested a predominance of the (+) enantiomer, the optical purity of the maackiain in root tissue of *S. japonica* was re-examined. Xylem and bark tissue from roots were separated and then extracted either immediately or after grinding and incubating in H_2O for 24 hr to allow endogenous enzymes to hydrolyse maackiain glycosides. Bark tissue extracted immediately after harvesting contained substantial amounts of free maackiain (0.9 mg/g fr. wt), while similarly extracted xylem tissue contained only 0.07 mg/g. The yield of maackiain increased greatly in both sample sources if they were extracted after stirring macerated tissue in H_2O for 24 hr (final concentrations = 3.5 mg/g for bark and 0.8 mg/g for xylem). The specific rotation values determined after purification by TLC of the bark and xylem samples obtained by both extraction procedures were all highly dextrorotatory ([α] = +224 to +229°). The free maackiain in root tissue was not racemic, contrary to what was expected from the previous work of Shibata and Nishikawa [9]. As with samples from the leaves, the specific rotation values of the samples from roots suggested the presence of minor amounts of the (−) isomer. No medicarpin was detected when these samples were analysed by HPLC.

Additional indication of the occurrence of both enantiomers in roots was obtained by crystallization procedures. When acetone–methanol was used as a solvent pair, a "crystalline" precipitate of maackiain was obtained that had an [α] value of +21° and a mp of 185–191°. Repeated recrystallization of maackiain from the mother fluid with ethanol–water yielded crystals of (+)-maackiain with a specific rotation identical to the (−)-maackiain standard but with a slightly depressed mp (178–179°) compared to the (−)-maackiain standard (179–180°). Shibata and Nishikawa [9] observed that the maackiain racemate had a higher mp (195–196°) than the pure enantiomers (180°) an observation consistent with the acetone–methanol precipitate of maackiain ([α] = +21°) being almost an equal mixture of (+) and (−) enantiomers.

Selective catabolism of maackiain and medicarpin enantiomers by N. haematococca

A previous study on the metabolism of (−)-medicarpin and (−)-maackiain had revealed that some isolates of *N. haematococca* MP VI hydroxylate the 6a carbon of these pterocarpans [12]. Since this alteration occurs at the site of asymmetry, such isolates might differ in their ability to metabolize enantiomers of these compounds. To test this, an isolate (T-95) of *N. haematococca* known to metabolize (−)-maackiain to 6a-hydroxymaackiain (3) and known to not metabolise (+)-pisatin (4), was compared for its ability to metabolize (−)- and (+)-maackiain. The (−)-maackiain was obtained from red clover and the (+)-maackiain was obtained from *S. japonica* by ethanol–water crystallization. All of the (−)-maackiain was metabolized within 24 hr and the only product detected was 6a-hydroxymaackiain, which was highly laevorotatory (Table 1). The specific rotation was close to that of (+)-6a-hydroxymaackiain obtained by the demethylation of (+)-pisatin (Table 1). *Ca* 50% of the (+)-maackiain was metabolized in the same time period but no 6a-hydroxymaackiain or any other known maackiain metabolite was detected in the culture extracts.

(+)-6a-Hydroxymaackiain might not be detected if it were readily metabolized to additional products, but neither (+)- or (−)-6a-hydroxymaackiain was significantly metabolized by this isolate under these culture conditions (Table 1). Thus, the detection of 6a-hydroxymaackiain during the metabolism of maackiain preparations by this isolate should be a direct indication of the presence of (−)-maackiain in the sample. As a further test of selective hydroxylation by this isolate, the nearly racemic maackiain ([α] = +21°) preparation obtained from *S. japonica* roots was employed as a substrate. 6a-Hydroxymaackiain was produced as a product and its [α] was similar to that obtained when (−)-maackiain was the sole substrate while the [α] of the residual unmetabolized maackiain was close to that expected for (+)-maackiain (Table 1). Similar results were obtained when the maackiain obtained as a phytoalexin from *S. japonica* was used as a substrate (Table 1), confirming the presence of (−)-maackiain in this sample. Unexpectedly though,

Table 1. Metabolism of optical isomers and enantiomeric mixtures of pterocarpans by *Nectria haematococca* isolate T-95*

Substrate and source	$[\alpha]_D^{25}$	% Residual substrate	$[\alpha]_D^{25}$	% 6aOH product	$[\alpha]_D^{25}$
Maackiain					
Red clover roots	−244	0	—	62	−328
Sophora japonica roots by EtOH–H_2O crystallization	+244	52	—	0	—
S. japonica roots by Me_2CO–MeOH precipitation	+21	30	+236	22	−305
S. japonica leaves as a phytoalexin	+152	57	+237	7	−110
Medicarpin					
Fenugreek	−195	0	—	60	−290†
S. japonica leaves as a phytoalexin	+120	65	+188	11	−200†
Residual substrate after T-95 metabolism of *S. japonica* phytoalexin sample	+188	36	—	0	—
6a-Hydroxymaackiain					
Demethylation of (+) pisatin	+331	85	—	—	—
T-95 metabolite of (−) maackiain	−328	99	—	—	—

*Cultures received 12 μg of pterocarpans/ml except cultures treated with 6a-hydroxymaackiain which received 30 μg/ml. These concns were noninhibitory to isolate T-95. Metabolic products and residual starting material in the cultures were collected after 24 hr of incubation. Yield data were determined on 6 ml cultures and are corrected for efficiency of extraction controls. Optical rotation values were obtained from pooled samples collected from several 12–15 ml cultures.

†The same ε as used for medicarpin was used for 6a-hydroxymedicarpin to obtain the reported [α] value.

the [α] of the 6a-hydroxymaackiain product from this source was substantially less than the 6a-hydroxy derivative produced from (−)-maackiain or the nearly racemic maackiain.

Analogous experiments (Table 1) on the metabolism of (−)- and (+)-medicarpin confirm that both enantiomers of medicarpin are also produced as phytoalexins by *S. japonica*. In this case the (+) enantiomer was obtained by recovering the medicarpin remaining after incubating the phytoalexin preparation with isolate T-95. (−)-6a-Hydroxymedicarpin (**5**) was detected as a metabolite of the incubation but, similar to the situation when the phytoalexin preparation of maackiain was used as a substrate, the [α] value of the 6a-hydroxy derivative was less than expected. When the recovered (+)-medicarpin was reincubated with isolate T-95, *ca* 60% of it was metabolized but no 6a-hydroxymedicarpin or other known medicarpin metabolites were observed.

DISCUSSION

It is not unexpected to find medicarpin and maackiain produced as phytoalexins by a plant species that contains a glycoside of maackiain as a natural constituent. The same phenomenon has been reported previously for red clover [13]. However, our study appears to be the first case in which (+) enantiomers of 6aH pterocarpans have been reported as phytoalexins [6]. Two (+)-6aOH pterocarpans (pisatin and tuberosin) have been identified as phytoalexins [6].

Studies by Dewick and associates [14, 15] indicate that the biosynthetic step which determines the stereochemistry of pterocarpans is the reduction of the isoflavone precursors. Biosynthesis of (−)-medicarpin by *Trigonella foenum-graecum* involves an *E* addition of hydrogen to the double bond of the isoflavone while the biosynthesis of (+)-pisatin by *Pisum sativum* involves *Z* reduction of the isoflavone precursor. If it is the normal pattern that synthesis of (−) pterocarpans involves exclusively *E* reduction and synthesis of (+) pterocarpans involves *Z* reduction, both types of reduction must be occurring during the synthesis of medicarpin and maackiain by *S. japonica*. An alternative possibility is that in *S. japonica* a single mechanism of reduction occurs but that the enzyme involved can initiate reduction from either side of the isoflavone molecule.

When the specific rotation values of the pterocarpans in the phytoalexin sample listed in Table 1 are compared to the magnitude of the specific rotation of the (−) standards, it is found that the (−)-medicarpin:(+)-medicarpin and (−)-maackiain:(+)-maackiain ratios are essentially the same (for medicarpin, $(195-120°)/(195+120°) = 0.238$; for maackiain $(244-152°)/(244+152°) = 0.232$). Subsequent analysis of other phytoalexin samples obtained from an individual *S. japonica* tree or of pooled samples obtained from several trees indicated that the (−):(+) enantiomer ratio can vary (0.34–0.48) as well as the medicarpin:maackiain ratio (1.5–7.3) [unpublished results]. However, the (−)-medicarpin:(+)-medicarpin ratio was always the same as the (−)-maackiain:(+)-maackiain ratio in samples isolated from the same source. Tracer studies [16–19] indicate that the substitution patterns on ring D of medicarpin and maackiain are determined at the isoflavone stage. A difference in the levels of enzymatic activity responsible for addition of specific substitutents to the isoflavones could explain why the ultimate maackiain:medicarpin ratio can vary. However, the observation that the (−):(+) enantiomeric ratio of medicarpin and maackiain vary together and always remains internally constant in *S. japonica* regard-

less of the maackiain: medicarpin ratio and the absolute (−):(+) enantiomeric ratio, implies that the same stereospecific enzymatic system is operating in the production of both medicarpin and maackiain.

The 6a-hydroxylation of 6aH-pterocarpans has been observed during the biosynthesis of 6a-hydroxypterocarpans by plants as well as during the catabolism of 6aH-pterocarpans by fungi [20, 21]. It has been previously suggested [12, 21] that these reactions in fungi might be catalysed by mono-oxygenases. The retention of configuration during the metabolism of (−)-medicarpin and (−)-maackiain (Table 1) by *N. haematococca* is consistent with, but does not prove, this suggestion. Aliphatic hydroxylation catalysed by mono-oxygenases is known to proceed with retention of configuration [22].

The extensively studied hepatic mono-oxygenases are well known for their broad substrate specificity, but much less is known about the substrate specificity of fungal and plant mono-oxygenases [23, 25]. Recently, Banks and Dewick [20] have shown that peas synthesize (−)-pisatin when (−)-maackiain is added exogenously to pea tissue, even though the plant normally synthesizes only (+)-pisatin. Such a synthesis could be mediated by a plant mono-oxygenase of low substrate specificity. The apparent lack of 6a-hydroxylation of samples of (+)-medicarpin and (+)-maackiain by *N. haematococca* (Table 1) suggests that the 6a-hydroxylation of the (−) enantiomers by this fungus is stereospecific for the pterocarpan substrates. However, the lower than expected specific rotation values of the 6a-hydroxy derivatives produced during the metabolism of unequal enantiomeric mixtures (the phytoalexin samples) suggest that some 6a-hydroxylation of the (+) enantiomers must have occurred even though none was observed when (+) enantiomers were used as the sole substrate. Thus, differential catabolism by isolate T-95 might not always be entirely stereospecific. Nevertheless, the degree of enantiomeric selectivity of these reactions was still sufficient to verify the presence of the (−) pterocarpans in the phytoalexin samples from *S. japonica*. Undoubtedly the stereospecificity of the reactions involved in the biosynthesis and catabolism of pterocarpans will be better understood when the enzymes responsible for these reactions can be studied in a purified form.

EXPERIMENTAL

Three *S. japonica* L. trees (15–20 cm diameters at base) growing on the Cornell campus were used as the source of fully differentiated leaves for the phytoalexin studies. Leaves from the shoots on the stump of a fourth tree were used as a source of young developing leaves. The roots of the latter tree also served as the source of *S. japonica* root tissue. *Helminthosporium carbonum* Ullstrup isolate # A was obtained from O. C. Yoder, Cornell University, and *Nectria haematococca* Berk. and Br. mating population (MP) VI isolate T-95 was from our own collection [25].

Phytoalexins were induced in leaves of *S. japonica* with *H. carbonum* using the drop-diffusate technique as described in ref. [26]. The metabolism of medicarpin and maackiain by *N. haematococca* was done in shake cultures as described previously [27] except that the cultures were incubated at 18–21°.

Isolation and separation procedures. Medicarpin and maackiain were extracted from drop diffusates by partitioning (twice) the diffusates with $CHCl_3$ (2 vol. to 1 vol. diffusate) and chromatographing (TLC) the $CHCl_3$ soluble residue on silica gel with toluene–EtOAc (1:1) [27]. The two pterocarpans have identical mobilities in this solvent system, but the quantity of the two compounds in the samples can be determined from their different UV absorption properties at 310 nm vs 287 nm [16]. Spot sampling and analysis of these preparations for medicarpin and maackiain content by HPLC confirmed the presence of these two pterocarpans in the phytoalexin samples.

HPLC was performed on a μPorasil (10 μm silica: 4.6 mm i.d. × 250 mm) column equipped with a guard column (2.1 mm i.d. × 70 mm) dry packed with silica pellicular packing material. Column eluent was monitored at 280 nm by an absorbance detector. The system was operated under isocratic conditions with 1,2-dichloroethane–*iso*-PrOH (0.13%, *iso*-PrOH) at a flow rate of 2.7 ml/min (Ret vol. maackiain = 15.0 ml; Ret vol. medicarpin 16.8 ml, solubility limit ≤ 2 μg of pterocarpan/μl of eluent). Some decomposition of the pterocarpan occurred when drying down the eluate, and was prevented by adding a drop of K-Pi buffer (pH 7) to the fractions before drying.

Medicarpin and maackiain were also obtained from *H. carbonum*-inoculated *S. japonica* leaves by extracting leaf tissue using a $CHCl_3$–NaOH procedure [28] and sequential TLC of the mixture in three solvent systems ($CHCl_3$–MeOH, 25:1; toluene–EtOAc, 1:1; C_6H_6–EtOAc–*iso*-PrOH, 90:10:1). The medicarpin–maackiain mixtures from the drop diffusates (after TLC in the above solvents) and the medicarpin–maackiain mixtures from inoculated leaf tissues were pooled and the pterocarpans separated by column chromatography on LH-20 Sephadex [28] using MeOH as eluant. The reported [α] values for the phytoalexin samples from *S. japonica* were determined on compounds purified in this manner.

Xylem and bark tissues from roots were separated and either ground in EtOH (1:1, w/v), for immediate extraction, or in H_2O followed by incubation with stirring for 24 hr. The tissue which was ground in H_2O was subsequently extracted with EtOH (1:1, w/v) and this EtOH and the aq. phase were combined. Further purification of all four extracts was accomplished using the same procedure as for leaf tissue except that the NaOH and LH20 Sephadex steps were omitted.

The following modified extraction procedures were used to obtain large amounts of maackiain for crystallization purposes from either *S. japonica* or red clover (*Trifolium pratense* L.) roots. Root tissue was ground in H_2O (1:2, w/v) and stored at room temp. for 24 hr. The solid debris was collected and extracted with EtOH (1–4 ×, 1:3, w/v). H_2O was added to the EtOH (1:4, v/v) and the EtOH removed by evaporation under red. pres. The aq. phase was partitioned against $CHCl_3$ (twice, 1:1, v/v) and after the $CHCl_3$ phase was partially decolorized with Norit A, it was extracted with 0.2 N NaOH (1:2, v/v). The NaOH phase was adjusted to pH 5–6, and NaCl added to a final concn of *ca* 20%. Crude maackiain pptd from this soln and was further purified by crystallization from Me_2CO–MeOH and EtOH–H_2O.

(−) Medicarpin was isolated from fenugreek (*T. foenum-graecum*) [15] and purified from a trace of maackiain by chromatography on LH-20 Sephadex. (+)-6a-Hydroxy-maackiain was prepared by fungal demethylation of (+)-pisatin [29]. Maackiain and medicarpin metabolites produced by *N. haematococca* were isolated and purified as described previously [27].

Identification and physical properties. Medicarpin and maackiain from *S. japonica* were identified by their identical R_fs on HPLC and LH-20 Sephadex, and identical UV (EtOH) and MS as authentic samples of medicarpin and maackiain obtained from fenugreek and red clover, respectively. The 6a-hydroxypterocarpans were identified by their TLC mobilities, UV and dehydration with acid to the corresponding pterocarp-6a-enes [12, 29]. Mp determinations of maackiain were recorded after

drying samples at 110° under vacuum for 24 hr. $[\alpha]_D^{25}$ values were recorded in EtOH at concns of 1–3 mg/ml except for the 6a-hydroxymaackiain (0.18 mg/ml) produced during the metabolism of the phytoalexin sample. The ε value for maackiain was redetermined from our sample of (−)-maackiain as 7930 at 310 nm (EtOH) while lit. values were used for the other pterocarpans [29, 30].

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